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Prepared by: Peter James[1], Diana Leemon[2], Steven Rice[2], Dalton Baker[1], Rosamond Godwin[1] and David Mayer[2].
[1]Queensland Alliance for Agriculture and Food Innovation (QAAFI), University of Queensland.
[2]Department of Agriculture and Fisheries (DAF), Queensland Government.

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Meat and Livestock Australia Limited
PO Box 1961
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Myco-insecticides for nuisance fly control in cattle feedlots

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Abstract

Nuisance flies (*Musca domestica* and *Stomoxys calcitrans*) are important arthropod pests on cattle feedlots with the potential to cause production loss, transmit disease and cause nuisance to surrounding communities. Fly control formulations based on insect-killing fungi may overcome some of the difficulties associated with chemical pesticides and enable treatment of flies in areas where chemicals are not acceptable. Two formulations, an ultra-low volume spray and a bait formulation, based on the entomopathogenic fungus *Metarhizium anisopliae* were developed and tested in laboratory tests, field cage studies and ultimately commercial cattle feedlots. When tested on feedlots the ULV formulation, applied to the vegetation away from feedlot pens, significantly reduced fly populations. The bait formulation gave results comparable to currently registered chemical bait formulations and provides a realistic option for use in areas, such as feed mills, where chemical methods are undesirable. Biopesticides based on *Metarhizium* could form an important component of integrated fly control programs on cattle feedlots and action towards registration of commercial formulations is recommended.

Executive summary

Flies are important arthropod pests in intensive animal facilities such as cattle feedlots with the potential to cause production loss, transmit disease and cause nuisance to surrounding communities. Control measures involve various integrated pest management (IPM) strategies including feedlot design, manure management and biological control, but also rely significantly on pesticide applications. However, excessive reliance on chemical pesticides is undesirable because of the development of insecticide resistance, potential environmental contamination and health and safety concerns. There is a general concern within the community regarding potential adverse effects from chemical use and there is often a market advantage for commodities produced in low chemical systems. This has led to increased pressure for the development of 'softer' control alternatives such as myco-pesticides. In addition, the use of a myco-pesticide may provide advantages on feedlots by enabling treatment of fly populations in areas where a chemical pesticide would not be acceptable.

Previous studies demonstrated that Queensland-isolated strains of the entomopathogenic fungus *Metarhizium anisopliae* infected and killed flies and that the efficacy of some strains was high (typically 80-100% mortality). This project built on the results of this work and developed ULV spray and bait formulations of *Metarhizium* suitable for use in the control of nuisance flies on cattle feedlots. The best strains from the previous feedlot project B.FLT.0326 were selected from the DAF fungal collection, passaged through flies and retested for efficacy. This confirmed M16 as the most effective strain, with attributes making it suitable for mass production. A number of modifications were made to optimise the spore production system and achieved yields in line with expectations for economic commercial production. The strain has also been shown to be effective against other veterinary pests including sheep lice, sheep blowflies, buffalo flies and cattle ticks, suggesting other markets and potentially increasing its attractiveness for commercialisation.

Spray formulation studies focussed on the development of very low volume (VLV) or ultra low volume (ULV) formulations as these offer the advantages of cost-effective carrier volumes, smaller droplet size facilitating better contact with the pest and foliage, and improved environmental persistence by providing a protective oil coating to the spores. A series of proprietary emulsifiable oils were investigated for VLV formulations and vegetable and mineral oils were investigated for ULV formulations. One of the advantages of VLV formulations is that the water phase enables incorporation of additives to enhance efficacy and offer spore protection, so a series of attractants and UV protectants was investigated. However none of these, with the possible exception of molasses, provided sufficient increase in efficacy to recommend their inclusion in the spray formulations.

As a result of laboratory testing, two ULV and two VLV formulations were selected for testing in field cage studies. The ULV formulations consisted of *M. anisopliae* spores suspended in canola oil or peanut oil mixed with Shellsol T (30:70) whereas the VLV formulations consisted of *M. anisopliae* spores suspended in 2% EAOs Horti Oil or Codacide, with 1% molasses. The ULV oil/Shellsol T formulations gave consistently better effect than the VLV emulsions and the Canola oil/Shellsol T ULV form marginally better effect than the Peanut oil/Shellsol formulation. As a result the 30:70 Canola/Shellsol formulation (30:70) (46g spores/L) was chosen for testing in feedlots.

Preliminary feedlot testing with a Micronex ULVA hand held spinning disk sprayer (Micron Group UK) confirmed ultra-low volume application as an efficient way of applying the *Metarhizium* mycopesticide to vegetation and flies. Measurement of vegetation from the spray area showed a 1000X increase in the concentration of spores and approximately 50% infection rate in flies.

Full feedlot testing was conducted using a Cifarelli mistblower (Series L3) fitted with the Micronair AU8000 ULV sprayer head (Enviromist Industries Pty Ltd/Micron Group UK). The sprayer released a targeted mist stream of ULV formulation atomised through the rotary spray head and propelled by a high volume blower. It was operated from the tray of a utility vehicle which drove parallel to the treatment areas and enabled full treatment of feedlot test areas within a couple of hours. The Cifarelli mistblower provided a droplet size of 50 – 100 µm likely to be in the optimal range for the desired application in the feed lots and delivered a higher volume of formulation over a greater area of vegetation than the Micronex applicator. Spraying was directed at vegetation away from the cattle pens and around the perimeter of the feedlots.

Four feedlots were selected for use in the field study, paired on the basis of similar environment and management practice. Spraying was conducted on one feedlot from each pair while the other two feedlots were left unsprayed for comparison. Fly populations were monitored on all feedlots for three seasons. Four methods of monitoring were used: spot cards and sticky cards because of their utility and because when left in place for a week, they account for within and between day variations in fly populations; a visual score, considered most likely to approximate a feedlot manager's assessment of fly numbers and Alsynite traps, used to assess the species composition of the fly populations. These measurements were combined into a fly index which was considered to give the most robust estimate of fly numbers. Years one and two were used to refine the sampling methodology and to assess the relative sizes and patterns of fly populations on the four feedlots. Data from the monitoring in these two years, as well as from the monitoring prior to spray testing in year three, were used to 'benchmark' fly populations on the four test feedlots for analysis of the effect of spraying.

Spraying led to a large increase in viable spore numbers on vegetation in the spray areas for at least 2 weeks post spraying and was shown to produce a measurable infection rate (42%) and mortality of flies (44%) collected immediately after spraying. Although significant mortality could not be demonstrated from the effects of spraying after this time, previous tests with flies exposed to treated vegetation one week and two weeks after spraying indicated mortality of 48% -75% and 15% respectively. The full analysis showed statistically significant reductions in fly numbers estimated as 20%, 23%, 31% and 9% by the fly index, visual scores, sticky cards and spot cards respectively. There was also a significant accumulative effect on fly mortality over the spraying period.

Large numbers of flies, attracted by steam flaked grain rations and cattle odours, were seen along feed bunks and on accumulated manure under fence lines at the edges of the pens. The results were extremely encouraging as no spray was applied to these areas and, it is likely that the majority of these flies were not exposed to the effects of spraying. In addition, an investigation of the effect of chemical spraying on feedlots in a previous project (B.FLT.0326) found no measurable reduction in fly numbers from the chemical treatments. This suggests that the effect from spraying the mycopesticide formulation was at least as

good, if not better, than that from currently registered chemical products. In contrast to chemical pesticides where residues are an issue, fungal formulations may be suitable for spraying closer to the cattle pens and along the sides of feedbunks. Applications of the formulations in these areas could be expected to significantly increase the effectiveness of mycopesticide spraying in reducing fly numbers.

The myco-pesticide bait formulation, derived from the same *M. anisopliae* isolate as used for the spray formulation, may provide a suitable and safer alternative to insecticide baits, particularly for use within areas such as feed stores and ration preparation areas, mills, equipment sheds, outbuildings and in other situations where the use of chemical methods may be undesirable.

M. anisopliae is produced commercially on a sterilised grain substrate which is friable and granular and similar in nature and appearance to many commercially produced bait forms. Three series of laboratory assays were conducted towards the development of a bait formulation derived from *M. anisopliae* conidia on whole rice. The first series of assays tested a range of additives thought to have attractive or protective qualities (raw sugar, molasses, canola oil, milk powder) while the second series of assays tested a range of potential accessory attractants (sugar, molasses, vinegar, milk powder, Envirosafe™ and Dynamic Lifter®). Raw sugar and Dynamic Lifter® were identified potential additives but the improvement from the inclusion of sugar was not great enough to warrant its use and testing focussed on the effect of Dynamic Lifter®.

The rice/spores formulation was tested with and without Dynamic Lifter®, and compared to the commercially available imidacloprid-based fly bait. The results indicated that the addition of Dynamic Lifter® did not increase efficacy of the formulation in comparison to the rice/spores bait. However, whilst the imidacloprid bait had a greater immediate knock-down effect than the *Metarhizium* bait, the *Metarhizium* bait was comparable over a longer term and caused a greater total mortality (53%) after a 7 day incubation period. The results indicate significant potential for development of a commercial *Metarhizium*-rice based fly bait.

This project has developed effective ULV and bait formulations of *Metarhizium* with suitability for use and demonstrated effectiveness in reducing fly numbers on cattle feedlots. Biopesticides based on *Metarhizium* could form an important component of integrated fly control programs on cattle feedlots and action towards registration of commercial formulations is recommended.

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1 Background

Nuisance flies are ubiquitous cosmopolitan pests of agricultural and public health importance that can breed in large numbers in waste associated with cattle feedlots when conditions become favourable. The major species of nuisance flies found breeding on southeast Queensland (QLD) and northern New South Wales (NSW) feedlots are house flies (*Musca domestica*) (86% of larvae) and stable flies (10% of larvae) (Hogsette et al., 2012). Bush flies (*Musca vestustissima*) are also found in significant numbers at some times of the year but breed outside of the feedlots and are blown there or attracted from surrounding areas by cattle and feedlot odours (Urech et al., 2012).

Cattle feedlots provide abundant carbohydrate and protein sources for adult flies while manure and spilled feed provide ideal breeding substrates for fly larvae (Skoda et al., 1993, Hogsette et al., 2012). During periods of warm, moist conditions in summer, large populations of flies can develop. High populations of flies can be annoying for workers and cattle and, when in large numbers, can cause difficulties with neighbours. In addition, house flies have been shown to be important links in the ecology of bacteria of food safety concern, most particularly enterohaemorrhagic *Escherichia coli.* Flies carrying this pathogen have been shown to be able to infect cattle through direct contact and/or by contamination of drinking water and feed (Ahmad et al., 2007). House flies are attracted to steam flaked cattle rations because of the high moisture and sugar content of flaked diets and can aggregate in large numbers in feed bunks. This is thought to be an important route for transfer of *E. coli* and other bacteria amongst flies and cattle (Ghosh and Zurek, 2014).

A survey of Australian feedlot managers in 2002 showed that 83% considered flies a problem and that working conditions, human health, animal welfare, the potential chemical residues from fly treatments and production losses were rated as the most important adverse effects of flies (FLOT.306). Previous MLA funded projects (FLOT.306, B.FLT.0326) investigated a range of fly control strategies and recommended an integrated program for control which included systematic fly monitoring, manure removal from beneath fence lines and other potential fly breeding sites, feedlot design to promote draining and avoid moisture pooling, the use of biological agents such as parasitoid wasps and strategic chemical applications.

The use of chemical pesticides remains an important component of control programs in most feedlots. However, *Musca domestica* has demonstrated a significant resilience to chemical control worldwide and has developed resistance to nearly all of the chemical groups used against it including; organophosphates, carbamates, pyrethroids, growth regulators and relatively new insecticides including spinosad, imidocloprid and nithiazine (Keiding, 1999, Wang et al., 2012, Scott et al., 2013). Studies on southeast Queensland and NSW feedlots in 2002 identified fly populations resistant to organophosphate pesticides, which were the predominant group used at that time, as well as reduced susceptibility to some fly baits (FLOT.306). In addition, there is a general concern within the community regarding the potential adverse effects from chemical insecticide use such as the effects of occupational exposure, the possibility of residues in the food chain and environmental effects. This has led to increased pressure for the development of ‘softer’ control alternatives such as myco-pesticides and often a market advantage for commodities produced in low chemical systems. In addition, use of a myco-pesticide may provide advantages on feedlots by

enabling treatment of fly populations in areas where a chemical pesticide would not be acceptable.

Previous project B.FLT.0326 demonstrated the benefits of augmentative release of parasitoid wasps as part of an integrated control program and included preliminary investigations into the feasibility of using fungal myco-pesticides for nuisance fly control. These studies demonstrated that Queensland strains of the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* infected and killed flies and that the efficacy of some strains was high (typically 80-100% mortality). Strains that provided high spore yields in culture and which were therefore most likely to be suitable for commercialisation were selected for further investigations. Laboratory bioassays showed that spore uptake from sprayed surfaces by flies was sufficient to kill flies and tests in feedlots showed that the levels of *Metarhizium* infection and mortality in flies netted after spraying were much higher than in flies from control feedlots, indicating that the flies were contacting and becoming infected with the fungus. The results were encouraging and further studies were recommended towards the development of optimal formulations of entomopathogenic fungi suitable for use in feedlots and potentially other intensive animal facilities, with a view to eventual commercialisation.

This project built on the results of the previous work. Laboratory research focussed on determining efficient methods for cost effective commercial production and tested the effectiveness of very low volume (VLV) and ultra low volume (ULV) spray formulations and a range of different bait formulations under conditions of controlled exposure. Ultra low volume or very low volume sprays have the advantage of using much smaller spray droplets and consequently much lower volumes of spray per hectare than high volume sprays (Burges, 1998). Smaller droplets are generally more efficacious for arthropod control than larger ones (Adams et al., 1990) and ULV and VLV sprays based on oils have the added advantage of more readily adhering to the lipophilic surfaces of insects and leaves. Oil also wets the surface of the hydrophobic conidia of *Metarhizium*, allowing the conidia to suspend easily in the oil (Burges, 1998).

A number of different additives were tested with the objective of maximising the viability of spores and the stability and utility of formulations, providing improved enhanced UV protection and increasing attraction and exposure of the flies to spores. Field research focussed on first developing a system for monitoring fly numbers, characterising the temporal and spatial patterns of flies within the test feedlots, developing an application strategy and evaluating the spray formulations for fly control under commercial conditions. This report details the results of these investigations and provides data which will assist potential commercial partners to assess the feasibility of investment towards the development of commercial spray and bait formulations of fungal myco-pesticides for nuisance fly control.

2 Project objectives

2.1 Improved and evaluated bait and spray formulations of a *Metarhizium* based myco-insecticide for *M. domestica* control in cattle feedlots.

2.2 Collated manifest of data and information suitable for:

- Circulation to companies interested in co-investing in the further development, registration and commercialisation of the myco-insecticide.
- Contribution to APVMA registration process.

3 Methodology

3.1 Spray formulation

3.1.1 Laboratory studies

3.1.1.1 Fungal isolate and spore production

All *Metarhizium* isolates used in these studies were from the Queensland DAF entomopathogenic fungal culture collection housed at the Ecosciences Precinct (ESP) Dutton Park. These isolates were obtained from either soil samples or dead insects, including adult flies, collected in Queensland. The fungal isolates were maintained on potato dextrose agar (PDA) (Difco) slopes held at 4°C and -20°C.

Three isolates (M10, M16, M54) which showed promise in the previous MLA project B.FLT.0326, along with two other isolates (M92, M93) from the DAF culture collection, were passaged through adult flies and the refreshed cultures were compared for spore production yields and pathogenicity to adult *M. domestica* in laboratory assays (section 3.1.1.2.2).

The spore production system was optimised for reliable spore yields and ten production runs provided bulk quantities of spores of the isolate M16 for the laboratory and field studies after this isolate was confirmed as the best isolate in terms of production and pathogenicity.

Spores were produced via a biphasic process. A liquid culture was first grown to inoculate solid media. The liquid culture consisted of 150 ml of sterile yeast peptone broth in 250 ml Erlenmeyer flasks inoculated with spores scraped from 14 day cultures on Oatmeal agar (Difco™) plates. Liquid cultures were grown for 5 days at 28°C on an orbital shaker. Mushroom spawn culture bags containing 1.5 kg steam sterilised rice were chemically sterilised with 180 ml 2.0% sodium metabisulphite for 24 hours, then neutralised with 36 ml saturated sodium bicarbonate. Each bag was inoculated with 150 ml of the liquid culture following the addition of 45ml of sterile 4% yeast extract. Extra sterile water was added to the bags to bring the total moisture to 40%. Inoculated bags were incubated for seven days at 28°C on wire racks; the solid cultures were then broken up and left for further 14 days of growth. Bags were opened and left to air dry for 4-5 days at 19°C in a de-humidified room. Spores were harvested from the dried grain through a series of sieves (1 mm, 300 µm and 150 µm) on an Endicott sieve shaker. Spore powder was stored at 4°C.

Quality assurance procedures were carried out to determine the number of spores per gram of product, percent moisture and viability of the spores. High moisture in *Metarhizium* spores is associated with a decrease in the viability of stored spores (Burges, 1998). Spore concentrations were determined using an improved Neubauer haemocytometer and the method outlined in Goettel and Inglis (1997). To determine moisture content a 1 gram sample of spores was placed in each of 5 unlidded glass Petri dishes (90 mm diameter). The Petri dishes were put into an oven at 100°C for 24 hours, after which the spores were transferred to a desiccator for 3-4 hours, to cool. The spores in the Petri dishes were weighed again and the difference in spore weight determined and expressed as percent moisture content. Spore viability was determined by suspending 0.01 g spores in 1ml of sterile distilled water containing 0.1% Tween 80, making a 1:10 dilution, then pipetting 2 lots of 20μl onto a Petri dish containing potato dextrose agar (Difco) and incubating at 25°C. Three replicate plates were used. Two coverslips per dish and 2 plates per spore batch were used. After 18 hours, lactophenol cotton blue was applied to each inoculated area to arrest growth and stain the germinating spores. Each stained area was covered with a 24 × 40 mm coverslip and the number of germinated and ungerminated spores in twenty fields of view under each coverslip was determined at 400X.

3.1.1.2 Laboratory bioassay methods

3.1.1.2.1 Background

Investigations were undertaken to develop liquid myco-pesticide formulations, based on the fungus *M. anisopliae*, for spray application to control flies in feedlots. Review of the literature indicated that the most economical spray regimes for applying fungal formulations are by the use as VLV and ULV sprays. VLV formulations are often oil/ adjuvant/water emulsions, with oils providing some protection from environmental conditions and the aqueous phase allowing the incorporation of additives such as feeding attractants. Additives such as carbohydrates (e.g. molasses or sugar) and proteins (e.g. milk powder) encourage flies to remain on treated surfaces, thereby increasing the chance of infection with fungal spores. ULV formulations are also oil-based but generally with no additives except the inclusion of an agent, often a petroleum derivative, which lowers viscosity to enable application through ULV spray equipment. Laboratory bioassays were undertaken to refine and test both VLV and ULV formulations of the fungus for potential use in field trials at feedlots.

3.1.1.2.2 Preliminary work

Preliminary mortality assays were undertaken with *M. anisopliae* (isolate M16) against adult *M. domestica* (flies) to determine a dose-response line. These assays entailed mixing a range of quantities of fungal spore powder in loose sugar and exposing flies to the mixture for a period of seven days in assay containers (three replicates per treatment, 20 flies each) under controlled conditions (27°C, 65%RH, 12:12 LD) and measuring cumulative mortality daily. The response lines from these assays informed selection of the doses used in the subsequent testing of VLV and ULV formulations.

During preliminary experiments, several key aspects of the fly bioassay system were refined to minimise control mortality and improve experiment repeatability. These included determining the most suitable fly-age for testing, identifying appropriate substrates for applying experimental formulations, and determining essential components of the assay test

arenas. In addition, dyes were mixed into formulations and fly frass examined to ascertain the time taken for fungal spores to be taken up by the flies.

3.1.1.2.3 Standard bioassay method

A standard bioassay method was developed for testing liquid formulations for potential use in VLV and ULV applications: 90mm Filter papers (Advantec® No. 1) were treated with fixed spore-doses and volumes of each formulation, allowed to dry in a fumehood, and placed in 90mm Petri dishes (Technoplas®) with twenty flies. The flies were then exposed to the filter papers inside the Petri dish for two hours before being transferred to 550 ml round plastic containers with perforated lids covered in gauze. In each container, flies were provided tap water in a 25ml vial with a cotton wick inserted through the lid and 2g of loose sucrose (sugar) in a small vial lid. The flies were incubated (27°C; 70% RH; 12:12 LD) and cumulative fly mortality measured every 24 hours for the duration (mostly seven days) of the assay.

3.1.1.3 VLV Investigations

3.1.1.3.1 VLV Formulation stability

Assays were undertaken to develop liquid formulations using commercially-available emulsifiable adjuvant oils (EAO) as carriers for applying *M. anisopliae* spores in VLV sprays. EAOs tested were Sacoa® Cropshield (mineral based), Kendon Codacide®, Synertrol Horti Oil®, and GrowGreen Xtend® (all canola based). Initially the suitability of each EAO for use with *M. anisopliae* spores was assessed: First, the maximum quantity of the spores that can be mixed with each of the EAOs was determined by incorporating increasing quantities of spores into oil (1×10^9 to 2×10^{10}spores/ ml of EAO) and making a 1% emulsion with water; Second, assays were undertaken to determine the proportion (1, 2, 3, 4 and 5 %) of each EAO with spores that could be made into a stable emulsion, with stability measured by observation immediately after mixing, then at five minutes, and again at three hours; Third, spore viability of *M. anisopliae* in EAO formulations was determined by leaving spores stand in EAOs for 24 hours, applying an aliquot of formulation to agar plates, incubating, and checking the proportions of germinating spores (section 3.1.1.1).

3.1.1.3.2 VLV Formulation efficacy

Cropshield, Extend, Horti oil and Codacide were tested in mortality assays with flies. Initial assays entailed testing a single dose of spores in 100% pure EAO (Experiment 1, Table 1) in comparison with no-spore controls and 0.1% Tween 80. Subsequently, three EAOs (Horti oil, Codacide, and Cropshield) were each tested against flies with a single dose of spores in 2% emulsions, which included 1 and 5% proportions of additives; molasses and milk powder (Experiment 2, Table 1). Samples of flies were taken from the assays and cultured on agar plates to measure infection rates as outlined in section 3.1.2.1.

All assays were performed using the standard assay method (section 3.1.1.2.3), whereby formulations were applied to filter paper, allowed to dry, and flies were exposed before transfer to plastic containers where daily cumulative mortality was measured.

Table 1: Treatments and parameters of assays with EAOs for VLV application

Exp.	Treatments	Dose/ Filter Paper			Drying Time (mins)	Reps
		Volume (ml)	No. Spores	Spores (g)		
1	Crop shield 100%	0.5	6.3×10^8	0.0084	30	3
	Extend 100%	0.5	6.3×10^8	0.0084	30	3
	Codacide 100%	0.5	6.3×10^8	0.0084	30	3
	Horti oil 100%	0.5	6.3×10^8	0.0084	30	3
	0.1% Tween 80	0.5	6.3×10^8	0.0084	30	3
	Crop shield 100%	0.5	0	0	30	3
	Extend 100%	0.5	0	0	30	3
	Codacide 100%	0.5	0	0	30	3
	Horti oil 100%	0.5	0	0	30	3
	0.1% Tween 80	0.5	0	0	30	3
2	EAO 2%	1	9×10^7	0.0048	30	3
	EAO 2% + 1% milk powder	1	9×10^7	0.0048	30	3
	EAO 2% + 1% milk powder + 1% molasses	1	9×10^7	0.0048	30	3
	EAO 2% + 1% milk powder + 5% molasses	1	9×10^7	0.0048	30	3
	EAO 2% + 5% milk powder	1	9×10^7	0.0048	30	3
	EAO 2% + 5% milk powder + 1% molasses	1	9×10^7	0.0048	30	3
	EAO 2% + 5% milk powder + 5% molasses	1	9×10^7	0.0048	30	3
	EAO 2% + 1% molasses	1	9×10^7	0.0048	30	3
	EAO 2% + 5% molasses	1	9×10^7	0.0048	30	3
	EAO 2%	1	0	0	30	3
	0.1% Tween 80 (control)	1	9×10^7	0.0048	30	3

3.1.1.4 ULV Investigations

3.1.1.4.1 ULV Formulation stability

Assays were undertaken to develop formulations using oil-based carriers for applying *M. anisopliae* spores in a ULV spray. Oils tested were Peanut (Crisco brand), Canola (Crisco brand) and mineral oil (Sigma®) mixed with Shellsol T®, a paraffinic oil used to lower the viscosity of oils for ULV applications. The ratios of Shellsol T and oil used were informed by the LUBILOSA program (Burges, 1998); 50:50 Shellsol T/mineral oil; and 70:30 Shellsol T/vegetable oil (canola or peanut). The viabilities of *M. anisopliae* in the ULV formulations were determined by leaving spores to stand in formulations for 24 hours, applying an aliquot of formulation to agar plates, incubating, and checking the proportion of germinating spores (section 3.1.1.1).

3.1.1.5 ULV Formulation efficacy

The three oils investigated as carriers for ULV spray application were each tested for efficacy against flies. Initial assays tested the oils at 100% with a single dose of spores and compared them to a positive control of 0.1% Tween 80 (Experiment 3, Table 2). In subsequent assays the oils were diluted at the appropriate proportions using Shellsol T (Experiment 4, Table 2). Finally, a comparison of the best ULV formulations was made with

the best VLV formulations (Experiment 5, Table 2). All assays were performed using the standard assay method (section 3.1.1.2.3).

Table 2: Treatments and parameters of assays with oil carriers for ULV application and for a comparison with best VLV formulations

Exp.	Treatments	Dose/ Filter Paper			Drying Time (mins)	Reps
		Volume (ml)	No. Spores	Spores (g)		
3	Canola 100%	0.5	6.3×10^8	0.0084	30	3
	Peanut 100%	0.5	6.3×10^8	0.0084	30	3
	Mineral 100%	0.5	6.3×10^8	0.0084	30	3
	0.1% Tween 80	0.5	6.3×10^8	0.0084	30	3
	Canola 100%	0.5	0	0	30	3
	Peanut 100%	0.5	0	0	30	3
	Mineral 100%	0.5	0	0	30	3
	0.1% Tween 80	0.5	0	0	30	3
4	Canola 30% + Shellsol T 70%	0.5	6.3×10^8	0.0084	30	3
	Peanut 30% + Shellsol T 70%	0.5	6.3×10^8	0.0084	30	3
	Mineral 50% + Shellsol T 50%	0.5	6.3×10^8	0.0084	30	3
	0.1% Tween 80	0.5	6.3×10^8	0.0084	30	3
	Canola 30% + Shellsol T 70%	0.5	0	0	30	3
	Peanut 30% + Shellsol T 70%	0.5	0	0	30	3
	Mineral 50% + Shellsol T 50%	0.5	0	0	30	3
	0.1% Tween 80	0.5	0	0	30	3
5	Canola 30% + Shellsol T 70%	0.5	6.3×10^8	0.0084	60	4
	Peanut 30% + Shellsol T 70%	0.5	6.3×10^8	0.0084	60	4
	Horti 2% + 1% molasses	2	1.8×10^8	0.0096	60	4
	Codacide 2% + 1% molasses	2	1.8×10^8	0.0096	60	4
	0.1% Tween 80	0.5	6.3×10^8	0.0084	60	4
	Canola 30% + Shellsol T 70%	0.5	0	0	60	4

3.1.1.6 Data analyses

All data was analysed using Generalized Linear Models (McCullagh and Nelder, 1989) in (GenStat, 2015). Data from formulation efficacy experiments were analysed by ANOVA with pairwise comparisons of mean fly mortality using Fisher's Protected Least Significant Differences at P=0.05.

3.1.2 Field studies

3.1.2.1 Cage trials at Pinjarra Hills

Field cage experiments were conducted to assess the rates of fly mortality from fungal infection after indirect exposure of flies to vegetation treated with different fungal formulations. The efficacy of the two best ULV formulations (oil and Shellsol T) and the two

best VLV formulations (EAOs) were evaluated against *M. domestica* in semi-controlled field conditions. The most promising fungal formulation was selected for field testing in commercial cattle feedlots.

Four replicate field cage experiments were conducted at the University of Queensland Pinjarra Hills Farm from December 2014 - February 2015. The ULV formulations consisted of *M. anisopliae* spores suspended in canola oil or peanut oil mixed with Shellsol T (30:70) with 1% Molasses. The VLV formulations consisted of *M. anisopliae* spores suspended in 2% EAOs Horti Oil or Codacide with 1% Molasses. These four treatments were tested against a negative control. The five treatment cages were arranged in a randomised complete block design with five replicates (Fig. 1D). All 25 cages were placed in a grassy field, in full sunshine (Fig. 1B).

For each test formulation, a fixed quantity ($1x10^9$) of *M. anisopliae* spores was applied to a single plant (a 40-50 cm high *Dianella caerulea* growing in 14cm pots) (Fig. 1A). The volume applied per plant was 2 ml for ULV formulations and 5.5 ml for the VLV formulations. The negative control plants were left untreated. Each plant (treated or untreated) was then placed inside a cage. The cages ($720mm^2$ base x 800 mm high pyramids) were constructed of poly pipe covered in mosquito netting (Fig. 1C). Each cage was then stocked with 200 flies. These flies were collected from a field population from the feedlots near Irvingdale and multiplied in the laboratory for one to two generations. Flies were exposed to the sprayed plants inside the field cages for 24 hours.

The caged flies were recovered after the 24 hour exposure and transported to the laboratory for 7 days incubation in bioassay containers. Fly mortality was measured after 7 days, then the level of *M. anisopliae* infection was assessed in both live and dead flies by plating samples of surface sterilized flies on water agar amended with 0.01% chloramphenicol media. Plates were incubated at 25°C for up to 10 days to allow sufficient time for sporulation on infected flies. Deposition of viable spores on vegetation was estimated by taking a random sample of four leaves from each plant 24 hours post-spraying. Spores were washed from a 2 cm section of each leaf and plated on CAD medium. This medium is selective for certain isolates of *Metarhizium* and per litre of distilled water consists of: Peptone (10g); Dextrose (10g); Chloramphenicol (1g); Cyclohexamide (0.5g); Dodine solution (10 ml) and agar (15g). The Dodine solution contains 1.65 g “Melprex” (65% a.i.) in 1 litre water).

All analyses were conducted using GenStat (2015). Spore counts were positively-skewed, so were transformed under log10(x+0.5) prior to analysis of variance. For presentation of equivalent means, the direct back-transformation (approximating the geometric means) was adopted. The counts data were subjected to a generalised linear model (McCullagh and Nelder, 1989) under the binomial distribution and logit link. These data consistently showed over-dispersion, so this was factored into the residual deviances for the statistical tests. Adjusted mean proportions, and their standard errors, were estimated.

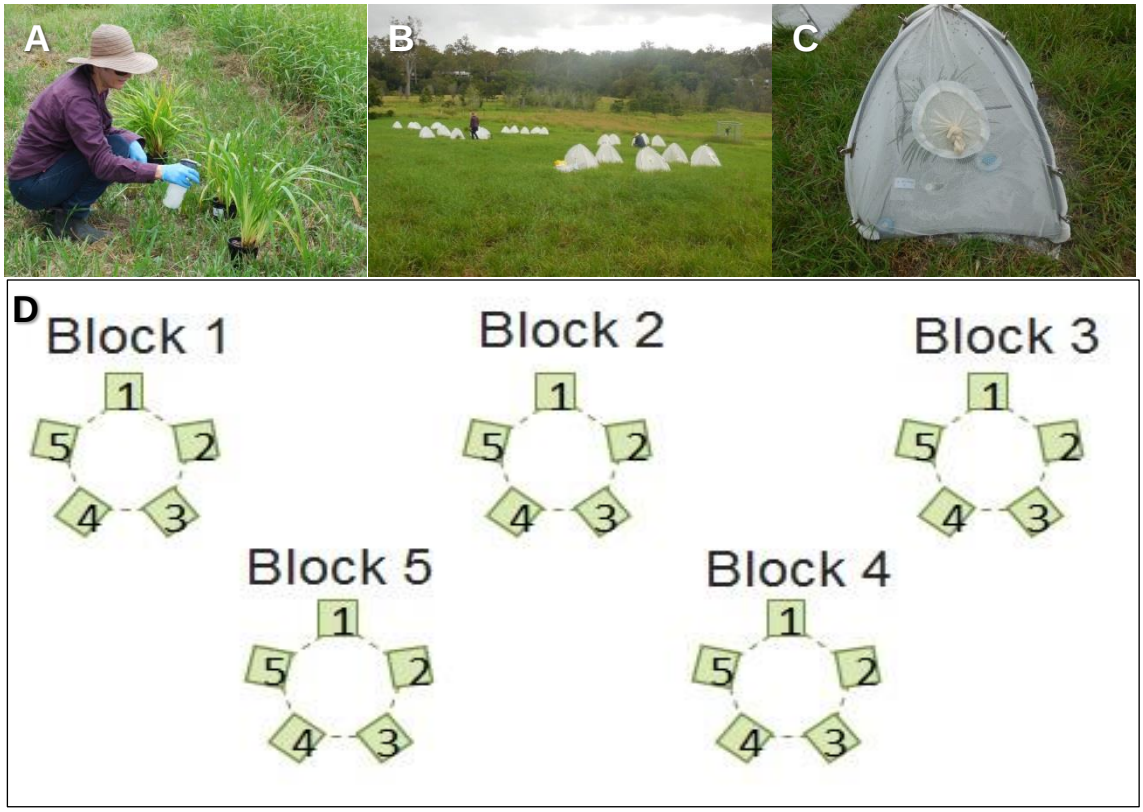

Figure 1: Cage trials at Pinjarra Hills methods. (A) Spraying of formulations, (B) arrangement of trial cages, (C) trial cage set up and (D) trial block design

3.1.2.2 Trial feedlots

Four feedlots were selected in the Quinalow and Irvingdale areas near Dalby for the field studies to minimise inter-farm variability due to geography, climate and management. Research was conducted in these feedlots across three fly seasons in the summers of 2013 - 2014; 2014 - 2015 and 2015 - 2016. Two different agricultural groups each ran two of the selected feedlots, thus providing paired feedlots with the same management practices, types of cattle, pen design and nutrition. Feedlots A and B (agricultural company 1) were designed to carry up 10,000 standard cattle units (SCU) while feedlots C and D (agricultural company 2) were designed to carry between 3,000 and 8,500 SCU. During season 2 and before season 3, feedlot B increased its carrying capacity to 14,000 SCU. However the new pens were within a section of the feedlot that was not used during the trial.

3.1.2.3 Fly monitoring methods

Fly populations were monitored weekly at the four feedlots over three consecutive summer seasons (season 1: 23/10/13 – 19/03/14, season 2: 15/01/15 – 30/04/15 and season 3: 19/11/15 – 17/03/16). Four different methods were used to monitor fly populations, spot cards, sticky cards, visual assessments and alsynite traps. The spot card method involved placing white system cards (102 × 152 mm) within plastic containers which were positioned at each of the monitoring sites (Fig. 2A). Flies that alight on the system cards may deposit fecal or regurgitated material that can be counted to estimate the relative number of flies at that position throughout the week. Sticky cards (102 × 152mm) (Starkeys® Genuine Glue

Boards) were placed at a number of the monitoring sites and the numbers of *M. domestica* stuck to the cards at the end of the week were counted (Fig. 2B). Visual assessments of fly numbers were taken at each of the monitoring sites during the feedlot visits. Visual assessments were made on a 0 – 6 scale whereby; 0 = no flies, 1 = few flies (1 – 5), 2 = low numbers (judged to be of no concern), 3 = moderate (higher than 2, but not worth treating), 4 = high (treatment needed), 5 = very high (above nuisance levels), 6 = extreme. Alsynite traps (Biting Fly Trap, Olson Products Inc.) were also used, primarily to assess species composition of the flies present (Fig. 2C). These traps function similarly to sticky cards however are larger and designed to attract flies.

In season one, feedlots A and B had 20 monitoring positions due to their larger size while feedlots C and D used 16 sites. However, previous studies indicated that 12 monitoring positions gave sufficient precision on large scale dairy operations (Gerry *et al.* 2011) and the number of monitoring positions was reduced to 12 per feedlot on the basis of fly counts and utility in the following two seasons. Efforts were made to avoid moving monitoring positions. However, unpredictable events such as the deconstruction of pens, cattle damage and excessive dust dictated that some monitoring positions needed to be relocated. The relocation of monitoring positions was recorded throughout the seasons and factored into the analyses.

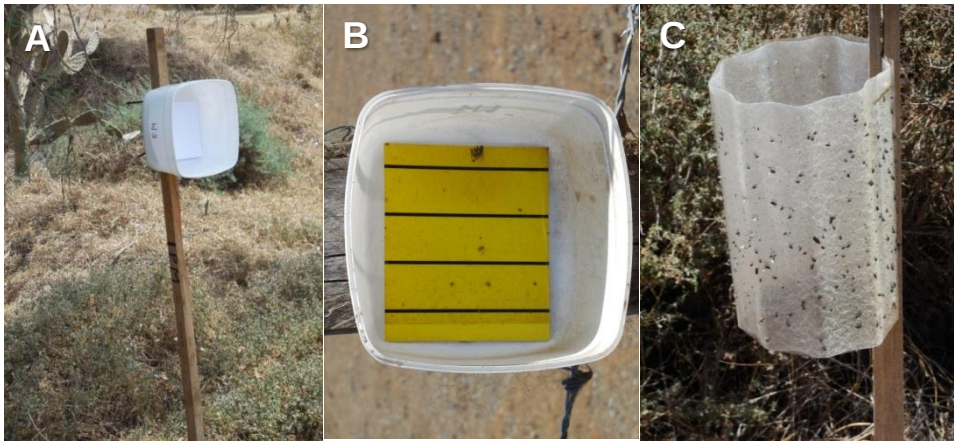

Figure 2: Fly monitoring methods. (A) Spot card, (B) sticky card and (C) alsynite trap

3.1.2.4 Pilot field trial in commercial feedlots

A small scale field trial was conducted at three of the four feedlots (feedlots A, B and C) from March to April 2015. The aim of this pilot study was to develop a methodology for further full scale testing of fungal formulations in commercial feedlots. The study also aimed to determine if flies became infected with *M. anisopliae* when vegetation was sprayed with a canola-based ULV formulation of *M. anisopliae* spores. The fly monitoring methods described in section 3.1.2.3 were used to detect reductions in fly numbers resulting from the spray. In addition, the deposition and persistence of spores on vegetation after spraying was assessed. The ULV formulation was tested in two of the feedlots (A and C) while the other untreated feedlot (B) acted as a control. The control feedlot was equidistant (approximately 5km) between feedlot A and feedlot C.

3.1.2.4.1 Formulation

M. anisopliae spores formulated in Canola oil/ Shellsol T (30:70) (46g spores/L of formulation) were applied to each test area in Feedlots A and C at a dose rate of 1 x 10^{13} spores/ha and application rate of 5L/ha.

3.1.2.4.2 Application

At each treated feedlot, a 0.175ha area of vegetation (approx. 350m long x 5m wide) was selected for spraying. A similar area which remained unsprayed was selected for monitoring and sampling in feedlot B (Fig. 3 - 5). The trial sites were determined on the basis of measured fly activity (section 3.1.2.3), proximity to the pens and suitability for application of the formulations to vegetation. The formulation was applied through controlled droplet application using a Micronex ULVA hand held spinning disk sprayer which delivered droplets in the 50 – 100µm range. The test feedlots were sprayed once per week for 4 weeks.

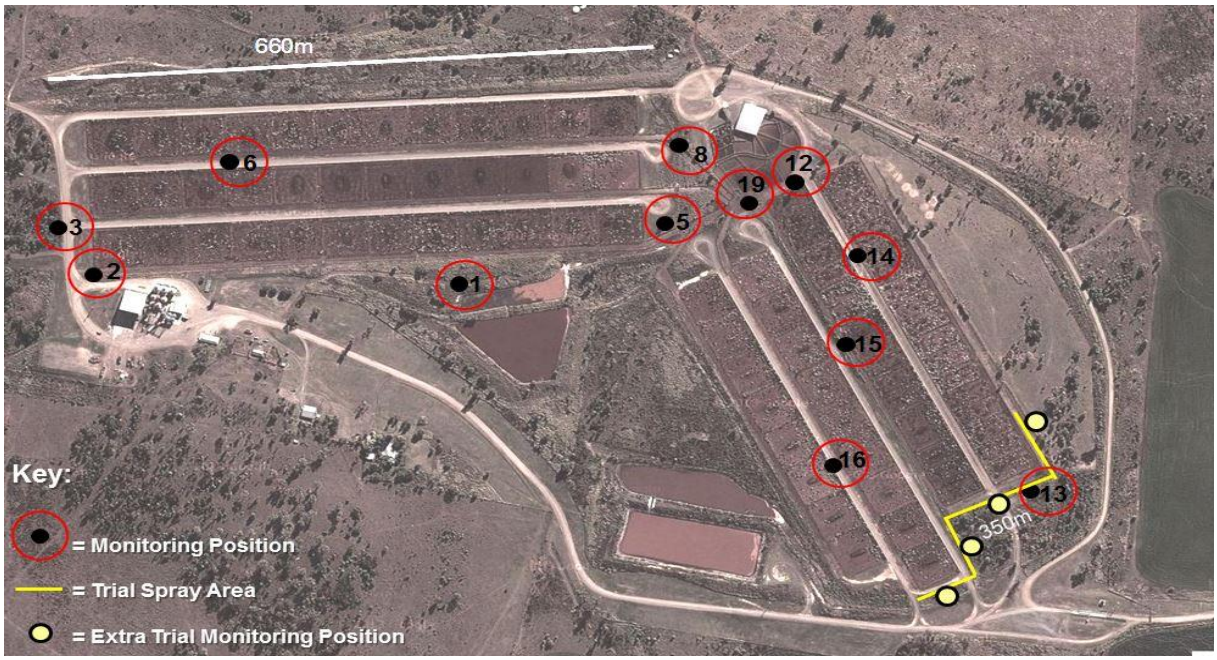

Figure 3: Map of feedlot A (season 2) displaying monitoring positions and pilot trial test areas. Satellite images taken from Google© Maps 2013

Figure 4: Map of feedlot B (season 2) displaying monitoring positions and pilot trial test areas. Satellite images taken from Google© Maps 2013

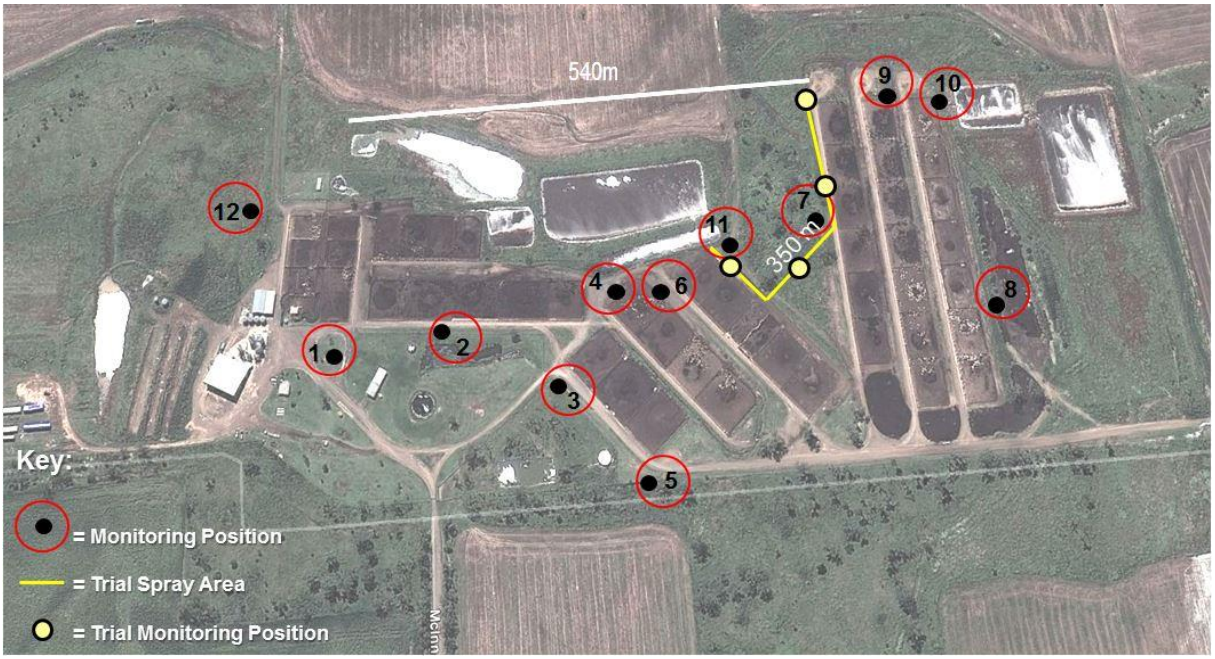

Figure 5: Map of feedlot C (season 2) displaying monitoring positions and pilot trial test areas. Satellite images taken from Google© Maps 2013

3.1.2.4.3 Fly Monitoring Sites

Twelve positions at each of the three feedlots used in the pilot trial were used for fly monitoring (Fig. 3 – 5). The spot card method was used across all twelve positions whereas sticky cards were used at four positions and alsynite traps at two. Four extra positions were

monitored along the selected spray lines during the four weeks of spraying to generate more accurate data in that subsection of each feedlot.

3.1.2.4.4 Fly infection

To assess the level of *M. anisopliae* infection within the fly population, flies were sampled across each of the trial feedlots. Approximately 200 flies were netted within each trial site at 30 minutes prior to and post spraying. Flies were transported to the laboratory in insulated coolers. Each fly sample was then subdivided into four standard bioassay containers (50 flies per container) and kept for 6 days in a controlled environment room (25°C, 65% RH and 12:12 LD). All cages and bioassay containers contained *ad libitum* sugar and water for fly sustenance. Fly mortality was recorded after day 6 and samples of flies both dead (all) and live (up to 21 per subsample) were used to assess *M. anisopliae* infection as per the methods outlined in section 3.1.2.1. Plated flies were incubated at 25°C for 7 days to allow sufficient sporulation of the infected individuals. The number of infected individuals within a subsample was recorded as a measure of infection.

3.1.2.4.5 Spore deposition

To assess deposition and viability of *M. anisopliae* spores on vegetation, approximately 300g of plant material was randomly sampled from each test area immediately prior to spraying and 30 minutes post spraying. A similar sample was taken from the test site in the untreated feedlot to provide an estimate of background levels of *M. anisopliae* spores. Samples were transported to the laboratory in insulated boxes where the vegetation was shredded before a random subsample (80g) was washed in 200ml of sterile 0.1% Tween 80. The resulting suspension was decanted and then diluted as necessary. 100µl aliquots from each dilution were plated onto three replicate plates of *M. anisopliae* selective CAD medium (section 3.1.2.1)). Plates were incubated for 14 days at 25°C after which the number of *M. anisopliae* colonies on each plate was counted and representative plates from each dilution were photographed. Colony counts at the various dilutions were used to estimate the number of spores per gram of vegetation.

3.1.2.4.6 Data ANALYSIS

Data analyses were conducted using GenStat (2015). Spore counts as determined by colony forming units (CFUs) were log10 transformed prior to analysis of variance. The time-series nature of the data was taken into account by an analysis of variance of repeated measures (Rowell and Walters, 1976), via the AREPMEASURES procedure of GenStat (2015). This forms an approximate split-plot analysis of variance (split for time). The Greenhouse-Geisser epsilon estimates the degree of temporal autocorrelation, and adjusts the probability levels for this.

3.1.2.5 Field trial in commercial feedlots

A full field trial to assess the efficacy of the *M. anisopliae* spore based ULV formulation was conducted in the four feedlots described in section 3.1.2.3 from November 2015 until March 2016. The aim of the study was to test the efficacy of the myco-insecticide formulation when applied with a high powered ULV sprayer to the vegetation around feedlots where flies are known to rest. A canola based formulation containing *M. anisopliae* spores was applied in defined areas across two feedlots (A and C). Feedlots B and D were used as untreated control feedlots. Fly populations were monitored weekly as per the methods described in

section 3.1.2.3. Measurements of the relative numbers and patterns in the fly population in seasons 1 and 2, as well as the first 3 monitoring weeks in season 3, were used to standardise the data before the effect of spraying was analysed to test the changes in relative fly numbers and patterns between sprayed and unsprayed feedlots. The deposition and persistence of *M. anisopliae* spores on vegetation was assessed via sampling and flies were collected to determine rates of infection and mortality from fungal infection. A full schedule of events is outlined in appendix I (section 9).

3.1.2.5.1 Formulation

M. anisopliae spores were formulated in Canola oil/Shellsol T (30:70). The Canola oil and spores component of the formulation was made in batches 18 – 24 hours before spraying. *M. anisopliae* spores (220g) were thoroughly mixed into 1200 ml of Canola oil (55g conidia/1000ml formulation) using a whisk to disperse the clumps of spores evenly through the oil. Equal quantities (300 ml) of this oil were dispensed into four 1L Schott bottles and packed into coolers in preparation for transport to the feedlots. Shellsol T (700 ml) was added to each bottle of formulation and shaken vigorously to disperse the spores before addition to the sprayer tank.

3.1.2.5.2 Application

Treated feedlots were sprayed seven times at fortnightly intervals from mid-December 2015 until mid-March 2016. There was a three week gap between the second and third sprays due to the Christmas break.

The formulation was applied by controlled droplet application using a Cifarelli mistblower (Series L3) fitted with the Micronair AU8000 ULV sprayer head (Enviromist Industries Pty Ltd/Micron Group UK) (Fig. 6A). The sprayer delivered droplets at the rate of 80 – 90 ml/min, with a reliable spraying range of approximately 10 – 15m parallel to the ground and a spray width of around 3 – 5m at the end of the range, under fair weather conditions. A preliminary calibration of the rate of spray delivery and distance of droplet dispersal was carried out in an open field at the University of Queensland's farm in Pinjarra Hills (Fig. 6B). The delivery rate of the sprayer was calibrated before each spray to assure consistent application throughout the field trial. Droplet size, when measured on system cards, averaged approximately 110µm however actual droplet size, when corrected for droplet spread, was estimated at between 50 – 100µm. The sprayer was mounted to the tray of a utility vehicle and manually operated from that position (Fig. 6C & Fig. 6D). The vehicle was then driven at approximately 5km/h along the predetermined spray lines (Fig. 7 & Fig. 9) with the sprayer head aimed laterally outwards from the vehicle at a constant height from the tray. Application of the formulation was suspended as the vehicle moved between spray lines or if undesirable targets such as cattle or feed bunks were in range of the sprayer.

Figure 6: Field trial ULV spray application method. (A) Mistblower fitted with ULV sprayer head, (B) sprayer calibration at Pinjarra Hills, (C) sprayer mounted to utility vehicle and (D) sprayer manually operated from vehicle.

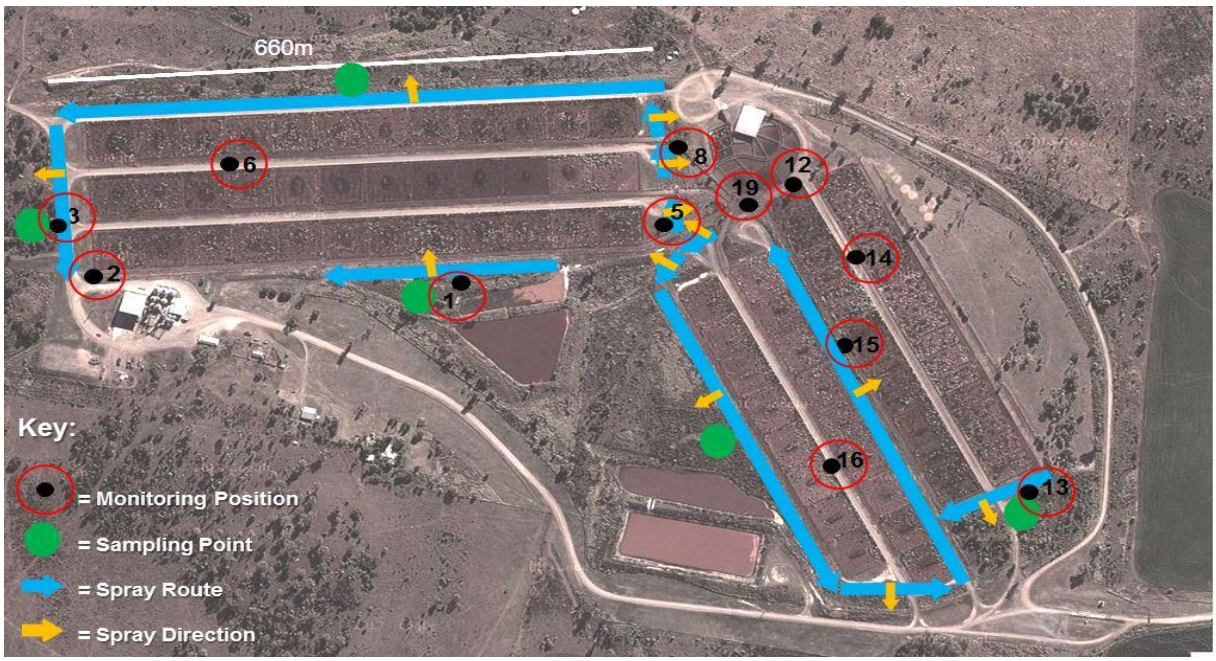

Figure 7: Map of feedlot A (season 3) displaying monitoring positions, vegetation sampling points and spray lines. Satellite images taken from Google© Maps 2013

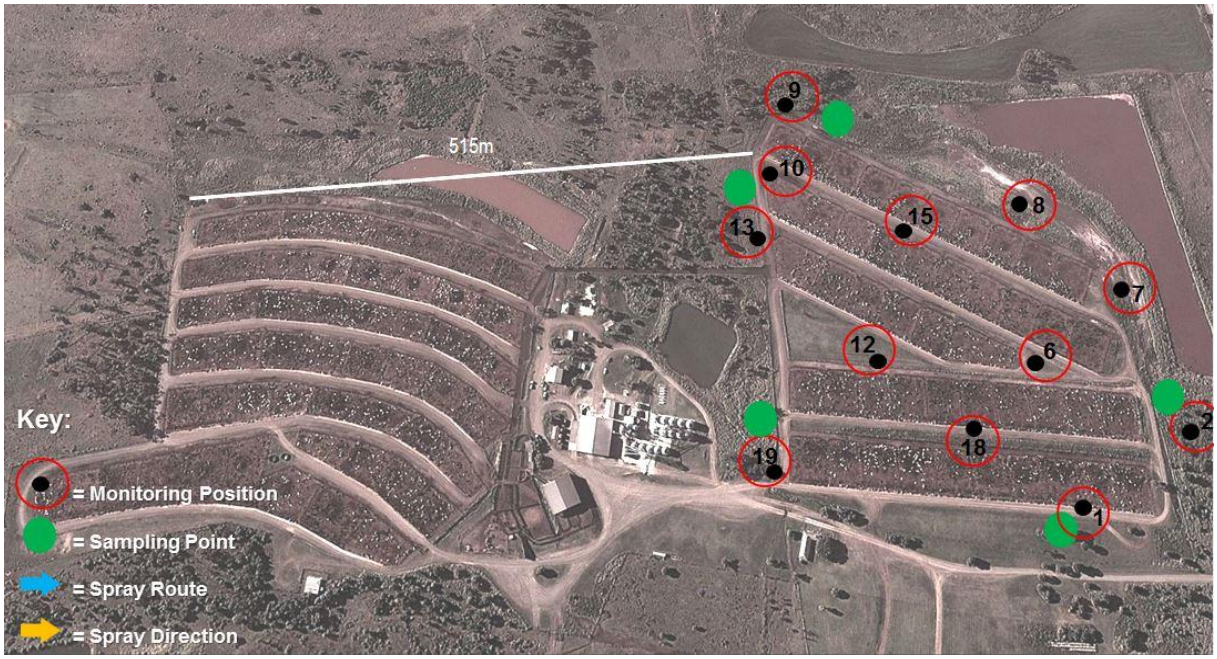

Figure 8: Map of feedlot B (season 3) displaying monitoring positions and vegetation sampling points. Satellite images taken from Google© Maps 2013

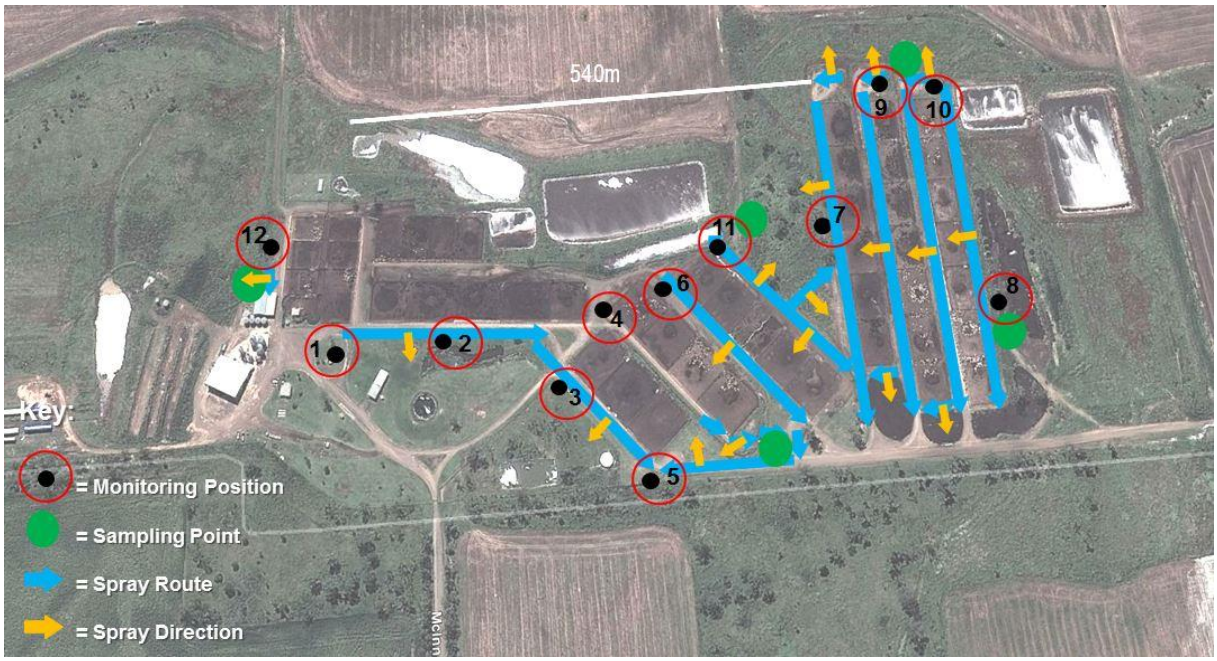

Figure 9: Map of feedlot C (season 3) displaying monitoring positions, vegetation sampling points and spray lines. Satellite images taken from Google© Maps 2013

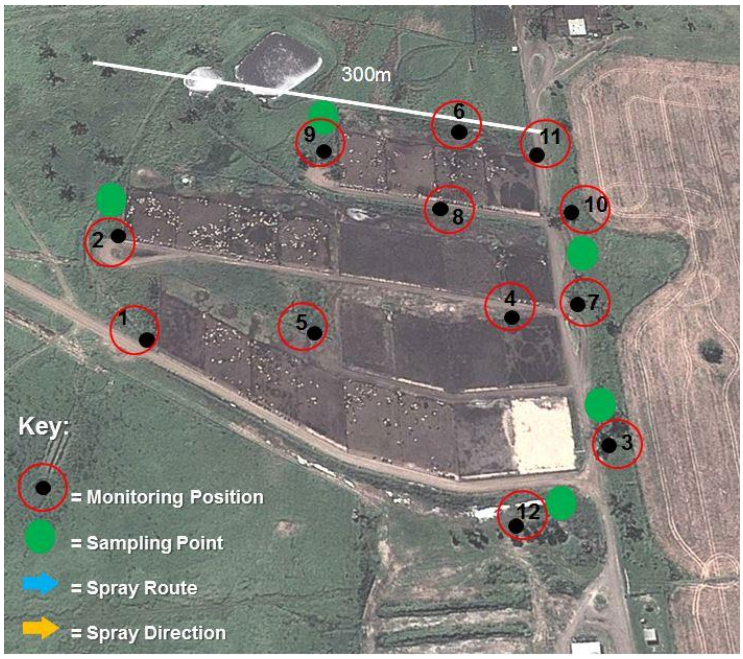

Figure 10: Map of feedlot D (season 3) displaying monitoring positions and vegetation sampling points. Satellite images taken from Google© Maps 2013

3.1.2.5.3 Fly monitoring sites

Twelve positions at each of the four feedlots were used for fly monitoring (Fig. 7 - 10) as per the methods outlined in section 3.1.2.3. The spot card method was used across all twelve positions and sticky cards were used at six positions per feedlot. Sticky cards were located at positions 1, 3, 6, 13, 15, 19 at feedlot A, 1, 2, 8, 9, 13, 19 at feedlot B, 1, 5, 7, 10, 11, 12 at feedlot C and 2, 3, 4, 5, 7, 9 at feedlot D.

3.1.2.5.4 Fly infection

To assess the uptake of *M. anisopliae* by flies within the trial sites, approximately 200 flies were netted from five defined areas (Fig. 7 - 10) in each of the treated feedlots prior to spraying and another 200 flies were netted 30 minutes post-spraying. Flies were similarly sampled, but only once, in the untreated control feedlots on the dates of spraying and on all four feedlots on intermediate weeks. Flies were transported to the laboratory and processed as per the Pilot Trial (section 3.1.2.4).

3.1.2.5.5 Spore deposition

To assess spore deposition and viability, vegetation samples were taken from five different areas of each feedlot. Vegetation samples were taken by walking a 10m transect, perpendicular from the spray line, and taking a hand sample every 2m. During spray weeks, vegetation samples were taken prior to spraying and approximately 30mins after spraying at the treatment feedlots. Samples were also taken from the untreated control feedlots. During intermediate weeks, one round of sampling was completed at all feedlots. The vegetation sampling points at each feedlot (Fig. 7 - 10) were the same as those used for fly sampling.

The samples were transported to the laboratory where the vegetation was shredded and thoroughly mixed before an 80g subsample was suspended in 200ml of sterile 0.1% Tween

80 solution and agitated to wash out any spores. The resulting washes were decanted, diluted and 100 µL aliquots were plated onto CAD (section 3.1.1.1). Plates were then incubated for 7-14 days at 25°C. *M. anisopliae* colonies were then counted and the number of *M. anisopliae* spores/g of fresh vegetation was calculated.

3.1.2.5.6 Data analysis

Visual scores were analysed untransformed, whereas spot-and sticky card counts were log10(x+1) transformed, to correct for skewness in these counts, stabilise the variance, as well as converting all data to a multiplicative relationship basis. In addition, a fly index which incorporated the information provided by the four different measurements of fly abundance was calculated, adjusting for missing values using a general linear model, but excluding dates with only one measure. Possible curvature in the effects was tested by inclusion of a quadratic term.

The full model fitted initially was;

$$Y_{i,j} = Feedlot_i + Spline(date_j) + \alpha\ Animals_{i,j} + \beta\ Spray_2 + \gamma\ Spray_3 + \varepsilon_{i,j},$$

where: $Y_{i,j}$ is the observed dependent variable (visual score, spot-card count, sticky-card count, or overall fly index, in feedlot i at time j; $Feedlot_i$ is the vector of the overall means for the feedlots (i = 1 to 4), $Spline(date_j)$ is a smoothing-spline over dates, using the appropriate degrees of freedom for each season; $Animals_{i,j}$ is the number of animals (relative to the feedlot capacity) at $feedlot_i$ and $date_j$; $Spray_2$ is the weeks since the pilot spraying treatment started (in year 2; zero for the other years and the unsprayed feedlots in year 2); $Spray_3$ is the weeks since the full spraying treatment started (in year 3; zero for the other years and the unsprayed feedlots in year 3); α, β and γ are the estimated slope coefficients; and $\varepsilon_{i,j}$ is the random error.

All variables were first analysed within each site to adjust for the missing observations (positions within each feedlot) across the dates. For the (few) time-gaps other than one week, all data were converted to ‘counts per week’. Cattle numbers for each date were converted to relative within each feedlot. This was used in preference to actual animal numbers, as under that approach the relatively large differences between the feedlots would result in considerable adjustments to the data, risking extrapolation. Possible curvature in these effects was tested with a quadratic term.

Dates were initially considered as discrete levels of a fixed effect and, as these values were sequential, autocorrelations were accounted for. Also, there is an expectation of a somewhat-smooth seasonal effect over time. Hence smoothing splines over the dates were adopted, first testing for an interaction with feedlots. The degrees of freedom (d.f.) for the splines were determined by the maximum variance ratio for the spline term, averaged across the five dependent variables (visual scores, spot-cards, stickies, alsynite traps and the overall fly index). Analysis of each measurement for fly abundance and the overall fly index was conducted within each season and then across all years.

In the first two years the primary aim was to estimate the season trend and baseline the differences between the feedlots. In year 1 there were no recorded differences in relative cattle numbers, so this term could not be tested. Nor were there any treatments applied, so the model only had terms for the spline of time (with 7 d.f.) and feedlot. In year 2 there were

only minor differences in the relative cattle numbers and this term was not significant, so it was again omitted. The model contained a spline of time (7 d.f.), feedlot and the 'pilot-spray' treatment. There was no evidence of curvature and no evidence that the pilot spray had any effect on overall fly numbers (p=0.17, 0.39, 0.12, 0.81 and 0.36 for the visual scores, spot-cards, stickies, alsynite traps and fly index respectively).

For the final analysis (Year 3) the fly index was adopted to take account of the information provided by the three measures of fly abundance made in this year (spot-cards, visual scores, sticky cards). The analysis used the "unsprayed data" from years 1 and 2, plus data from the three monitorings prior to spraying in year 3, to characterise and standardise the intrinsic differences between feedlots, and tested the effect of spraying against this.

3.2 Bait Formulation

3.2.1 Background

A medium of *M. anisopliae* conidia on whole rice was used as the principal component for bait formulation development. *M. anisopliae* is produced commercially on a sterilised grain substrate which is friable and granular, like many commercially produced bait forms, and requires little modification thus reducing production costs. To increase the principal bait formulation's attractiveness to adult flies, and to increase its persistence in the environment, a number of additives and accessory attractants were investigated. These included molasses (Organic), canola oil (Crisco® brand), milk powder (Coles brand), loose sucrose (CSR® brand, refined sugar), Chinkiang vinegar (Jiangsu Hengshun Vinegar Industry Co. Ltd), Dynamic lifter® (Yates Pty Ltd) and a commercially available fly attractant (Envirosafe™). The first three of these are thought to provide protection against ultraviolet radiation as well as to act as phago-stimulants (Burges, 1998). After the effects of additives and accessory attractants had been explored, the final bait formulation was tested for efficacy in a further series of assays.

3.2.2 Bait Additives

The aim of this series of assays was to assess the efficacy of bait formulations based on whole rice and spores combined with a number of additives at varying levels (Table 3 - 5). For assays one and two, each bait treatment was spread evenly over the surface of a Petri dish (90mm) and placed within a mesh cage (600 x 600 x 600mm) inside a controlled environment room (27°C, 65% RH, 12:12 LD). Forty, unsexed, *M. domestica* adults (flies*)* were then put in each cage. Instantaneous counts of the number of flies resting on each bait were made three times at five minute intervals beginning on the hour at four, five, six and 23 hours after the flies were added to cages. This gave a total of four sets of counts per treatment over the exposure period. After 24 hours, each group of 40 flies was removed from its cage, split equally into two small bioassay containers, and kept for a period of eight days (27°C, 65% RH and 12:12 LD). Daily cumulative mortality of the flies was recorded. Both assays were repeated three times. Assay three differed slightly in that fifty, unsexed, *M. domestica* were used per cage, there were four replicates for each treatment and total mortality was recorded after six days rather than cumulative mortality over eight days.

3.2.2.1 Assay 1

Table 3: Bait formulation additives tested in Assay 1.

Treatment	Rice + Spores (g)	Canola Oil (ml)	Sugar (g)	Molasses (g)
1	-	-	-	-
2	20	-	-	-
3	20	4	-	-
4	20	-	2.5	-
5	20	-	5	-
6	20	-	10	-
7	20	4	-	2.5
8	20	4	-	5
9	20	4	-	10

3.2.2.2 Assay 2

Assay one results indicated that bait formulations containing oil and molasses were less effective than those containing dry sugar. This lack of efficacy might have been caused by the relatively high volume of oil used. Therefore this assay aimed to test bait formulations with lower levels of oil.

Table 4: Bait formulation additives tested in Assay 2

Treatment	Rice + Spores (g)	Canola Oil (ml)	Molasses (g)
1	20	1	2.5
2	20	1	5
3	20	1	7.5
4	20	2	2.5
5	20	2	5.0
6	20	2	7.5
7	20	3	2.5
8	20	3	5.0
9	20	3	7.5
10	20	2	-
11	-	-	-

3.2.2.3 Assay 3

In assays one and two, the bait formulations containing oil and molasses were not as effective as formulations with raw sugar. In previous assays, canola oil was used to allow molasses to be mixed into the formulation without clumping. Canola oil is also known to offer a level of protection against ultraviolet light, which could be beneficial for bait formulations with a fungal active ingredient. Therefore, the aim of this assay was to determine if the addition of raw sugar to bait formulations containing a low level of oil increased efficacy. This assay also tested another potential UV protectant, milk powder.

Table 5: Bait formulation additives tested in Assay 3

Treatment	Rice + Spores (g)	Canola Oil (ml)	Sugar (g)	Milk Powder (g)
1	20 (sterile rice only)	-	-	-
2	20	-	-	-
3	20	3	-	-
4	20	3	10	-
5	20	-	-	10

3.2.3 Attractant studies

A series of assays were conducted to assess the possibility of including an accessory attractant to the bait formulation in order to increase its efficacy. Fly assays were conducted in replicate mesh cages (600 × 600 × 600 mm). Fly traps each containing a potential attractant were randomly placed upright in a circular arrangement on the floor of each cage. Fly traps consisted of cylindrical plastic jars (125 × 95mm) with plastic lids, each inserted in the centre with a tapering plastic tube (40mm L x 10-6mm diam.) flush with the lid top surface and extending into the jar. Attractants were contained in 50mm dia. vial lids placed in the bottom of the traps. Control traps contained empty vial lids. A 90mm petri dish lid with dampened sponge (Wettex®) was placed in the centre of each cage to provide the flies with water. The cages were placed within a controlled environment room (27 °C, 65% RH, 11:13LD) before 50, unsexed, adult *M. domestica* were released into each cage. The number of attractants and quantities used varied between assays, as did the exposure time.

3.2.3.1 Assay 1

This assay tested five treatments (molasses, vinegar, sugar, wetted milk powder and a nil control) in five cages, using 2.5g of each potential attractant in each trap jar. The flies were allowed free range in cages overnight for about 20 hours and the assay was repeated twice.

3.2.3.2 Assay 2

Six treatments were tested in this assay; a commercial attractant containing (Z)-9-tricosene (Envirosafe™), Envirosafe™ with the addition of dead flies, water with the addition of dead flies, a wetted commercial fertiliser derived from composted poultry manure (Dynamic Lifter®), molasses and a nil control. Three grams of each potential attractant was used in each trap jar. The flies were exposed for 48 hours and the assay was repeated once.

3.2.3.3 Assay 3

Dynamic Lifter® showed the most promise as a potential attractant in assay 2. In order to confirm its attractive properties, 3g of Dynamic Lifter® was tested alone against a nil control. Six replicate cages were used and flies were exposed for 48 hours.

3.2.4 Bait efficacy

3.2.4.1 Assay 1

Previous assays that tested UV protectants as adjuvants (oil and milk powder) have indicated that the addition of these adjuvants causes a significant decrease in the efficacy of the bait. As a result of this, these adjuvants were omitted from the final bait formulation. Without the presence of an UV protectant the longevity of the bait formulation could be

affected, but baits may be suitable for use indoors or in shaded bait stations. This assay aimed to test this premise by comparing the decrease in spore viability between shaded baits and those under natural UV exposure for 24 hours.

Viability assays were conducted in twelve replicate plastic containers with lids. Six of these containers were coated externally with a black spray paint (White Knight Squirts®, Flat Black). Five grams of rice and *M. anisopliae* bait media was placed in each of the containers. The containers were then fixed to a wooden board and placed in an exposed area of the roof at the EcoSciences Precinct. After 24 hours, 0.01g samples of spores were sieved out of each replicate and the percent viability of spores in each sample was determined using the process outlined section 3.1.1.1. Viability of the stored spores used in the assay was also bot not included in the experiment was used as a control.

3.2.4.2 Assay 2

The bait additive and accessory attractant assays indicated that the most cost efficient and effective bait formulation would be the dry bait formulation consisting of whole rice and *M. anisopliae* spores, with the possible addition of Dynamic Lifter® as an accessory attractant. The aim of this assay was to undertake a final evaluation of the efficacy of the bait formulation and to compare it with results from a commercially available bait.

Fly assays were conducted in fifteen replicate mesh cages (600 × 600 × 600mm) within a controlled environment room (27°C, 70% RH and 12:12 LD). Bait trays consisting of a white plastic tray (150 × 150mm), centre fitted with 90 mm Petri dishes via adhesive Velcro® dots were used to expose each bait. The treatments used were 10g of sterile rice, 10g of sterile rice with Dynamic Lifter®, 10g of rice and *M. anisopliae* spores, 10g of rice and spores with Dynamic Lifter® and finally 2g of Quickbayt®. Rice or Quickbayt® components of the respective treatments were spread around the outside of the Petri dish while Dynamic Lifter® components were added to the Petri dish and moistened with 10ml of water. One hundred, unsexed, *M. domestica* were added to each of the mesh cages and allowed 2.5 hours of exposure to the baits before the bait trays were removed from the cages. The flies were then split equally into two small bioassay containers and kept for a period of 7 days in order to record the mortality. Both the small bioassay containers and the mesh cages contained *ad libitum* water and sugar to sustain the flies. There were three replicates for each of the five treatments and the assay was repeated once.

3.2.5 Data analysis

Cumulative mortality data was analysed by an analysis of variance of repeated measures (Rowell and Walters, 1976), via the AREPMEASURES procedure of GenStat (2015). This forms an approximate split-plot analysis of variance (split for time). The Greenhouse-Geisser epsilon estimates the degree of temporal autocorrelation, and adjusts the probability levels for this. Total percentage mortality and spore viability data was square root arcsine transformed before being analysed by a one-way analysis of variance (ANOVA). For the evaluation of different attractants in cages, data was square-root arcsine transformed and then analysed using a two-way ANOVA for experiment and treatment. Post-hoc analyses for all data sets were conducted using Fisher's Protected Least Significant Differences (LSD's). Results are presented as back transformed data. All analyses were performed using GenStat (2015).

4 Results

4.1 Spray formulation

4.1.1 Laboratory studies

4.1.1.1 Fungal isolate and spore production

The two *Metarhizium* isolates with the highest rates of pathogenicity to *M. domestica* were M10 and M16 (Fig. 11). These two isolates also gave the best spore yields. Isolate M16 had a history of consistency in performance in previous projects, and was the isolate used in the previous project B.FLT.0326. The results supported continuing with this isolate. The use of enriched media to grow liquid starter cultures and inclusion of yeast extract in bags of solid growth media (rice) further boosted the spore production with isolate M16. Over the project life the average spore yield was 6.55% and the highest yield was 9.65%. These yields compare favorably for commercial production (R. J. Milner; personal communication). During this project more than 7.08 kg of spores were produced for both the laboratory and field studies over 15 production runs. The viability of the spores used for the field trials was ≥ 90% and the moisture level ranged from 13% to 20%. Although this is on the high side for long term storage at 4°C, spores were stored at -20°C. In one gram of M16 dried spores there were approximately 4 $\times 10^{10}$ spores.

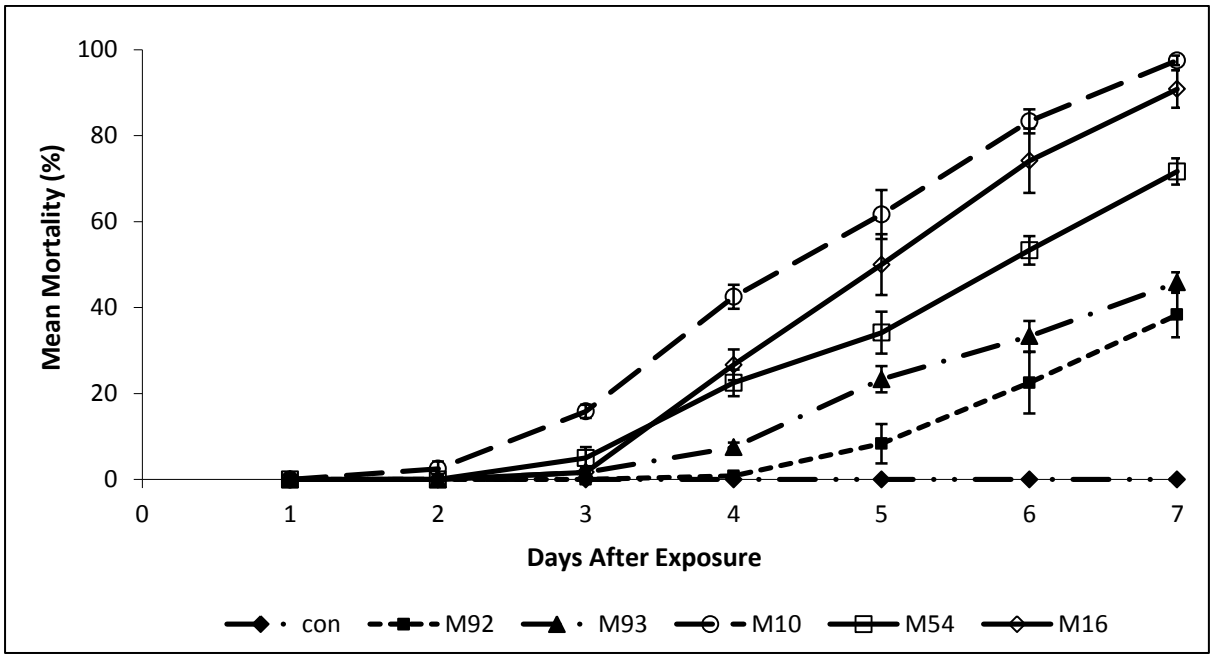

Fig 11: Mean mortality (%) (±SEM) in flies exposed to different *Metarhizium* isolates

4.1.1.2 Spray formulation development

4.1.1.2.1 Preliminary work

A dose response line for M16 was achieved by mixing a range of quantities of spore powder into sugar (Fig. 12). The assay determined that a dose of $3x10^8$ - $6.3x10^8$ (0.0048 – 0.0083g) spores per container was suitable for fly mortality studies using either loose sugar or liquid formulations to carry spores.

Container type and bioassay methodology was refined to minimise fly mortality in control treatments. Young flies <5 days old were found to be better for assays than older flies (7-10 days old), because of their longer survival time, and plastic containers with liners that minimised accidental fly entrapment were chosen.

Filter paper was found to be the best surface in fly exposure experiments with 0.5mL formulation the optimal quantity for 9 cm filter papers and an exposure time of 2 hours.

Assays with dyes indicated that at least 2 hours was needed for flies to take up test formulations.

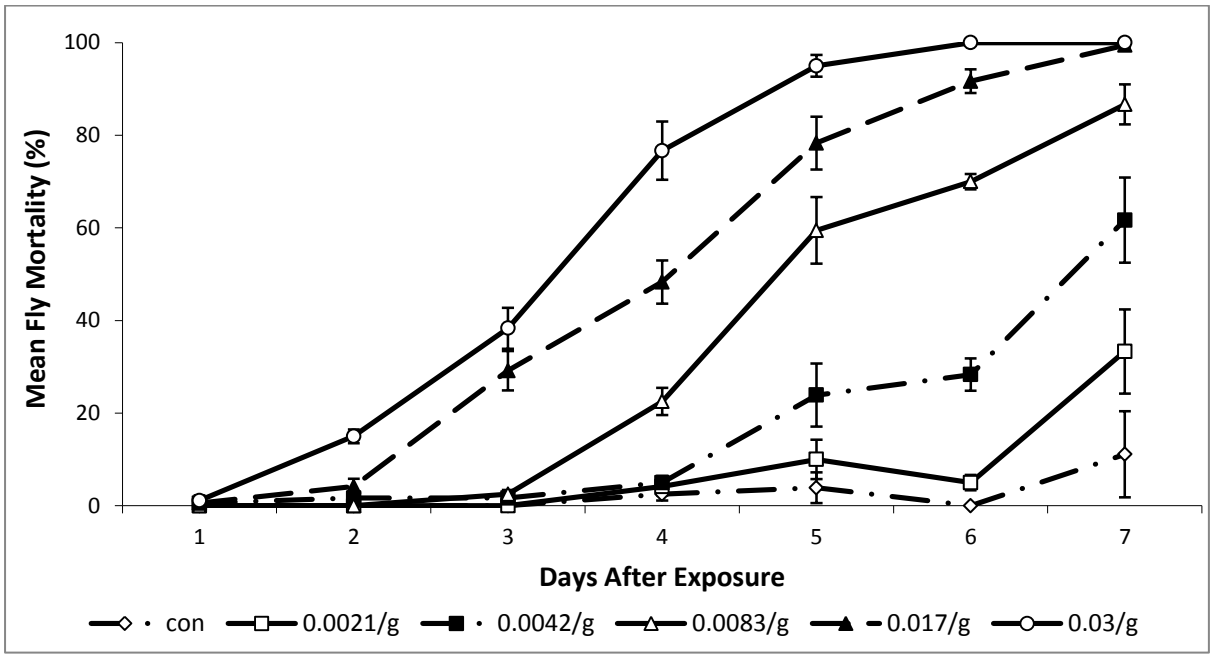

Figure 12: Mean mortality (%) (±SEM) in flies exposed to a range of doses (number of spores per container) of *Metarhizium* isolate M16 spores formulated in sugar.

4.1.1.3 VLV Investigations

4.1.1.3.1 VLV Formulation stability

The highest concentration of spores that could be suspended in an emulsion with 1% EAO was 9 x10^7 spores/ mL of emulsion. This is because the maximum of spores/ mL that can be suspended in the oil fraction is 9 x10^9 spores/ mL oil if the oil is then used at 1%.

By comparing the stability of EAOs for formulating spores when used at 1-5 %, it was found that the optimal level for use in an emulsion was 2 %.

The most stable emulsions were obtained with Horti Oil, while Cropshield gave the least stable followed by Xtend. Emulsions with Codacide were intermediate in their stability.

No detrimental effect on germination was observed when spores were formulated in 2% EAOs Codacide (89% germination) and HortiOil (84% germination). Germination appeared to be enhanced compared to the Tween 80 control (79% germination).

4.1.1.3.2 VLV Formulation efficacy (100% EAO)

In experiment 1, Cropshield seemed to have some toxic effect on flies even without spores, sometimes causing 20% mortality soon after contact. None of the other EAOs appeared to be toxic to flies (Fig. 13).

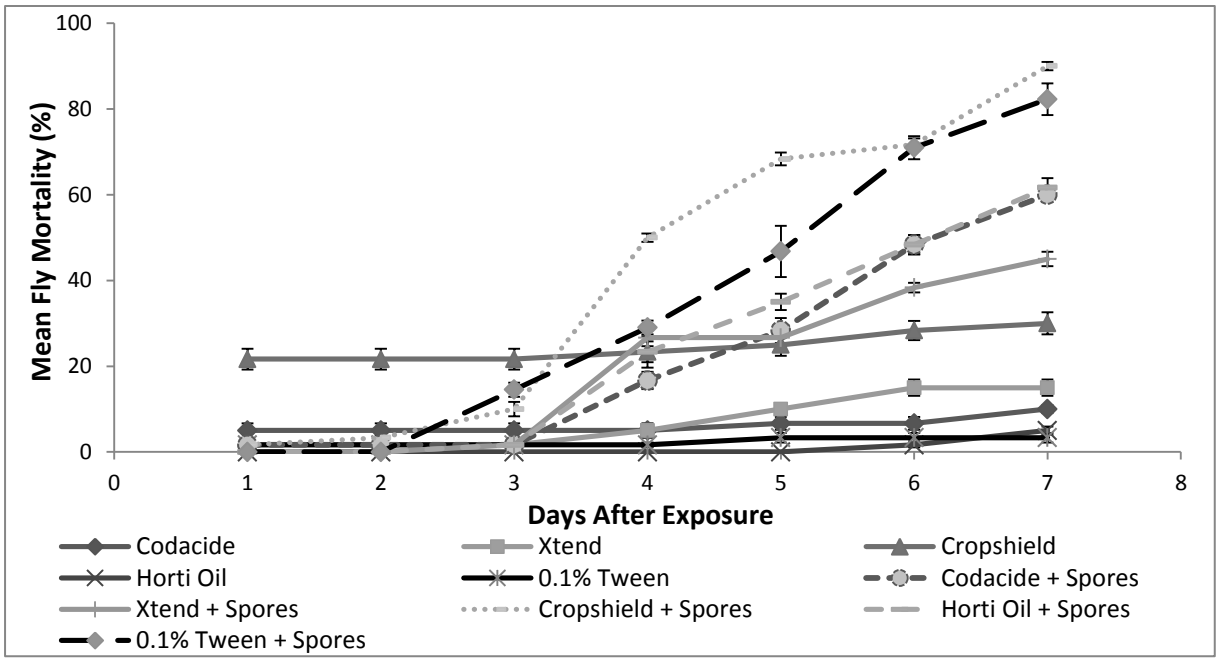

Figure 13: Mean fly mortality (%) (±SEM) after exposure to four 100% concentration commercial emulsifiable adjuvant oils (EAO) and Tween 80 as a control with and without *M. anisopliae* M16 spores at 6.3 x 10^8.

When spores were formulated in 100% EAOs, 94% fly mortality was observed in the Cropshield + spores treatment after seven days. This was higher than any of the other EAOs + spores but not significantly different to the Tween 80 formulation (82% Mortality). The Codacide + Spores (60%), Horti Oil+ Spores (61%), and Xtend + Spores (45%) were significantly lower than Tween 80 + spores (P=0.032).

There was a significant relationship between the infection rate with *Metarhizium* and fly mortality rates. This was most pronounced four days after plating out the flies. There was a significant linear regression (Fpr<0.001) R^2 = 89.1 when all treatments were included and Fpr = 0.017, R^2=84.8 for only the +spore treatments. This means that in the plus spore treatments, approximately 85% of the mortality can be explained by *Metarhizium* infection.

4.1.1.3.3 VLV Formulation efficacy (2% EAO + Additives)

In experiment 2, none of the emulsions on their own were significantly toxic to flies indicating that most of the mortality was a result of the fungus. This was supported by the observation of a significant relationship between the *Metarhizium* infection rates and mortality rates. For example, for the Cropshield emulsions, R^2 =80.5 (P>0.001) indicating that 80% of the variation in mortality could be accounted for by infection with *Metarhizium*.

Spores formulated in the three EAOs were less effective at killing flies than spores formulated in the Tween 80 control. Mortality rates achieved with spores formulated in Horti

Oil, Codacide and Cropshield emulsions were 56%, 53% and 70% respectively of that achieved with spores in Tween 80.

Emulsion stability was not affected when formulated with1% molasses and/or milk powder, but was significantly reduced when 5% molasses and/or milk powder was used. The efficacy of the emulsions appeared to be marginally improved with 1% molasses, but no additional benefit was provided by increasing the molasses concentration to 5%. Milk powder in the emulsion did not enhance efficacy, and if anything, resulted in a reduction in efficacy.

No detrimental effect on germination was observed when spores were formulated in 2% EAOs Codacide (89% germination) and HortiOil (84% germination). In fact, germination appeared to be enhanced compared to the Tween 80 control (79% germination).

4.1.1.4 ULV Investigations

4.1.1.4.1 ULV Formulation efficacy (100% Oil)

In experiment 3, 100% oils (Peanut, Canola or Mineral) alone had no detrimental effect on pathogenicity of the fungus to flies and no toxicity to the flies (Fig. 14).

Spores formulated with both vegetable oils produced similar mortality rates to the Tween 80 + spores. Spores formulated with mineral oil were significantly more effective than with the vegetable oils ($P<0.05$), with around 25% more flies dead within 6 days than in the peanut or canola oil treatments.

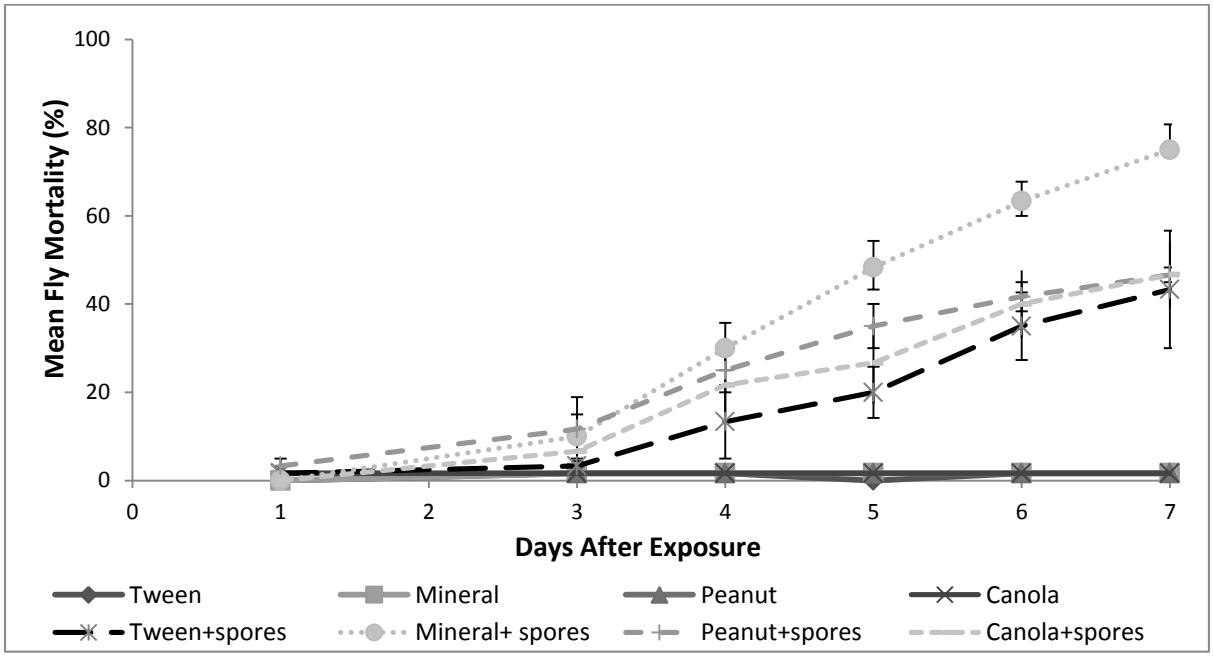

Figure 14: Cumulative daily fly mortality (%) (±SEM) for *M. anisopliae* isolate M16 spores 6.3 x 10^8 formulated in different oil-based carriers with 0.1% Tween 80 controls.

4.1.1.4.2 ULV Formulation efficacy (Shellsol T Mixtures)

In experiment 4 with oils formulated with Shellsol T to reduce viscosity there was also no significant difference between the oil/ Shellsol T/spore mixtures and the control (Tween80 +

spore) in respect to their pathogenicity to flies (Day 7; P=0.618). None of the formulations without spores had a significant toxic effect to flies (Day 7; P=0.734) (Fig. 15).

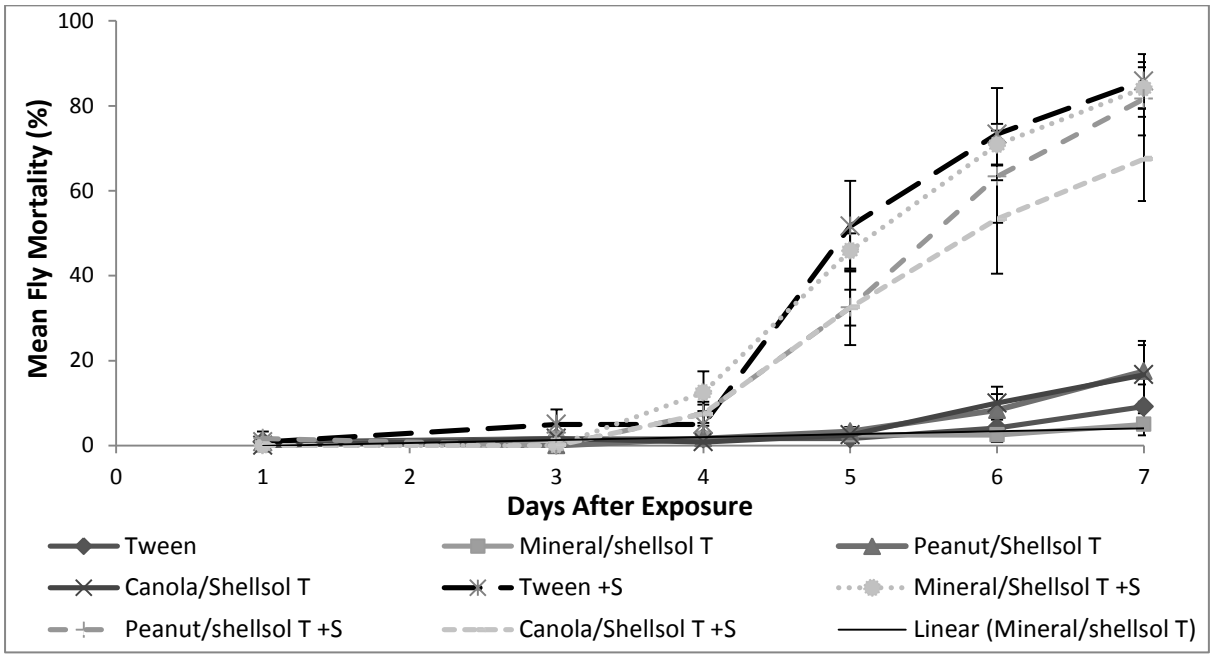

Figure 15: Cumulative daily fly mortality (%) (±SEM) for *M. anisopliae* M16 spores 6.3 x 10^8 suspended in different oil/Shellsol T formulations with 0.1% Tween 80 control.

All of the oil/ Shellsol T mixtures tested appeared suitable for ULV application and were equally effective in killing flies when freshly mixed.

4.1.1.4.3 ULV Formulation stability

After 24 hr storage in oil/Shellsol T mixtures, spore germination rates were only 15% in the mineral oil/ Shellsol T mixture compared to 90% in the Canola/shellsol and peanut/ shellsol mixtures, and 70% in the Tween 80 control (Table 6). Mineral oil appeared to have a detrimental effect on spore germination since storage in the (50:50) shellsol/oil produced greater inhibition to germination than the 70:30 shellsol/oil formulation.

Table 6: Germination studies of *M. anisopliae* M16 spores after 24 hr storage in Shellsol T/oil formulations

Formulation	Rep A # spores counted	Germination (%)	Rep B # spores counted	Germination (%)	Average Germination (%)
Control 0.1% Tween80	525	71.6	597	69.2	70.4
70:30 Shellsol T/Peanut	436	96.3	797	90.1	93.2
70:30 Shellsol T/Canola	928	91.2	643	89.7	90.4
50:50 Shellsol T/Mineral	223	3.1	-	-	3.1
70:30 Shellsol T/Mineral	422	13	351	17.9	15.5

4.1.1.4.4 Comparison of best VLV and ULV formulations

In experiment 5, there was a significant difference in fly mortality between the various *Metarhizium* formulations at four days post-exposure (P<0.001). The oil/Shellsol T formulations generally had higher mortalities than the emulsion formulations. Fly mortality in the Canola/Shellsol T formulation was significantly higher than the emulsion formulations and not significantly different from the Tween 80 control from Day four (Fig. 16).

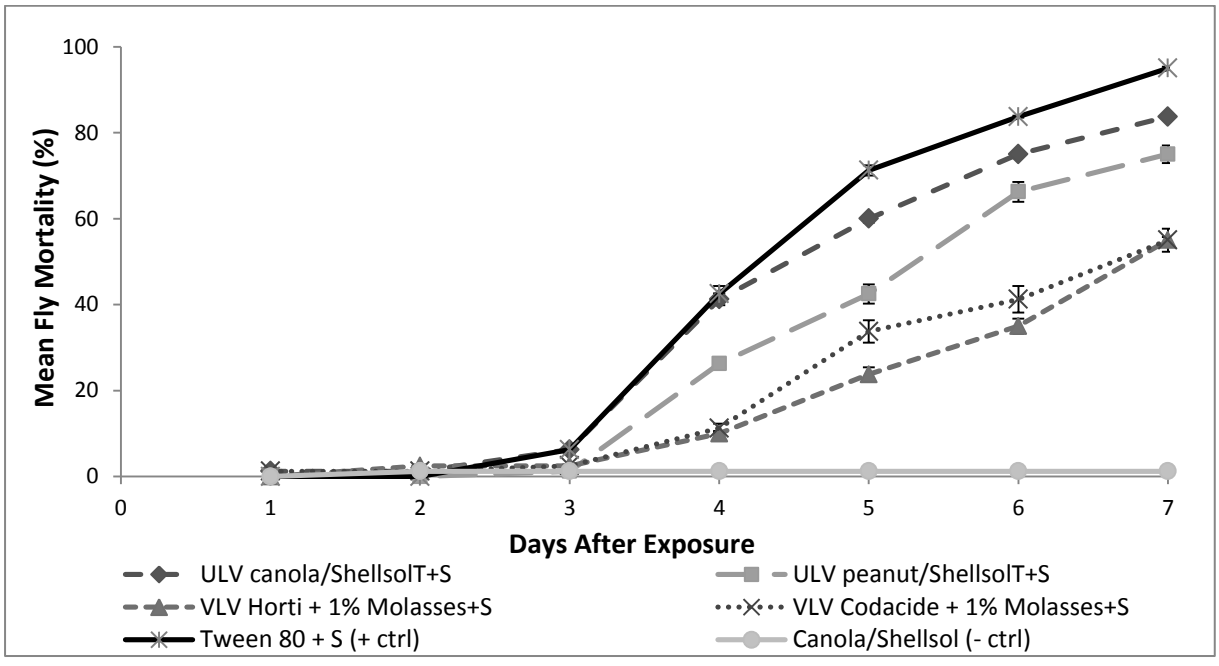

Figure 16: Cumulative daily fly mortality (%) (±SEM) after exposure to two oil- based formulations (ULV Canola or Peanut/Shellsol T) and two emulsion based formulations (VLV Codacide or Horti) plus spores (+S) in a laboratory bioassay.

Fly mortality in Peanut/Shellsol T formulation was intermediate, not significantly less than the Canola/Shellsol T formulations or significantly different to the emulsion formulations. There was little difference in fly mortality between the two emulsion formulations, whose use tended to result in around half the mortality observed for the oil based formulations.

4.1.2 Field studies

4.1.2.1 Cage trials at Pinjarra Hills

Three of the four cage trials conducted provided meaningful results (trial 2; Dec and trials 3 & 4; Feb). In trial 1 experimental methodologies were still being developed and as a consequence, little data was obtained. This trial was therefore excluded from the analyses (Table 7).

Table 7: Summary statistics for each of the three cage trials whose data was used for analysis

Trial	Number of flies per trial	Control mortality (%)	Whole experiment mortality (%)	No. spores applied per plant
2	2208	22	33	Oil/ Shellsol T: 2.3 x10^9 Emulsions: 1 x 10^9
3	3274	8	31	1 x 10^9
4	3086	8	12	1 x 10^9

4.1.2.1.1 Fly mortality

Results from trials 2, 3 and 4 showed that there was a significant difference between the formulations in their ability to infect and kill flies. Infection rates and mortality rates were generally higher for the oil/ Shellsol formulations than the emulsion formulations, matching results from the laboratory studies. Mortality recorded for the emulsion formulations was generally half to a third of that recorded for the oil/ Shellsol formulation across all three cage experiments.

In terms of overall fly mortality, the oil/Shellsol formulations performed significantly better than the rest in trial 2 and Canola/Shellsol was significantly better than the rest in trial 3 (Table 8). There was little difference between the two oil/ Shellsol formulations in trial 4 although Peanut/Shellsol was significantly better than the emulsions. Overall fly mortality was lower in trial 4 which reduced the differences between the formulations. In all three trials the fly mortality rates observed for emulsion formulations were not significantly different from one another.

Across the three trials, higher fly mortalities were generally observed after exposure to the oil/ Shellsol formulations than the emulsions formulations with results suggesting Canola oil as the best oil carrier.

Table 8: Ranked means for each formulation for fly mortality 7 days post exposure.

Ranking (H to L)	Trial 2	Mean (%)	Trial 3	Mean (%)	Trial 4	Mean (%)
1	Canola/Shellsol	49.0[a]	Canola/Shellsol	48.4[a]	Peanut/Shellsol	16.9[a]
2	Peanut/Shellsol	45.4[a]	Peanut/Shellsol	36.5[b]	Canola/Shellsol	15.6[ab]
3	Horti Oil	30.2[b]	Codacide	30.0[b]	Codacide	10.9[bc]
4	Codacide	23.0[b]	Horti Oil	29.4[b]	Horti Oil	10.7[bc]
5	Negative Ctrl	20.9[b]	Negative Ctrl	8.4[c]	Negative Ctrl	6.8[c]

N.B. means within columns with the same superscript following are not significantly different at the P=0.05 level.

4.1.2.1.2 Fly Infection

The rate of *M. anisopliae* infection in flies mirrored the mortality rates suggesting a direct link between infection with the fungus and death of the flies.

In trial 2 the fungal infection rates in flies exposed to the Canola/ Shellsol formulation were significantly higher than all the other formulations (Table 9). The higher level of infection in trial 2 may have been because twice the number of spores was applied per plant in the oil-based formulations compared to the emulsion formulations. However, an equivalent dose of spores was applied in both oil/ Shellsol formulations suggesting that canola oil is a more efficacious carrier than peanut oil. Infection rates in the peanut oil/Shellsol formulation did not differ significantly from the emulsion formulations.

In trials 3 and 4 infection rates were significantly higher in flies exposed to the Canola/Shellsol formulations than the emulsion formulations. Infection rates in flies exposed to the Peanut/ Shellsol formulation were also significantly higher than the emulsions in trial 4.

The general trend across all three trials was for higher infection rates following exposure to the oil/Shellsol formulations than with emulsion formulations, where 20-30% lower infection levels were observed. The best infection levels were observed for the Canola/Shellsol formulation.

Table 9: Ranked mean infection rates in flies exposed to four *M. anisopliae* formulations.

Ranking (H to L)	Trial 2	Mean (%)	Trial 3	Mean (%)	Trial 4	Mean (%)
1	Canola/Shellsol	41.5[a]	Canola/Shellsol	40.1[a]	Peanut/Shellsol	9.6[a]
2	Peanut/Shellsol	27.9[b]	Peanut/Shellsol	37.4[ab]	Canola/Shellsol	9.2[a]
3	Horti Oil	19.1[bc]	Horti Oil	29.9[bc]	Horti Oil	5.6[b]
4	Codacide	16.1[c]	Codacide	28.2[c]	Codacide	3.5[b]
5	Negative Ctrl	0[d]	Negative Ctrl	15[d]	Negative Ctrl	0[c]

N.B means within columns with the same superscript following are not significantly different at the P=0.05 level.

4.1.2.1.3 Spore deposition

In trial 2 approximately twice the number of spores was applied/ plant in the oil/ shellsol formulations than in the emulsion formulations (Table 10). This was because the concentration of spores was found to be too high for the minimum volume able to be applied

per plant with the hand held sprayer. In subsequent trials (3 and 4) the concentration of spores in the oil/ Shellsol formulations was reduced to allow an equivalent dose of spores to be applied per plant for all the formulations.

In trial 3 and 4 where the spore dose / plant was equivalent for all four formulations, there was little difference between the treatments in spore deposition. This indicates that our method of application was consistent for each formulation. In trial 3 the Canola/Shellsol treatment had the least number of spores per plant despite having a high infection percentage and high rate of infection. The method of sampling leaves may have contributed to these results. Spores were only obtained from a 2 cm section taken from each of 4 randomly sampled leaves/ plant. A better indication of spore deposition may have been achieved by taking a larger sample such as 10-20 leaves /plant.

Table 10: Ranked means showing Log10 of *M. anisopliae* spores washed from leaf samples from treated plants as an estimate of spore deposition.

Ranking (H to L)	Trial 1	Log10 spores	Trial 2	Log 10 spores	Trial 3	Log 10 spores	Trial 4	Log 10 spores
1	Peanut/ Shellsol	2.2^{a}	Canola/ Shellsol	2.6^{a}	Horti Oil	2.4^{a}	Codacide	2.5^{a}
2	Horti Oil	1.8^{ab}	Peanut/ Shellsol	2.2^{b}	Codacide	2.1^{a}	Peanut/ Shellsol	2.5^{a}
3	Codacide	1.4^{b}	Horti Oil	2.2^{b}	Peanut/ Shellsol	2.1^{a}	Horti Oil	2.5^{a}
4	Canola/ Shellsol	0.8^{c}	Codacide	1.5^{c}	Canola/ Shellsol	1.7^{b}	Canola/ Shellsol	2.5^{a}
5	Negative Ctrl	0^{d}	Negative Ctrl	0^{d}	Negative Ctrl	0^{c}	Negative Ctrl	0.3^{b}

N.B. means within columns with the same superscript following are not significantly different at the P=0.05 level.

4.1.2.2 Pilot field trial in commercial feedlots

In this experiment a limited area (350 m x 5 m) in each of two feedlots was treated with a Canola oil/Shellsol T *M. anisopliae* formulation. The formulation was applied once a week for four weeks using controlled droplet application at an Ultra Low Volume rate. Estimates of spore deposition, fungal infection rates in flies and fly mortality were made over the 4 weeks of spraying (Table 11)

Metarhizium does occur naturally in the environment as shown by the spores detected on vegetation at the control feedlot throughout the spray trial and in the treated feedlots prior to the first spray. This would include numerous wild type strains which may vary in their virulence and specificity for different insects. Along the perimeter of the feedlot background levels appeared to be in the order of hundreds of spores per gram of fresh vegetation Random samples of vegetation taken within the test zones of the treated and untreated control feedlots demonstrated that spraying with *M. anisopliae* caused a 1000 fold increase in spores/ g of vegetation immediately after spraying with a 100 fold higher numbers of viable spores still being present in the test area a week later. Although the treated feedlots were sprayed weekly for 4 weeks there did not seem to be an accumulation of viable spores, but live spores persisted in the environment at higher than background levels for at least two weeks after spraying finished.

There were also significantly higher infection rates in flies captured 30 minutes post-spray than in those captured just prior to spraying. The effect of spraying on the mortality of sampled flies was just outside the P=0.05 level of significance. Greater replication or spraying over a larger area may give increased power to make comparisons between treatments. Approximately 50% of flies netted after spraying were infected with *Metarhzium* with approximately 40% of the sampled flies dying within 6 days. Of the flies that died, 75% were infected with the fungus. More flies may have died from *Metarhzium* infection if they had been incubated longer as around 20% of the live flies were also found to be infected.

Although the numbers of viable spores in the treated feedlots were approximately 100 fold greater than in the control feedlot one to two weeks post-spraying, this did not equate to significantly higher infection and mortality in the flies sampled immediately prior to spraying. The treated feedlots sampled pre-spray were not significantly different in infection rates and fly mortalities to the control untreated feedlot (P>0.05).

The number of spots per card and the visual fly scores within each test zone were extremely variable in the first three weeks of the trial. No significant effect of spraying on fly populations within experimental areas of the treated feedlots could be detected. Fly numbers generally declined in the last 3 weeks of monitoring in both the treated and the control feedlots probably as a result of the seasonal decrease in overnight temperatures.

Table 11: F-probabilities and the comparison of means for feedlots pre and post spraying during the pilot trial

Treatment Means	Spore Deposition (Log10 spores)	Total Infection (%)	Infection in Dead Flies (%)	Infection in Live Flies (%)	Fly Mortality (%)
F probability	0.001	0.007	0.028	0.011	0.067
Control	2.1^{a} ± 0.06	2.2^{a} ± 2.91	2.4^{a} ± 9.05	0.44^{a} ± 1.49	15.7^{a} ± 5.17
Post Spray	5.4^{c} ± 0.05	48.5^{b} ± 2.06	76^{b} ± 6.40	19.04^{b} ± 1.05	41.6^{b} ± 3.66
1 Week Post Spray	4.1^{b} ± 0.05	3.8^{a} ± 2.06	10.7^{a} ± 6.40	0.7^{a} ± 1.05	17.2^{a} ± 3.66

N.B. Means within columns with the same superscript are not significantly different at the P=0.05 level.

4.1.2.3 Field trial in commercial feedlots

4.1.2.3.1 Fly monitoring

Fly monitoring with the alsynite traps in the first two seasons indicated that *M. domestica* was overwhelmingly the most abundant species on the monitored feedlots comprising 82% overall of all flies caught compared to 13% stable flies and 5% bush flies (Fig. 17).

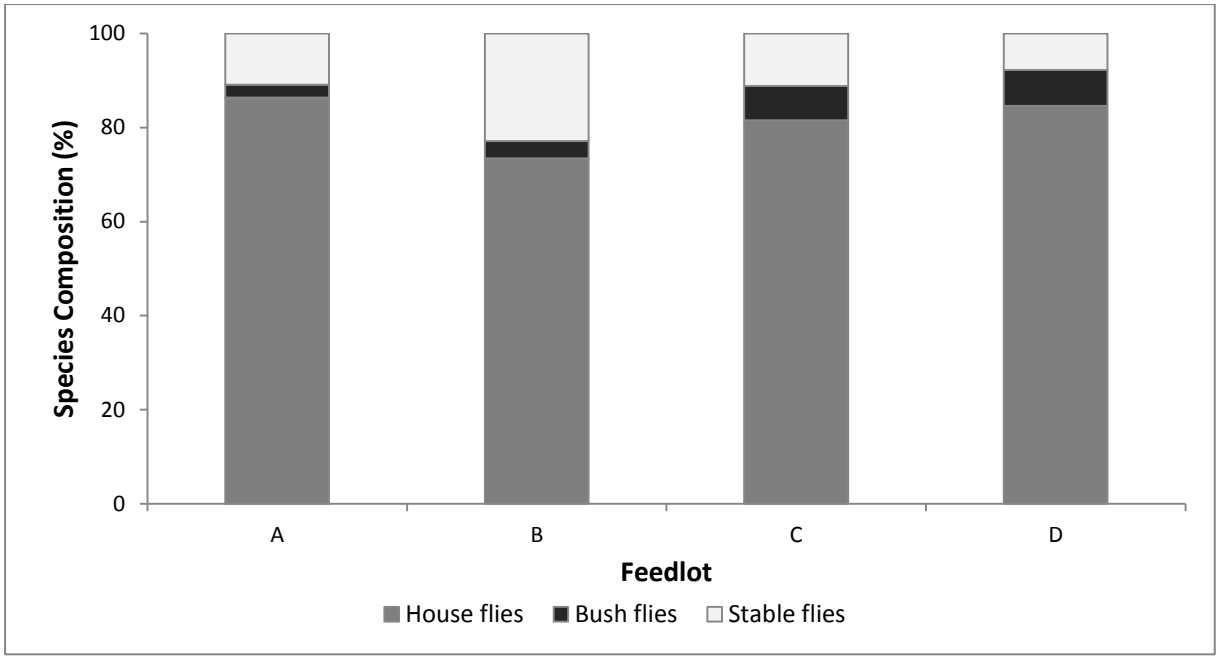

Fig 17: Species composition of fly populations measured using alsynite trips over two years on the test feedlots

Higher numbers of stable flies were trapped on feedlot B than on the other feedlots and this is thought to be due to the closer proximity of alsynite trap sites to the feed mill area on this site than on the other properties. Bush flies were trapped mainly early in the fly season (November and December) and numbers were higher on Feedlots C and D which had extensive cattle grazing pastures nearby than on Feedlots A and B which were mainly surrounded by cropping areas. The fly count data was variable but patterns assessed by the different measurement methods over the three seasons were in general agreement (Fig. 18). There was significant correlation between fly numbers as assessed by the three methods used in all three seasons (via spot cards, sticky cards and visual assessment) when assessed within farm (Table 12). In particular, the correlation between visual scores and spot card values where at least 12 sites were monitored on each property in each year were 0.6 or above on all except property D where the correlation was 0.57 (Table 12). The correlation of values from alsynite traps with those from the other methods of measurement was generally lower, which was unsurprising given that there were only two alsynite traps on each farm.

Table 12: Correlation (r values) amongst fly numbers assessed by spot cards, sticky cards, visual assessments and alsynite traps across sites within feedlots

Feedlot A				
	Spot Card	0.2379		
	Sticky Card	0.2959	0.6518	
	Visual	0.1257	0.6483	0.4331
		Alsynite	Spot Card	Sticky Card
Feedlot B				
	Spot Card	0.3579		
	Sticky Card	0.3545	0.5206	
	Visual	0.4173	0.6009	0.4148
		Alsynite	Spot Card	Sticky Card
Feedlot C				
	Spot Card	0.0721		
	Sticky Card	0.2905	0.7001	
	Visual	0.0472	0.6019	0.5821
		Alsynite	Spot Card	Sticky Card
Feedlot D				
	Spot Card	0.3523		
	Sticky Card	0.6349	0.4938	
	Visual	0.3964	0.5704	0.4105
		Alsynite	Spot Card	Sticky Card

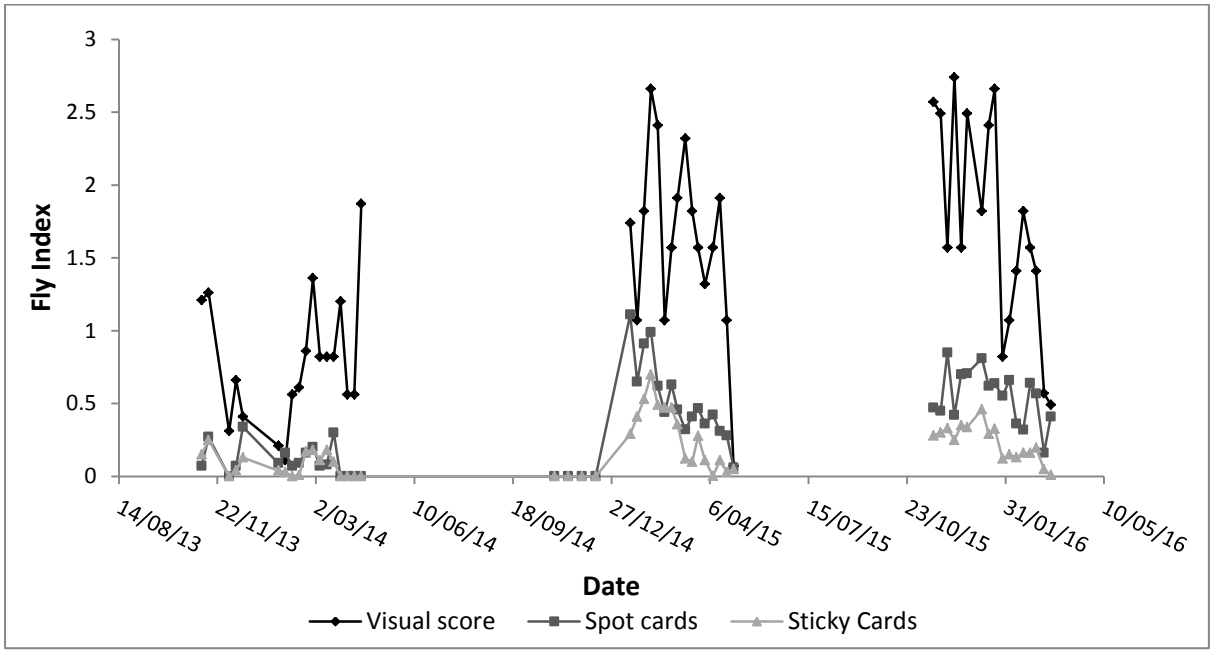

Figure 18: Pattern in fly counts as assessed by the different monitoring methods over the three seasons. (Spot card and sticky card catches are represented as $\log_{10}(x+1)$ transformed counts.)

The patterns of fly numbers in years 1 and 2 on the four feedlots as assessed by the fly index and following fitting the model predicted smoothing splines for fly index over time are shown in Figures 19 and 20. In season 1 as there were no records of animal numbers the model only had terms for the spline of time and feedlot. In year 2, animal numbers were

available but had no significant effect and this term was dropped from the model, leaving terms for the spline of time, feedlot and the pilot spray treatment. There was no evidence of curvature from the final model and no evidence that the pilot spray treatment had any effect on fly numbers (probability values of 0.17, 0.39, 0.12, 0.81 and 0.36 for the visual scores, spot-cards, stickies, alsynite traps and fly index respectively). This was not unexpected given that only relatively small areas were sprayed on each of the test feedlots in this year.

Whilst there were some notable differences between the feedlots over the seasons, there was also a degree of consistency. Using data from across all seasons is expected to give the best overall standardisation of the feedlot differences. Although there was high variability, the general pattern in fly numbers in both years was similar at all four feedlots (Fig. 19 and Fig. 20). The average values for each of the measured variables within each season and across all seasons are shown in Table 13. Overall fly numbers at feedlots A and B tended to be lower than at feedlots C and D.

Table 13: Mean values for different measures of fly abundance within season and across all seasons

	Fly Index	Visual scores	log10 (spot)	log10 (sticky)	log10 (alsynite)
Season 1					
Feedlot A	0.84^{ab}	0.81^{a}	0.16^{a}	0.13^{a}	2.38^{b}
Feedlot B	0.82^{a}	0.78^{a}	0.19^{a}	0.14^{a}	2.14^{ab}
Feedlot C	0.94^{bc}	1.29^{b}	0.22^{a}	0.34^{b}	1.90^{a}
Feedlot D	1.02^{c}	1.60^{c}	0.37^{b}	0.28^{b}	1.88^{a}
Season 2					
Feedlot A	1.12^{ab}	$1.58a^{b}$	0.52^{ab}	0.29^{ab}	1.84^{b}
Feedlot B	1.24^{b}	1.99^{b}	0.62^{b}	0.26^{a}	1.88^{b}
Feedlot C	0.97^{a}	1.38^{a}	0.45^{a}	0.38^{b}	1.57^{a}
Feedlot D	1.13^{ab}	1.98^{b}	0.58^{b}	0.31^{ab}	1.47^{a}
Season 3					
Feedlot A	1.19^{a}	1.82^{a}	0.54^{a}	0.26^{a}	
Feedlot B	1.36^{b}	2.20^{ab}	0.65^{ab}	0.28^{a}	
Feedlot C	1.50^{c}	2.38^{bc}	0.69^{b}	0.49^{b}	
Feedlot D	1.58^{c}	2.52^{c}	0.87^{c}	0.41^{b}	
All seasons					
Feedlot A	1.06^{a}	1.38^{a}	0.42^{a}	0.23^{a}	
Feedlot B	1.14^{ab}	1.59^{b}	0.49^{b}	0.24^{a}	
Feedlot C	1.16^{b}	1.68^{b}	0.47^{ab}	0.40^{c}	
Feedlot D	1.25^{c}	2.00^{c}	0.61^{c}	0.34^{b}	

N.B. Means within columns with the same superscript are not significantly different at the P=0.05 level.

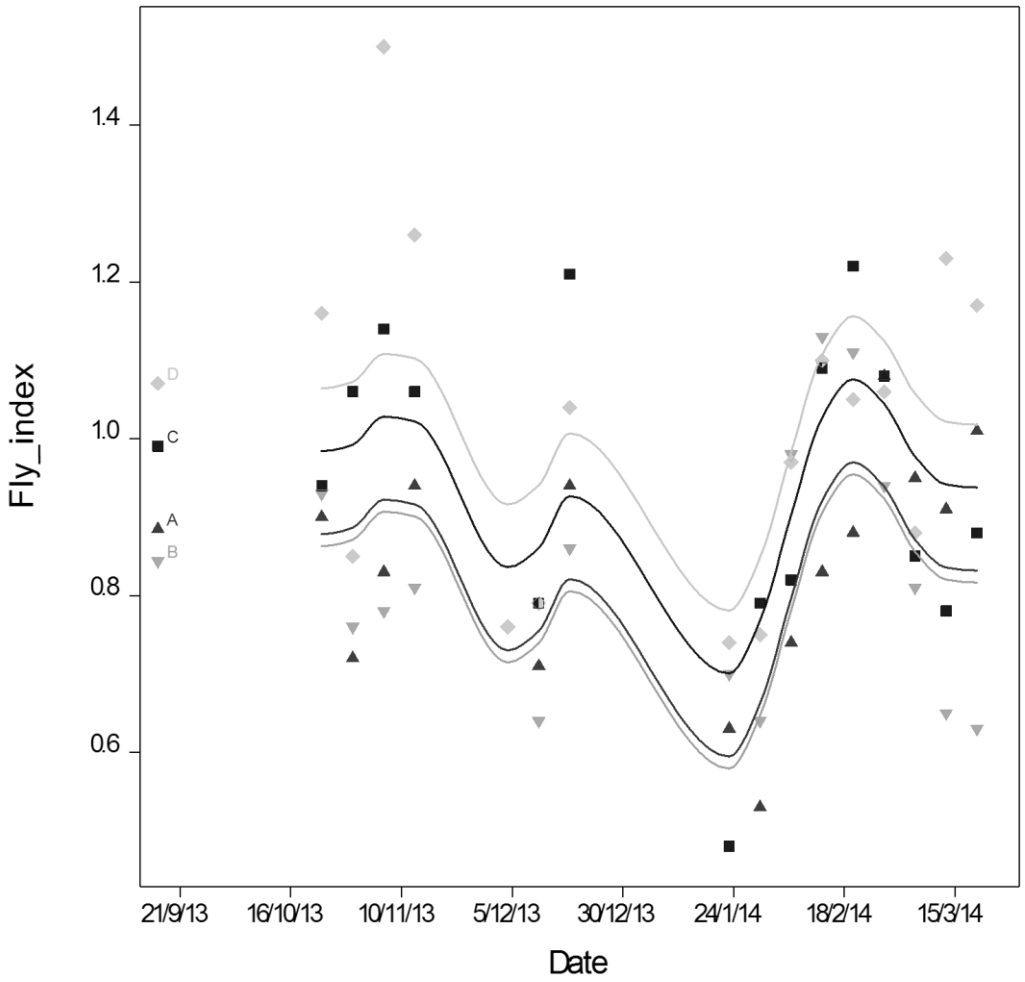

Figure 19: Fly numbers (fly index) and the fitted splines for patterns in fly numbers on the four feedlots (A, B, C and D) in season 1

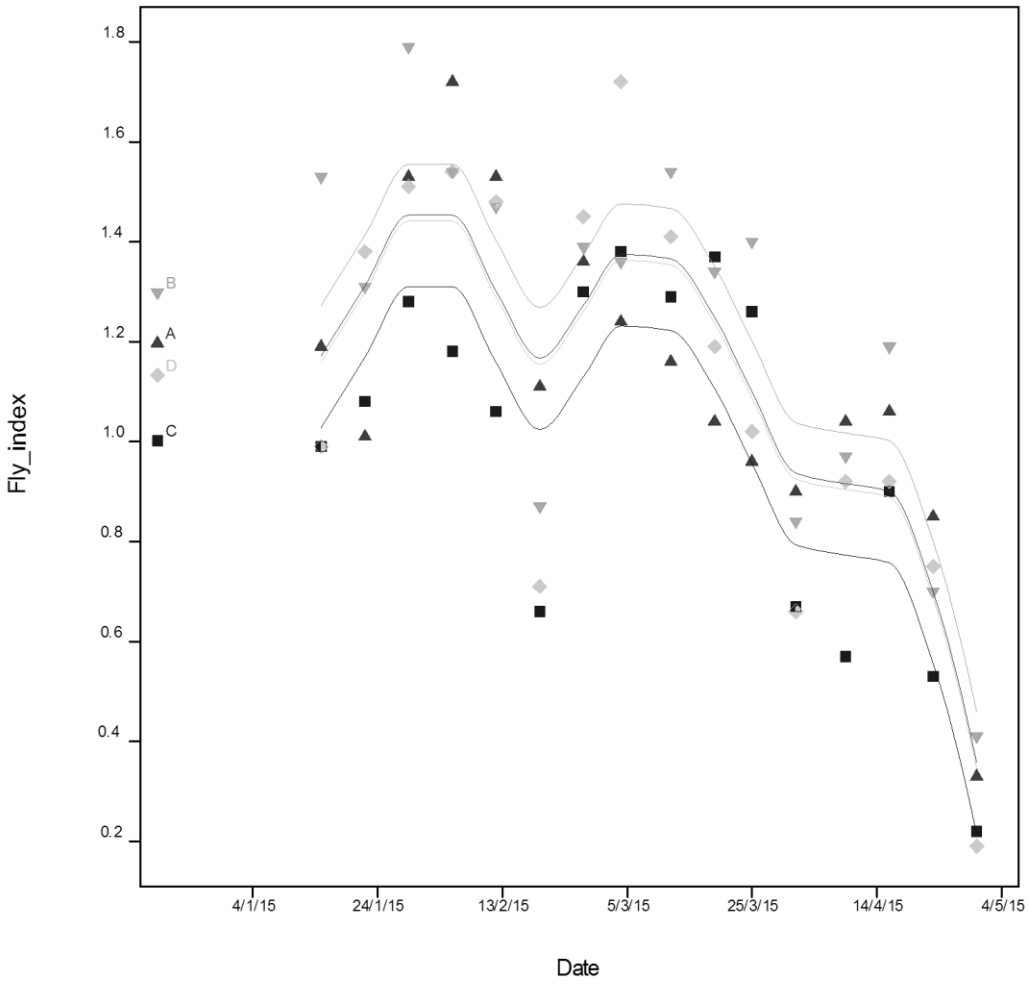

Figure 20: Fly numbers (fly index) and the fitted splines for patterns in fly numbers on the four feedlots (A, B, C and D) in season 2

The pattern of fly numbers in season 3 is shown in Figure 21. Feedlots C and D had the highest fly numbers, as in the previous years, but there was a larger separation between the splines for these two feedlots with Feedlot D, the unsprayed feedlot, having clearly higher fly numbers than Feedlot C. Similarly, in the other paired feedlots, Feedlot B the unsprayed feedlot had higher fly numbers than Feedlot A, which had mycopesticide applied.

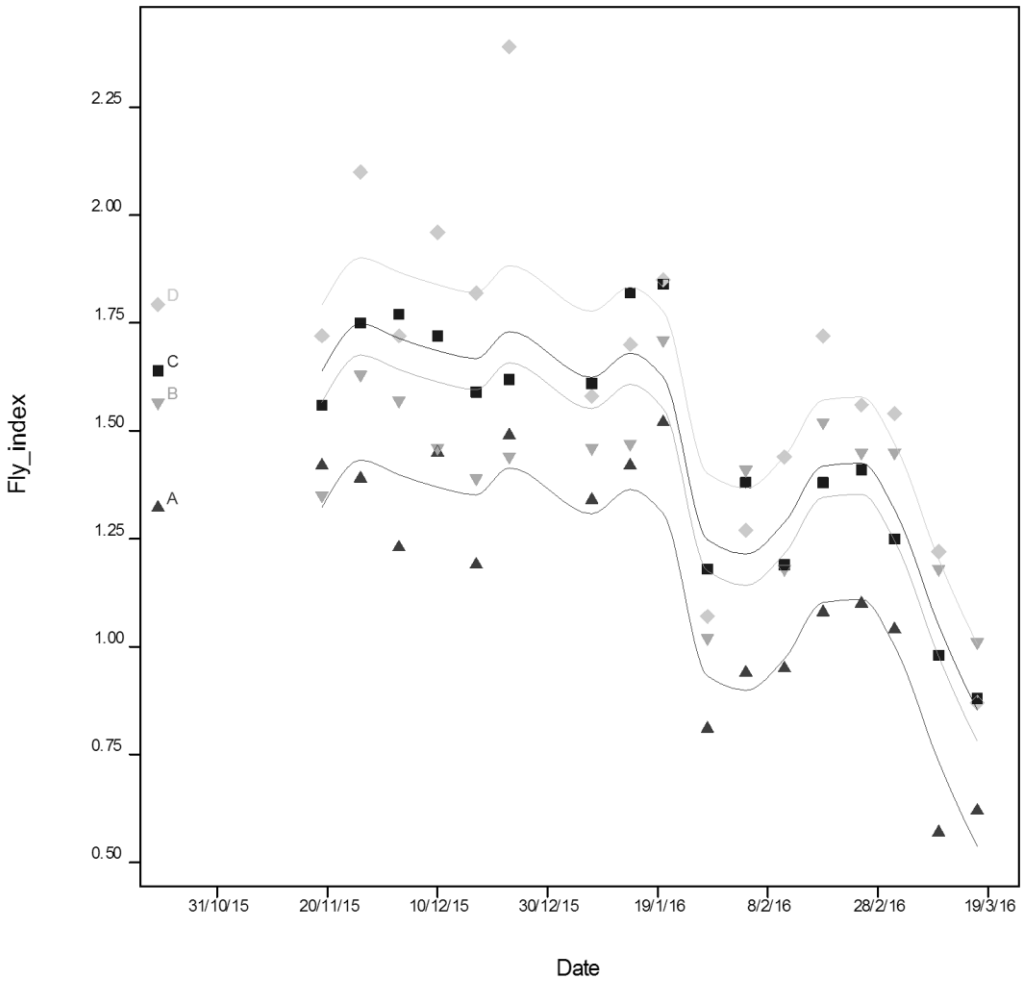

Figure 21: Fly numbers (fly index) and the fitted splines for patterns in fly numbers on the four feedlots (A, B, C and D) in season 3

The analysis to examine the effect of spraying used the “unsprayed data” from years 1 and 2 as well as data from the three monitorings prior to spraying in year 3 to characterise and standardise the intrinsic differences between feedlots and to provide a baseline against which to assess the effects of spraying. Two analyses were conducted, the first using only the results of the three monitorings before spraying in year 3 as a basis for comparison and the second using the combined results of ‘unsprayed’ monitoring from years 1, 2 and 3.

Using just the data from the unsprayed monitorings in year 3 in the analysis showed some evidence of an effect , with reductions 13.3%, 20.6%, 0% and 30.1% for visual scores, spot-cards, stickies and fly index respectively, but these results were not significant (p= 0.24, 0.16, 0.67 and 0.16). However, the sensitivity of the analyses for these measures were reduced by only having three sampling dates before spraying to benchmark the differences between the feedlots.

More powerful and appropriate tests come from using the data from all three seasons, as the intrinsic differences in unsprayed fly populations are better estimated. Summarising the effects over the period of spraying (Table x) the estimated reductions from spraying as measured by fly index, visual cards and sticky cards were 20.5%, 23.3% and 31.0% respectively (p= 0.002, .047, 0.004). When fly numbers were assessed using spot cards the

trend was the same with a predicted 9.3% decrease in fly numbers, but the effect was not significant (P>0.05).

Although there was considerable variability in the data, the results also showed a significant linear accumulative effect of spraying on fly numbers as assessed by three of the four fly assessment methods (fly index, visual scores and sticky card counts (Table 14).

Table 14: Summary statistics for the final analysis of the effect of spraying on the four measures of fly abundance

	Fly Index	Visual scores	log10 (spot)	log10 (sticky)
R^2 (%)	**73.9**	**67.6**	**73.1**	**58.3**
***Spray effect* -**				
P-value	0.002	0.047	0.485	0.004
Coefficient	-0.0185	-0.0303	-0.00325	-0.01239
s.e.	0.0059	0.0152	0.00464	0.00421

This trend, summarised by averaging the two untreated feedlots (Feedlots A and C) and the two treated feedlots, is shown in Figure 22. Whilst the data is quite variable the results show an accumulative reduction in fly numbers from the effects of spraying and confirm the appropriateness of the linear model adopted for the analysis.

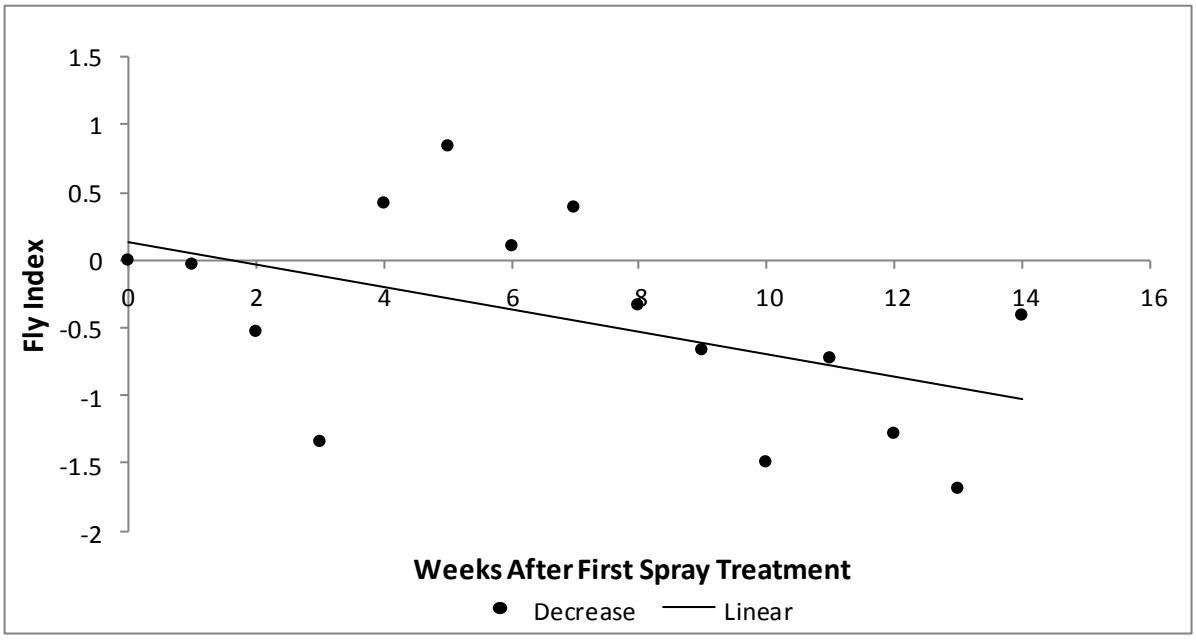

Figure 22: Mean decrease in fly index across the treated feedlots (A and C) during the season 3 field trial

4.1.2.3.2 Fly infection and vegetation sampling

The spraying of formulation was found to have significant effect on the amount of *M. anisopliae* infection and mortality within the sampled fly populations (p<0.001). Infection in flies sampled 30 minutes post-spraying that died within the 7 day incubation period averaged at 73.4%. Infection in live flies sampled 30 minutes post spraying was significant however at a much lower level, averaging 3.5% infection. No significant level of infection was found

within the sampled fly populations after a week or more post-spray. The mortality rate of flies sampled 30 mins post-spray and held for a 7 day incubation period averaged at 43.7% ($p<0.001$) (Table 15).

Table 15: F-probabilities and the comparison of means for feedlots pre and post spraying during the field trial

Treatment Means	Spore Deposition (Log10 spores)	Total Infection (%)	Infection in Dead Flies (%)	Infection in Live Flies (%)	Fly Mortality (%)
F probability	<0.001	<0.001	<0.001	<0.001	<0.001
Control	$1.3^{a} \pm 0.12$	$0.7^{a} \pm 0.15$	$1.9^{a} \pm 0.48$	$0.0^{a} \pm 0.00$	$16.0^{a} \pm 1.00$
Post Spray	$4.6^{c} \pm 0.15$	$41.9^{b} \pm 1.00$	$73.4^{b} \pm 1.33$	$3.5^{b} \pm 0.56$	$43.7^{b} \pm 2.17$
1 Week Post Spray	$3.4^{c} \pm 0.23$	$1.1^{a} \pm 0.43$	$4.8^{a} \pm 2.01$	$0.0^{a} \pm 0.02$	$21.0^{a} \pm 4.49$
2 Weeks Post Spray	$2.2^{b} \pm 0.16$	$0.3^{a} \pm 0.15$	$1.00^{a} \pm 0.54$	$0.0^{a} \pm 0.00$	$17.2^{a} \pm 1.74$
3 Weeks Post Spray	$2.0^{ab} \pm 0.39$	$0.9^{a} \pm 0.63$	$3.6^{a} \pm 2.77$	$0.0^{a} \pm 0.00$	$16.4^{a} \pm 4.52$

N.B. Means within columns with the same superscript are not significantly different at the P=0.05 level.

Vegetation sampling showed there was a background level of *M. anisopliae* spores in feedlots of approximately 22 spores per gram of vegetation. After spraying the level of spores detected increased one thousand fold to approximately 23,000 spores per gram of vegetation. However this level gradually decreased over time so that after 3 weeks the level was approaching the level found in the control feedlots (Fig. 23) (Table 15).

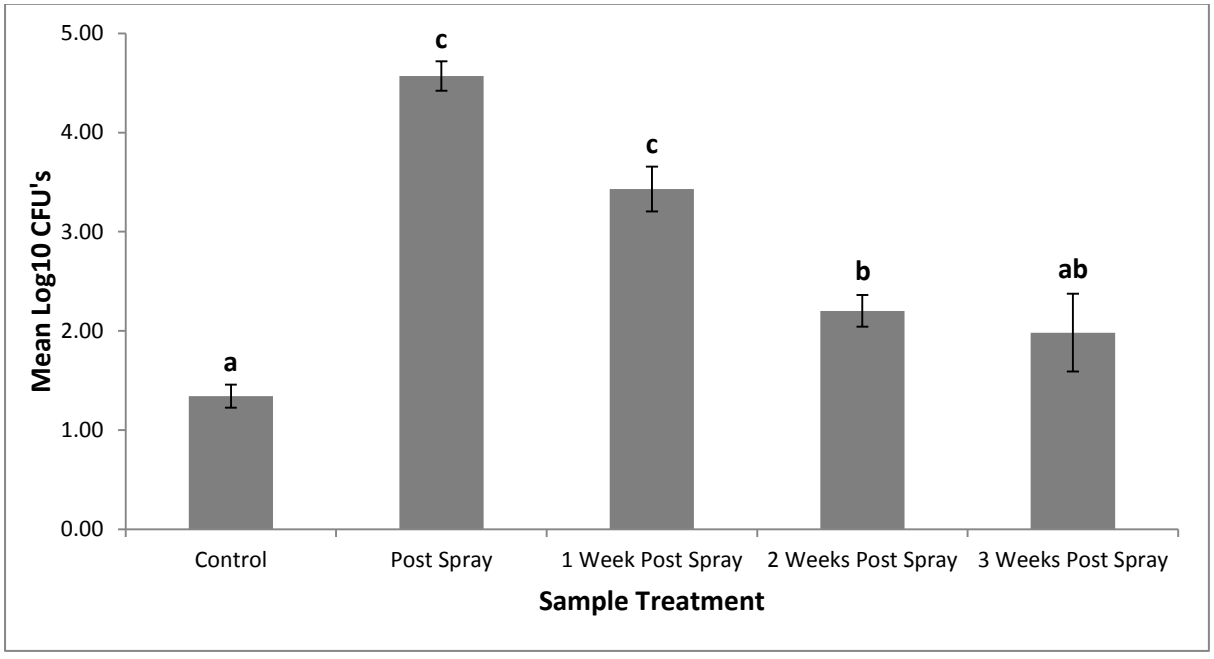

Figure 23: Mean log10 CFU's (±SEM) across sample treatments within the field trial. Constants represent values obtained from post-hoc analysis (Fisher's Protected LSD's). Means of treatments with the same constant are not significantly different at the 5% level.

4.2 Bait formulation

4.2.1 Bait additives

4.2.1.1 Assay 1

Dry baits containing rice and *M. anisopliae* spores alone or incorporating raw sugar were found to be the most effective, reaching 90% - 100% mortality within six days post exposure. The average cumulative mortality for these baits was significantly higher than the control and oil based baits from the second day post-exposure onwards ($p<0.001$) (Table 16). Baits containing oil and molasses however, only reached ~50% mortality within the same period although the average cumulative mortality was higher than the control six days post-exposure onwards (Fig. 24) (Table 16).

Table 16: Mean total fly mortality (%) after 2 and 6 days post exposure to bait formulations containing a range of different additives

Treatment	Day 2 Mean Mortality (%)	Day 6 Mean Mortality (%)
Control	0.87^{a}	16.09^{a}
Rice + Spores + Oil	1.73^{a}	47.29^{c}
Rice + Spores	12.83^{bc}	84.05^{d}
Rice + Spores + Sugar L	20.67^{cd}	90.73^{de}
Rice + Spores + Sugar M	28.39^{d}	100.00^{f}
Rice + Spores + Sugar H	19.97^{cd}	98.18^{ef}
Rice + Spores + Oil + Molasses L	3.68^{ab}	42.36^{bc}
Rice + Spores + Oil + Molasses M	5.37^{ab}	37.56^{bc}
Rice + Spores + Oil + Molasses H	1.66^{a}	32.14^{b}

N.B. Means within columns with the same superscript are not significantly different at the P=0.05 level.

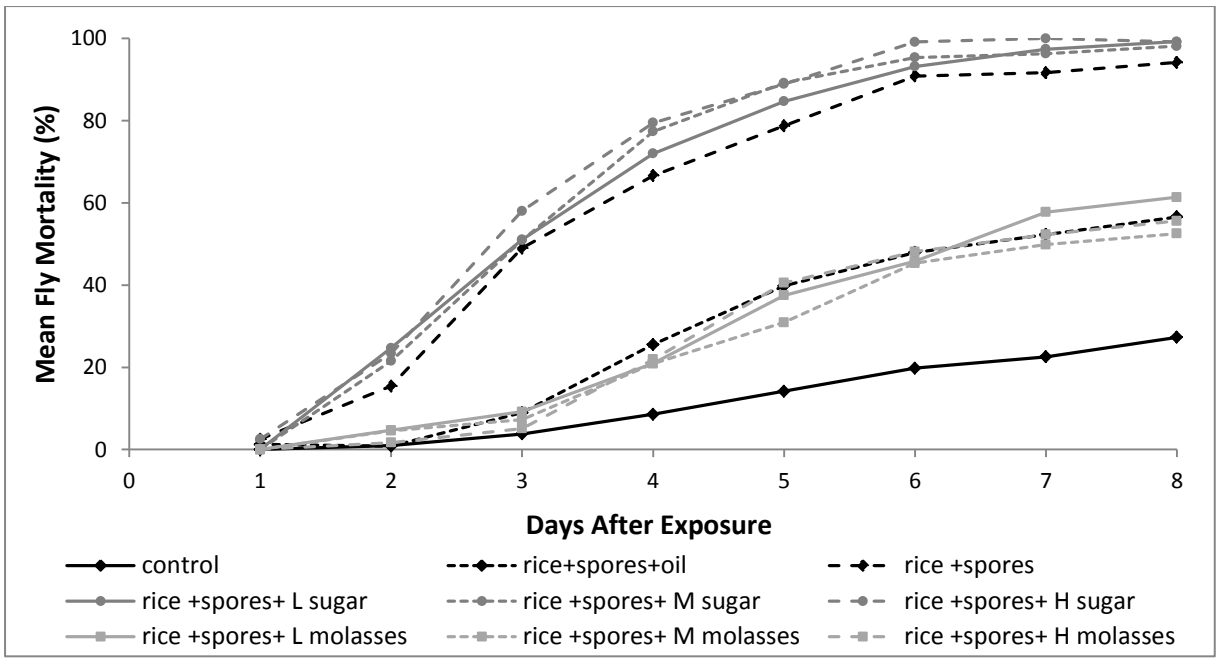

Figure 24: Mean cumulative daily fly mortality (%) after exposure to bait formulations containing a range of different additives.

4.2.1.2 Assay 2

The bait formulation containing a medium level of oil and no molasses had the highest efficacy with 34% mortality by the sixth day after exposure (p=0.019). There appeared to be no advantage to adding molasses to the bait formulation as the highest mortality achieved by a bait containing molasses was only 20% by day six (Table 17).

Table 17: Mean total fly mortality (%) after 6 days post exposure to bait formulations containing a range of different additives.

Treatment	Day 6 Mean Mortality (%)
Control	3.50[a]
Medium Oil	34.30[d]
Low Oil + Low Molasses	19.72[cd]
Low Oil + Med Molasses	12.18[abc]
Low Oil + High Molasses	5.13[ab]
Medium Oil + Low Molasses	17.33[bcd]
Medium Oil + Medium Molasses	6.00[ab]
Medium Oil + High Molasses	11.88[abc]
High Oil + Low Molasses	15.58[abc]
High Oil + Medium Molasses	9.79[abc]
High Oil + High Molasses	13.23[abc]

N.B. Means within columns with the same superscript are not significantly different at the P=0.05 level.

4.2.1.3 Assay 3

The addition of raw sugar to bait formulations containing low levels of oil had no beneficial effect on the efficacy of the bait. The dry bait formulation containing milk powder was slightly more effective than those containing oil however, rice and spores alone had the highest efficacy at 70% mortality after six days (p<0.001) (Fig. 25).

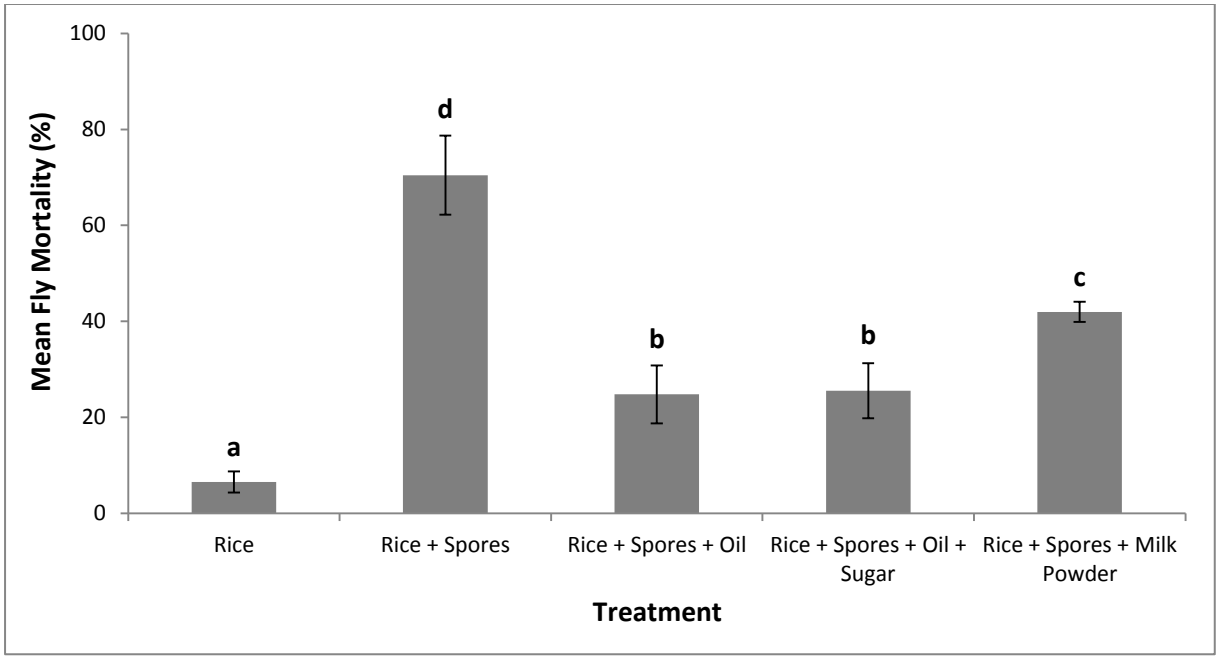

Figure 25: Mean fly mortality (%) (±SEM) six days after exposure to bait formulations (n = 50). Constants represent values obtained from post-hoc analysis (Fisher's Protected LSD's). Means of treatments with the same constant are not significantly different at the 5% level.

4.2.2 Accessory attractants

4.2.2.1 Assay 1

Molasses showed the most promise as a potential accessory attractant to the bait formulation with a mean entrapment of ~35%. The remaining treatments did trap some flies however not enough to be significantly different from the control ($p<0.001$) (Fig. 26).

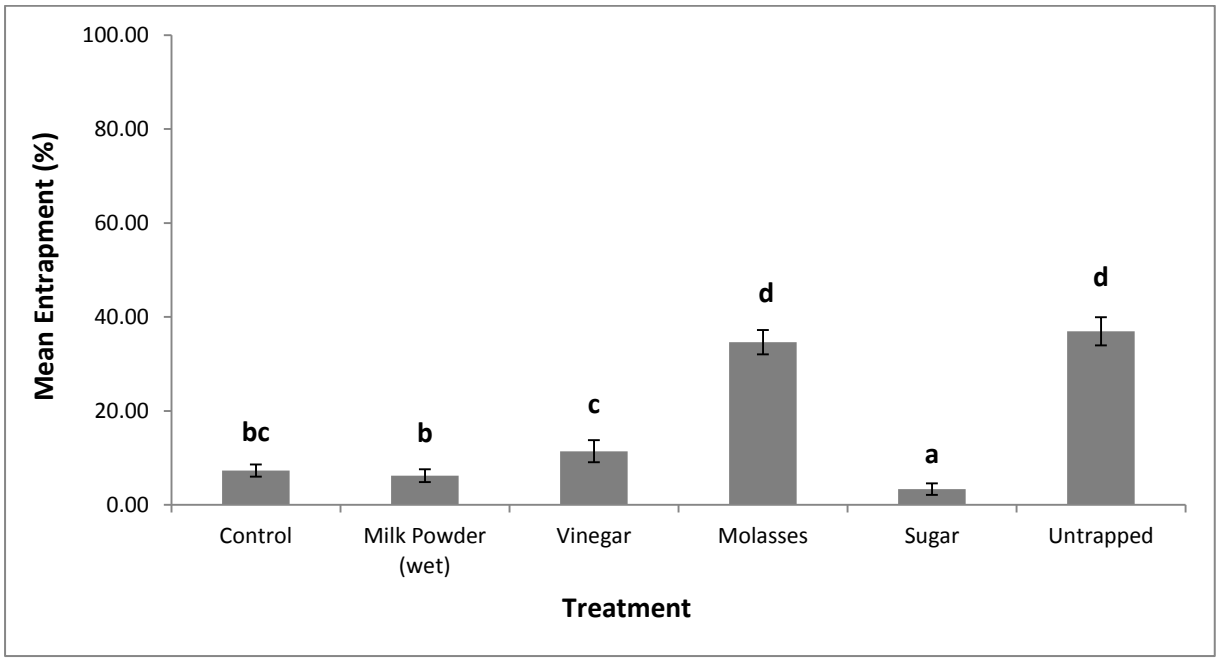

Figure 26: Mean fly entrapment (%) (±SEM) after 20 hours (n = 50). Constants represent values obtained from post-hoc analysis (Fisher's Protected LSD's). Means of treatments with the same constant are not significantly different at the P=0.05 level.

4.2.2.2 Assay 2

Dynamic Lifter® was the only potential attractant tested to show a significant level of attraction with a mean entrapment of ~21% ($p<0.001$). Molasses, identified as a reasonable attractant in Assay 1, failed to exhibit any attraction in this assay as did the commercial fly attractant containing (Z)-9-tricosene (Envirosafe™) (Fig. 27).

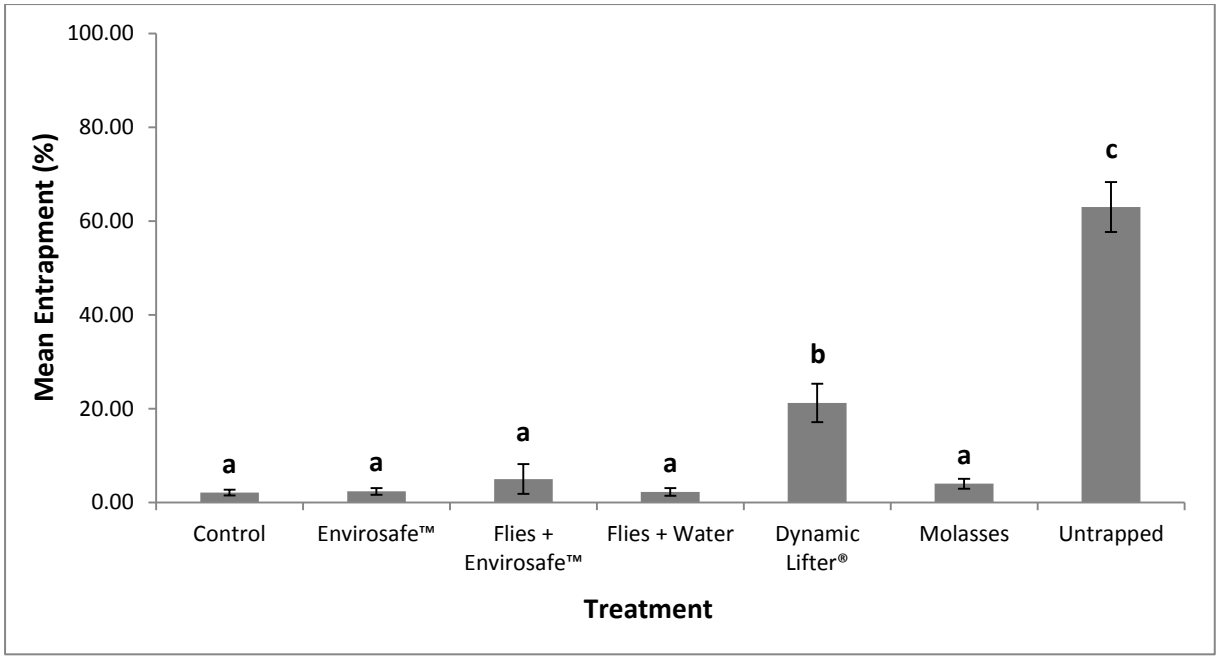

Figure 27: Mean fly entrapment (%) (±SEM) after 48 hours (n = 50). Constants represent values obtained from post-hoc analysis (Fisher's Protected LSD's). Means of treatments with the same constant are not significantly different at the 5% level.

4.2.2.3 Assay 3

The attractive qualities of Dynamic Lifter® were further confirmed in a three-choice test with an significant average fly entrapment of ~24% ($p<0.001$) (Fig. 28).

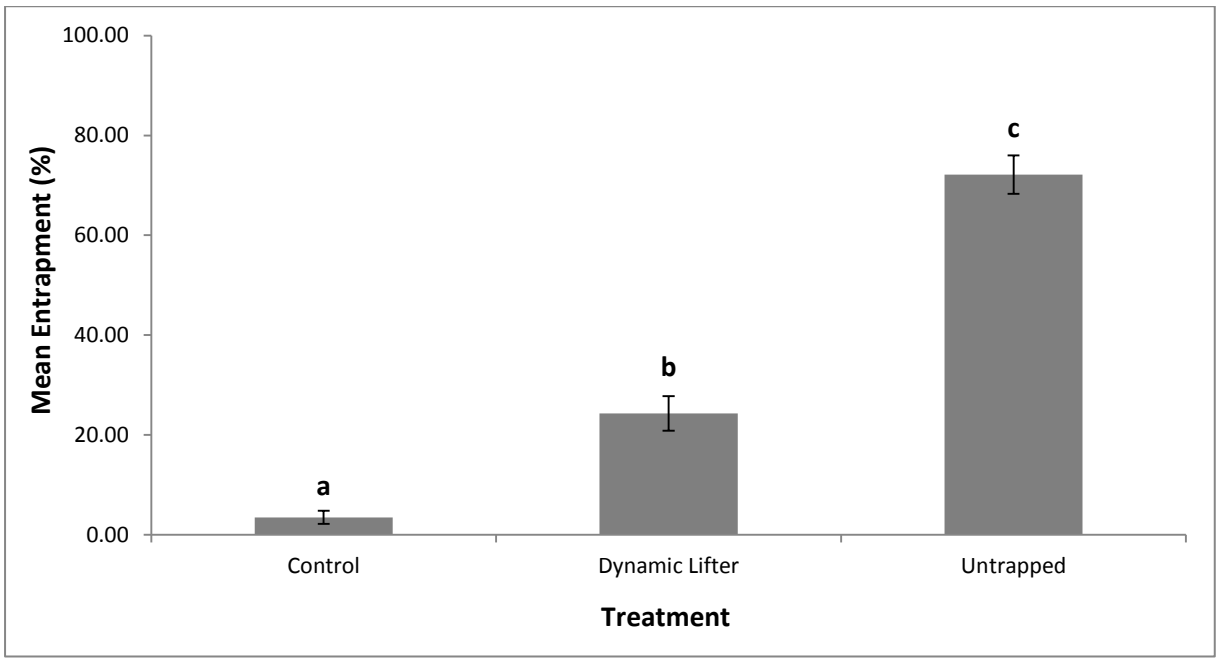

Figure 28: Mean fly entrapment (%) (±SEM) after 48 hours (n = 50). Constants represent values obtained from post-hoc analysis (Fisher's Protected LSD's). Means of treatments with the same constant are not significantly different at the 5% level.

4.2.3 Bait Efficacy

4.2.3.1 Assay 1

Exposure to low levels of natural UV radiation appeared to have no significant effect on the viability of *M. anisopliae* spores (p=0.756) (Fig. 29).

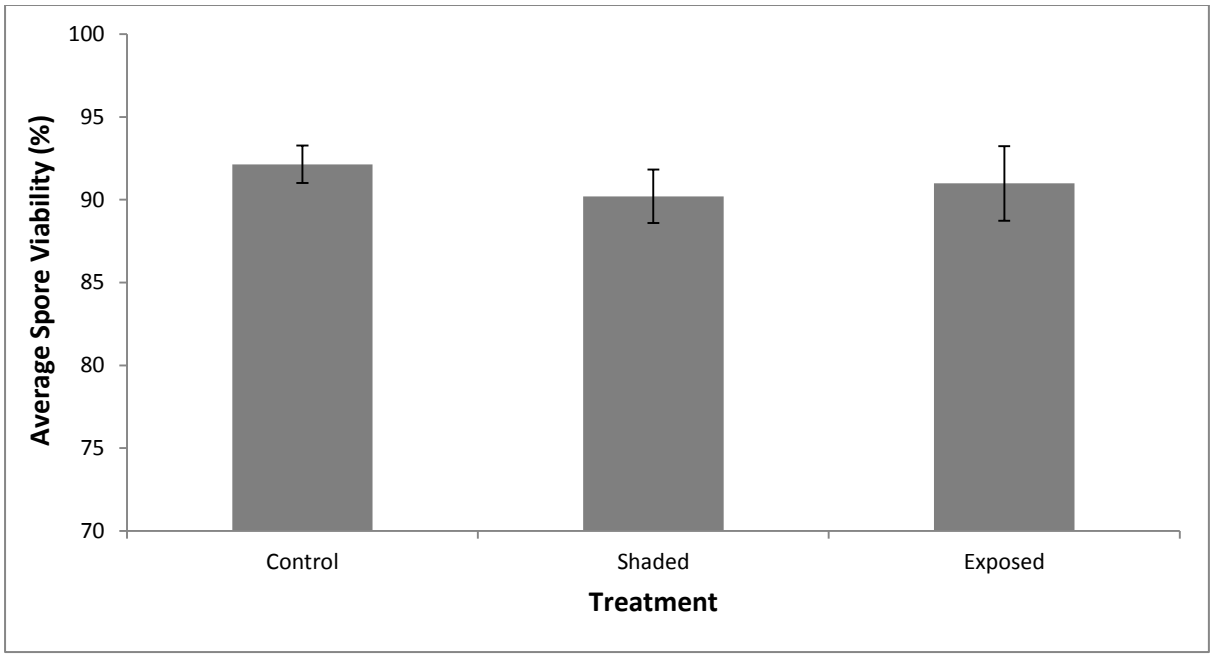

Figure 29: Mean spore viability (%) (±SEM) after 24 hours exposure to natural ultra-violet radiation. Control viability measured directly from stored *M. anisopliae* spores.

4.2.3.2 Assay 2

This assay determined that there was no benefit in using Dynamic Lifter® as an accessory attractant to the bait formulation. Mean fly mortality 7 days following a 2.5 hour exposure time was between 49% - 54% for both the bait formulation on its own and with Dynamic Lifter® as an accessory ($p<0.001$). Both of these treatments outperformed the commercial insecticide bait (Quickbayt®) at ~25% mortality (Fig. 30).

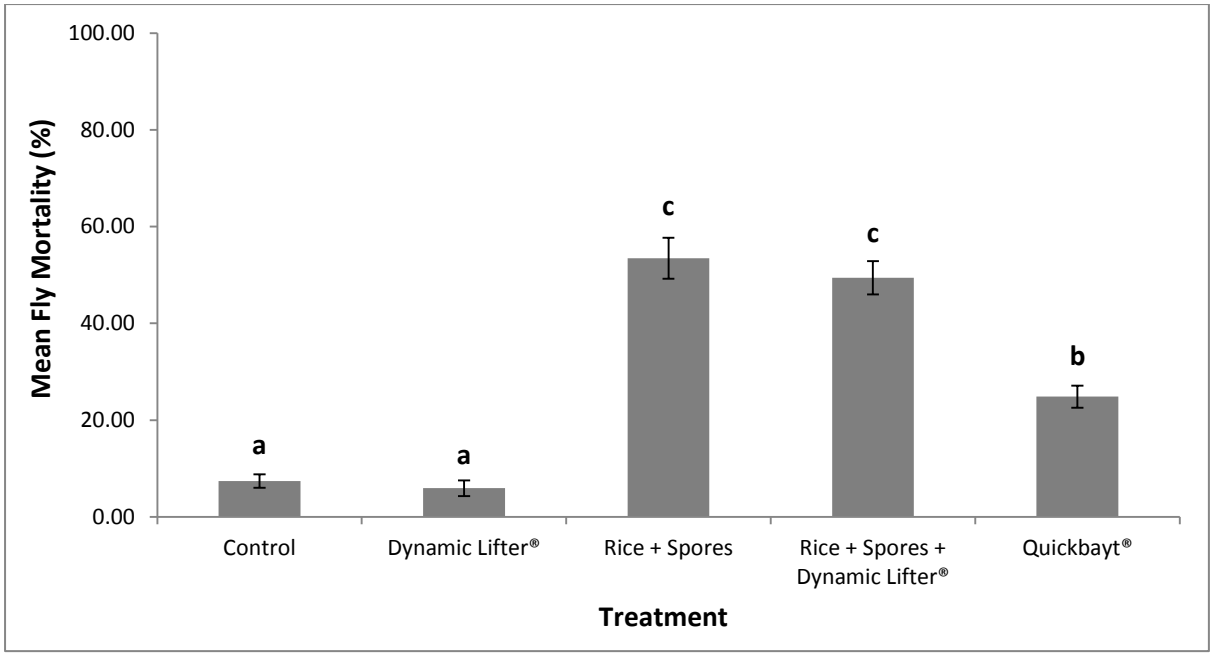

Figure 30: Mean fly mortality (%) (±SEM) 7 days post exposure to treatments (n = 100). Constants represent values obtained from post-hoc analysis (Fisher's Protected LSD's). Means of treatments with the same constant are not significantly different at the 5% level.

5 Discussion

The advantages of a fungal formulation have been well discussed. There is no known mammalian toxicity, the spores will not impact on the health of animals, are rapidly inactivated if ingested and will not cause meat residues. A review of safety data for warm blooded animals reported no toxicological or pathological symptoms when *Metarhizium* spores were applied by different methods to birds, mice, rats, guinea pigs or rabbits (Zimmerman, 1993) and the likelihood of resistance developing to biological controls is considered to be low. To date, only allergies and no other adverse medical effects have been reported in association with the production and use of *Metarhizium* biocontrol agents (Siegel & Shadduck, 1990; Zimmerman, 2007; Cook et al, 1996; Goettel et al, 2001) except for extremely rare infections in immune-compromised individuals (Vestergaard et al., 2003). The spores will be inactivated in the digestive tract if ingested by cattle and are too large to be absorbed across the epidermal barrier into animal tissues. In addition, although a wide host range increases the feasibility of effects against non-host arthropods, the spores were relatively short lived in the environment and preliminary observations suggested little impact on parasitoid wasps or other predators and parasites that help suppress fly numbers, making it well suited to use in an integrated fly control program. The adverse effects of microbial control agents such as *Metarhizium,* which are a natural part of the ecosystem, are likely to be far less than that of any chemical sprays.

This project has demonstrated that the application of a ULV spray formulation of *Metarhizium* spores caused a significant reduction in fly numbers in treated feedlots over a summer fly season. Moreover sampling of flies and vegetation demonstrated a significant increase in the number of viable *Metarhizium* spores on vegetation in treated feedlots, along with an increase in fly mortality resulting from *Metarhizium* infection. These results are

consistent with those achieved in a previous MLA project B.FLT.0326, but the improved formulation and application methodology has produced much better results despite applying significantly lower amounts of spores, increasing the cost competitiveness of the formulation. In addition, a potentially low cost, non-chemical bait for use in areas where chemicals are not favoured has been developed and shown to give results comparable to a currently registered bait formulation.

5.1 Spore Production

The strain of *Metarhizium* ultilised for these studies was M16 isolated from flies in Queensland from the DAF fungal collection housed at the EcoSciences Precint in Brisbane. It was passaged through adult *M. domestica* flies before multiplication for use in this project. Testing confirmed its superior pathogenicity to *M. domestica* and suitability for commercial production. A number of modifications were made to optimise the spore production system and the average yield of 6.5% *Metarhizium* spores per kg of substrate achieved was well within the range expected for an Australian commercial production unit (Milner, Pers. Comm.) and way above the 4.5 % yield reported in the successful LUBILOSA locust control program (Gryzwacz et al., 2014). The strain has also been shown to have effect against other veterinary pests including sheep lice, sheep blowflies, buffalo flies and cattle ticks (Leemon and Jonsson, 2012). The potential for use against a range of pest species adds to the feasibility for commercial production.

5.2 Spray Formulation Development

Whereas previous project B.FLT.0326 used high volume sprays with large droplets, judged to be poorly effective because of high runoff, this project focussed on developing effective VLV or ULV formulations. Different droplet sizes have different dispersal and impaction characteristics and smaller droplets in the size range of 10-80 µm and 30-80 µm have been determined to be optimal for flying insects and insects on foliage respectively, while droplets in the range of 40 -100 µm are optimal for foliage (Jones and Burges, 1998). The final size of a droplet reaching the target also depends on the amount of evaporation that occurs. Evaporation rate increases as droplet size increases due to increasing surface to volume ratio however, oil evaporates much less than water as a result of different vapour pressure and viscosity (Jones and Burges, 1998).

A major breakthrough for formulating fungal spores for application under dry conditions occurred when it was discovered that suspending fungal spores in some oils, rather than killing them as previously believed, actually made the spores more efficient under dry conditions (Prior et al, 1988). Oil readily wets the hydrophobic, lipophilic surfaces of insects and leaves as well as wetting the extremely hydrophobic spores of *Metarhizium,* allowing them to be easily suspended in a formulation without clumping (Burges, 1998). Therefore the application of small droplet sprays with oil as a carrier has many advantages including a high work rate enabling large areas to be treated efficiently with an economic level of formulation. A further advantage of an oil formulation is the protection of the fungal spores from desiccation and to a small degree, from degradation by ultra violet light, so that they remain viable until contact with flies which rest on the treated vegetation.

This project first investigated a series of oils suitable for both ULV and VLV spray formulations. A range of proprietary emulsifiable oils were investigated for VLV formulations

and vegetable and mineral oils were investigated for ULV formulations. The laboratory investigations looked at the effect of the oils on spore viability and fly infectivity. The capacity of the oils to suspend high levels of *Metarhizium* spores was also measured.

Overall, the ULV oil formulations performed better than the VLV emulsifiable oil formulations. Formulation stability studies showed that the VLV formulations were limited in the number of spores/ml that could be put into stable emulsion and that direct comparison of the best formulations showed that ULV had greater efficacy against flies. ULV formulations offer benefits to agricultural spray regimes such as cost-effective carrier volumes, smaller droplet size facilitating better contact with the pest and foliage, and improved environmental persistence by providing a protective oil coating to the spores (Burges 1998, Busvine 1957, Bateman et al. 1997).

In ULV studies, formulation of mineral oil with spores was significantly more effective than those with peanut or canola oil. Further assays showed that there was no difference in efficacy between the three oils when formulations included the thinning agent, Shellsol T. Germination studies, however, showed that mineral oil in Shellsol T markedly reduced spore viability by 24 hours at room temperature. Burges (1998) indicates that *M. anisopliae* spores can maintain high germination rates when stored in mineral oil, but at low temperatures. Despite formulations with peanut and canola showing no decline in spore viability at room temperature, as a precautionary measure Shellsol T was not added to the oil and spores until just before use.

Initial VLV investigations showed that the EO Cropshield had inherent toxicity to flies, but was very unstable in emulsion with spores. The other EOs had little inherent toxicity and variable stability. A benefit of VLV formulations is that the water phase enables incorporation of additives to enhance efficacy and offer spore protection (Burges, 1998). However, the milk powder and organic molasses included as feeding stimulants to increase fly attraction and encourage ingestion of the formulation provided little improvement in efficacy. There are a variety of molasses types on the market, and studies indicate that some are more attractive to flies than others (Geden, 2005). A study of various molasses types may be needed to fully elucidate its potential as an additive.

The two vegetable oils (peanut and canola; 30%) with Shellsol T (70%) were the most effective formulations. Despite the comparative lower efficacy of the best two EO formulations (2% Codacide and 2% Horti oil) they were not abandoned, as EO formulations with fungi have shown to be efficacious in many field studies (Jenkins & Thomas, 1995 and Javaid et al., 2000). In addition, it has been suggested that VLV formulations with nutrient additives may have an advantage over ULV in wetter climates (Burges, 1998).

Two ULV and two VLV formulations were chosen for further testing in cage trials under field conditions. The results of the cage trials corroborated the conclusion from the laboratory studies that ULV formulations were superior. Thus a ULV formulation of *Metarhizium* spores in canola oil was selected for testing in a pilot trial in commercial feedlots applied with a Micronex ULVA+ (Micron Group UK) hand held spinning disk sprayer. Although the ULVA+ is designed to treat vegetation, application was passive, relying mostly on wind drift to take the formulation into the treatment area. In addition, the tank capacity was limited and the operators were on-foot which required them to wind through the treatment area to achieve a

consistent application. These limitations were acceptable in the small scale trial, but subsequent full feedlot trials required more powerful and time-efficient spraying methods

The positive results in the pilot trial led to upgrading the spray program to a full spray trial in two feedlots. The sprayer used in the full trials was the Cifarelli mistblower (Series L3) fitted with the Micronair AU8000 ULV sprayer head (Enviromist Industries Pty Ltd/Micron Group UK). The sprayer released a targeted mist stream of ULV formulation atomised through the rotary spray head and propelled by the blower. It was operated from the tray of a utility vehicle which drove parallel to the treatment areas and enabled full treatment of feedlot test areas within a couple of hours. The Cifarelli mistblower provided a droplet size of 50 – 100 um likely to be in the optimal range for the desired application in the feed lots.

5.3 Field Trials

Accurately estimating the effects of different nuisance fly treatments in cattle feedlots is difficult. Flies are motile and numbers of flies at different monitoring sites within feedlots are not independent. This means that feedlots must be considered as the experimental unit in statistical examination of the effects of treatments and helps explain the lack of reported studies assessing the effects of chemical insecticides or other fly control methods when applied in cattle feedlots. In addition, the numbers and activity of flies are variable across feedlots and influenced by factors such as differences in moisture, the availability of food and oviposition sites, shade and resting sites. Environmental factors such as temperature, wind and intense solar radiation cause daily and seasonal variations in fly numbers and their activity with flies resting in different sites on different days and at different times of the day. All of these factors make accurately estimating fly numbers and assessing changes in the size of populations within feedlots problematic (Godwin et al., submitted (appendix IV, section 9)).

Different methods have been used to monitor fly populations in intensive livestock systems. The advantages and disadvantages of each of these are discussed by various authors (Lysyk and Axtell 1986, Beck and Turner 1985, Gerry et al 2011). Following a consideration of these studies we used spot cards and sticky cards, most often recommended for monitoring fly numbers because of their accuracy and utility and, because when left in place for a week, they account, at least in part, for the within and between day variation in distribution and activity of flies. We also used subjective scoring at each monitoring point, which although it only gave a point in time estimation in each week, was likely to most closely approximate feedlot managers assessment of fly numbers. Alsynite traps were used in the first two seasons to provide an estimate of the species composition of flies on each feedlot. Eventually the different monitoring methods were combined to a fly index which we believe gave the best assessment of variations in fly numbers as it utilised information from all of the monitoring information available. However, we also analysed the results as measured by the spot cards, sticky cards and visual scores individually.

Maximising the number of monitoring sites generally increases the accuracy of estimating variations in fly populations. Between 7 and 25 spot cards, depending on fly densities, were recommended to provide estimates of fly numbers in poultry houses with a coefficient of variation of 0.15 (Lysyk and Axtell, 1986) whereas, Gerry et al. (2011) recommended the use of 12 monitoring sites on large scale dairy operations to provide a similar level of precision. We decided to adopt this recommendation, particularly considering that our objective was to

determine relative fly densities and seasonal patterns on the four farms, rather than to compare absolute values or indicate thresholds for treatment.

On feedlots, monitoring apparatus is subject to the vagaries of environment and potential loss from the impacts of curious cattle, feedlot management activities and the effects of native or pest animals and birds. Thus in the early seasons we established approximately 20 monitor sites which we eventually reduced to 12 per feedlot on the basis of resilience of equipment at different sites and omission of sites that consistently gave zero or very low counts and which therefore were considered unlikely to provide a significant contribution to the measurement of variations in fly numbers.

Although the monitoring data collected in year 1 and year 2 and three monitorings in year 3 (prior to spraying) was variable, it provided an acceptably consistent profile of differences and temporal patterns in fly numbers over farms, and enabled a reasonably sensitive assessment of the effects of spraying on fly populations. Analysis using the fly index as the best estimate of fly populations showed an estimated reduction in fly numbers of 20% ($p=0.002$). Analysing the individual fly monitoring measures separately also indicated a reduction in fly numbers in all cases (9.3% with spot cards, 31.0 % with sticky cards and 23.3% with visual scores) with the reduction statistically significant ($P<0.05$) for sticky cards and visual scores, although not for spot cards. In addition, there appeared to be a significant accumulative reduction in fly numbers from the effects of successive sprayings (Fig. 22).

Isolations from the vegetation (Tables 11, 15; Figure 23) showed that there is already an average background level of *Metarhhizium* in the feedlot environments of up to 126 viable spores per gram of vegetation (as measured by colony forming units (CFUs). *Metarhizium* has a worldwide distribution and has been isolated from soils and insects from the arctic to the tropics (Zimmermann, 2007). The source of the *Metarhizium* washed from the vegetation samples taken from the control feedlots is most likely the dust that is blown onto vegetation and thrown up from animal and vehicular movement around the feedlots. The background level of *Metarhizium* varied through the trials as did the amount of dust observed on the vegetation. Other reports of *Metarhizium* levels in the soil show wide variation. Vestergaard and Eilenberg (2000) reported natural levels of *Metarhizium* in soils in Denmark ranging from 0 up to 300 ± 200 cfu per g of soil whereas. Vanninen (1995) reported a range from 0 to 9000 CFUs per g of soil in different ecosystems and soil types in Finland.

Spraying led to a large increase in viable spore numbers on vegetation in the spray areas. Spore numbers were significantly higher than in control plots up to at least 2 weeks post spraying. At 3 weeks, spore numbers were also higher than background levels but neither the difference between week three levels and controls, nor between week 3 levels and week 2 levels was significant (Fig. 23). The lack of significance at 3 weeks is not surprising since vegetation was only sampled twice on each feedlot at this time, resulting in low statistical power. The persisting number of spores in the environment at one and two weeks post spraying did not translate to higher fly infection rates or higher fly mortality rates at these times. Many of the flies initially infected would have been dead or close to death by this time and less likely to be trapped. In addition, even though flies may still have been becoming infected from the remaining viable spores in the environment at these times, the dilution effects of newly emerging and immigrant flies may have meant the level of infection in the overall population was too low to be detected with our sampling methodology. That spores sprayed on leaves may still be causing mortality up to 2 weeks after application is also

supported by the results of previous studies in B.FLT.0326 under more controlled conditions in which flies were exposed in the laboratory to vegetation from the field previously sprayed with spores. One week after spraying mortality of flies exposed to the treated leaves was between 48% and 75% and at 2 weeks mortality was 15% compared to 6% in flies exposed to untreated leaves.

Persistence in the environment is a two edged sword. On one side, it is expected that elevated numbers of spores in the environment up to 2 weeks post spraying may have continued to lead to an elevated, or early, mortality of flies and pre-lethal infections may have reduced reproduction (Acharya et al., 2015). On the other side, short persistence in the environment will limit the possibility of infections in non-target arthropod populations.

These results were extremely encouraging as, in comparison. Analysis of the effect of chemical sprays on feedlots in a previous project found no measurable reduction in fly numbers. This is not to suggest that spraying did not kill flies, but simply that the overall reduction in fly populations was not sufficient to be measured. This suggests that the effect from spraying the mycopesticide was at least as good, if not better than that from the application of chemical sprays.

It should be noted that in this study spraying was not conducted near the cattle or feed bunks or along the laneways close to the pens (Fig. 7 - 10). However very large numbers of flies were consistently seen in these areas, in and along the sides of feed bunks and on accumulated manure under fence lines at the edges of the pens. Recent studies have demonstrated the steam flaked grain cattle rations frequently used on feedlots are particularly attractive to flies (Ghosh and Zurek, 2014). With abundant food, oviposition sites, and shade on the sides of feed bunks, it is likely that a large proportion of these flies did not migrate far from their breeding areas and were unlikely to have been directly impacted by spray droplets or exposed to secondary pick-up of spores from the sprayed vegetation. That a large proportion of the fly population was not exposed to spores and yet a measurable reduction in fly numbers could still be measured, further reinforces the effectiveness in reducing fly numbers of the mycopesticide formulation and spray methodology we have developed.

5.4 Bait Formulations

The use of bait formulations as part of an integrated pest management program (IPM) has been shown to be an effective method for controlling housefly populations in contained environments (Ahmad and Zurek, 2009). However, most commercially available baits are insecticide based and frequent use of the same or similar insecticides has been shown to cause selective pressure within fly populations, leading to the development of insecticide resistance (Scott and Wen, 1997). A myco-pesticide bait formulation derived from the same *M. anisopliae* isolate used to develop the spray formulation in this project may provide a suitable and safer alternative to insecticide baits. Mycopesticide bait formulations may also provide an effective option for controlling flies within areas such as feed stores and ration preparation mills, equipment sheds, outbuildings and in other situations where the use of chemical methods is undesirable.

A series of laboratory assays was conducted with the intention of developing a bait formulation derived from *M. anisopliae* conidia on whole rice. *M. anisopliae* is produced

commercially on a sterilised grain substrate which is friable and granular and similar in nature and appearance many commercially produced bait forms. It was shown to be attractive to flies and would require little modification or processing, thus reducing production costs.

A number of additives and accessory attractants were investigated.to increase attractiveness to adult flies and to improve the persistence of activity. Of the additives tested, only raw sugar caused a significant increase in the efficacy of the bait (5% to 15% increase in mortality by 6 days after exposure). However this increase was not great enough to warrant the use of sugar in the final bait formulation. Canola oil and molasses were also tested but caused major decreases in the efficacy of the bait. It is suspected that the canola oil had a repellent effect on the flies and molasses reduced the accessibility of the spores, though these hypotheses were not tested. The bait formulation containing milk powder, thought to be a fly arrestant and feeding stimulant and suggested to reduce UV degradation (Burges, 1998) was also largely ineffective at killing flies. Due to these findings, the final bait formulation did not contain a UV protectant. Although bait efficacy Assay 1 indicated no detrimental effect to spore viability under low, natural levels of UV radiation, exposure to high UV levels could be expected to reduce the period of efficacy, suggesting that the formulation may be most suitable for use indoors or in shaded situations.

The second series of assays tested a range of potential attractants for inclusion in the bait formulation. Previous studies have shown molasses to be attractive to flies, although the level of attractiveness appears to depend on the source and type of the molasses (Geden 2005) and Assay 1 of the attractant series in our test also identified molasses as a potential attractant. However, molasses was subsequently outperformed as attractant in assay 2 by a commercial fertiliser based on composted poultry manure (Dynamic Lifter®). Dynamic Lifter was ultimately selected as the best of the potential attractants tested. Interestingly, a commercially available fly attractant containing the female housefly sex pheromone (Z)-9-tricosene (muscalure), was found to have no attractive qualities to a mixed sex sample of *M. domestica* under our test conditions. Variability in effect from the use of muscalure has been noted in a number of other studies and is discussed by Hanley et al. (2009).

A bait formulation incorporating Dynamic Lifter® was evaluated for efficacy in a final assay, alongside a commercially available insecticide bait (Quickbayt®) for comparison. However, addition of Dynamic Lifter® provided no additional increase in efficacy compared to the rice-spores bait alone. Quickbayt® gave a much greater knock-down effect than the myco-pesticide bait, but after 7 days the net mortality caused by the myco-pesticide bait was significantly higher than the insecticide product (53% compared to 25% respectively).

Baits with a high knock-down effect are generally favoured by consumers due to the visible impact of seeing dead flies near the bait. However, a slower acting but more effective bait may provide a greater effect in reducing fly populations in the longer term. In addition, accumulations of dead flies on the bait surface may reduce access of flies to baits and has been suggested to reduce effectiveness of fast knock-down chemical baits (Geden, 2005). The myco-pesticide bait has the added advantage of leaving zero residues in the environment and being completely harmless to non-arthropod animals, making it ideal for areas such as feed mills and storage areas where the use of chemical pesticides may be undesirable. Recent research shows that *M. anisopliae* infection in female *M. domestica* reduces lifetime fecundity and egg viability substantially (Acharya et al*.,* 2015). This

suggests that a *M. anisopliae* bait could have a significant longer term effect in suppressing fly populations.

5.5 Conclusions

This project has developed an effective ULV formulation of *Metarhizium* and demonstrated that application in commercial cattle feedlots produced a measurable reduction in fly numbers. This was despite the fact that no spray was applied near feed bunks or cattle pens where large numbers of flies were seen. These results are consistent with those from laboratory and field cage testing of the ULV formulation and with those achieved in a previous MLA project. However, the improved formulation and application methodology has produced much better results despite applying significantly lower amounts of spores, suggesting the feasibility of commercial development. In addition, a potentially low cost, non-chemical bait based on *Metarhizium* was developed which gave results comparable to a currently registered chemical bait formulation and provides a realistic option for use in areas where chemical methods are not desirable. Biopesticides based on *Metarhizium* could form an important component of integrated fly control programs on cattle feedlots and action towards registration of commercial formulations is recommended.

6 Key Messages

- The field trials demonstrated that the ULV spray formulation comprised of *Metarhizium anisopliae* spores in a Canola/Shellsol T (30:70) carrier successfully infected and reduced *M. domestica* fly populations at cattle feedlots.
- *Metarhizium anisopliae* formulations meet Australian Beef Industry objectives because they are environmentally friendly, leaving no residues and having little to no threat to non-arthropod animals.
- The formulation developed was cost-effective due to the increased spore yield achieved as part of the laboratory studies and the more effective droplet size and efficiency of formulation use offered by ULV technology.
- The ULV formulation developed in this project would complement an IPM program incorporating good feed lot management practices such as; regular fence line cleaning, good water drainage and use of parasitoids.
- The efficacy of the ULV formulation is highly dependent on the sprayer technology being used to deliver it into the environment. The backpack sprayer used in this project was effective however; new sprayer technology may be more cost effective and increase the efficacy of the ULV formulation.
- The bait formulation derived from the *Metarhizium anisopliae* spores and growth medium had comparable efficacy to commercially available chemical baits. The non-chemical bait would be suitable for areas such as feed mills and other outbuildings where chemical use is undesirable.

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9 Appendix

Appendix I: Schedule of events during the field trial in commercial feedlots (season 3).

Date	Spray Week	Fly Monitoring	Spraying	Fly Sampling	Vegetation Sampling	Comments
19-11-2015	1 (-2)	✓				
26-11-2015	1 (-1)	✓				
03-12-2015	1	✓	✓	✓	✓	
10-12-2015	1 (+1)	✓				
17-12-2015	2	✓	✓	✓	✓	
23-12-2015	2 (+1)	✓				
30-12-2015	2 (+2)					Christmas Break
07-01-2016	3	✓	✓	✓	✓	
14-01-2016	3 (+1)	✓				
20-01-2016	4	✓	✓	✓	✓	
28-01-2016	4 (+1)	✓				
04-02-2016	5	✓	✓	✓	✓	
11-02-2016	5 (+1)	✓		✓	✓	
18-02-2016	6	✓	✓	✓	✓	
25-02-2016	6 (+1)	✓		✓	✓	
02-03-2016	7	✓	✓	✓	✓	
10-03-2016	7 (+1)	✓		✓	✓	
17-03-2016	7 (+2)	✓		✓	✓	

Appendix II: Raw data from pilot trials at commercial feedlots.

Feedlot	Spray	Average CFUs/g vegetation	log 10 CFUs	Fly Mortality (%)	Infection in Dead Flies (%)	Infection in Live Flies (%)	Total Infection (%)
Feedlot A Pre Spray	1	247	2.39	2	0	0	0
Feedlot A Post Spray	1	1,098,765	6.04	34	91	17.9	72.1
Feedlot C Pre Spray	1	371	2.57	16	3.2	0	2.8
Feedlot C Post Spray	1	423,529	5.63	65.5	94.0	22.7	77.8
Feedlot B Control	1	224	2.35	6.5	7.1	1.2	8.5
Feedlot A Pre Spray	2	54,878	4.74	27.5	18.2	1.2	7.9
Feedlot A Post Spray	2	117,647	5.07	53.5	81.9	18.8	50.9
Feedlot C Pre Spray	2	25,000	4.40	38	1.8	0	0.8
Feedlot C Post Spray	2	71,951	4.86	56.9	40.5	19.7	30.7
Feedlot B Control	2	125	2.10	29.5	1.7	0	0.7
Feedlot A Pre Spray	3	58,294	4.77	6.5	15	0	2.1
Feedlot A Post Spray	3	302,500	5.48	16	81	12	31.0
Feedlot C Pre Spray	3	56,625	4.75	7.5	6	0	1.0
Feedlot C Post Spray	3	350,617	5.54	18	53	8	21.7
Feedlot B Control	3	494	2.69	4.5	0	0	0.0
Feedlot A Pre Spray	4	25,183	4.40	10	10	0	1.9
Feedlot A Post Spray	4	208,750	5.32	37	78	21	47.4
Feedlot C Pre Spray	4	42,625	4.63	13	16	1	4.6
Feedlot C Post Spray	4	283,125	5.45	36	80	21	46.6
Feedlot B Control	4	24	1.38	16	3	1	1.7

Appendix III: Raw data form field trials at commercial feedlots.

Feedlot	Spray	Mean CFUs/g vegetation	log 10 CFUs	Fly Mortality (%)	Infection in Dead Flies (%)	Infection in Live Flies (%)	Total Infection (%)
Feedlot B Control	-2	-	-	16.00	0.00	-	-
Feedlot D Control	-2	-	-	46.00	2.00	-	-
Feedlot A Treatment	-2	-	-	18.50	0.00	-	-
Feedlot C Treatment	-2	-	-	15.00	0.00	-	-
Feedlot B Control	-1	-	-	11.33	0.00	-	-
Feedlot D Control	-1	-	-	10.00	0.00	-	-
Feedlot A Treatment	-1	-	-	26.00	0.89	-	-
Feedlot C Treatment	-1	-	-	43.50	0.00	-	-
Feedlot B Control	1	183	2.26	9.74	0.00	0.00	0.00
Feedlot D Control	1	178	2.25	33.37	1.39	0.00	0.64
Feedlot A Pre Spray	1	350	2.54	14.65	0.00	0.00	0.00
Feedlot A Post Spray	1	121111	5.08	23.86	61.55	0.00	22.50
Feedlot C Pre Spray	1	-	-	31.66	0.00	0.00	0.00
Feedlot C Post Spray	1	16667	4.22	35.90	45.96	0.00	21.61
Feedlot B Control	2	275	2.44	2.47	0.00	0.00	0.00
Feedlot D Control	2	175	2.24	2.49	11.11	0.00	1.04
Feedlot A Pre Spray	2	167	2.22	2.90	0.00	0.00	0.00
Feedlot A Post Spray	2	34542	4.54	34.12	65.03	1.19	30.34
Feedlot C Pre Spray	2	83	1.92	9.01	0.00	0.00	0.00
Feedlot C Post Spray	2	20167	4.30	30.42	61.38	1.19	26.50
Feedlot B Control	3	158	2.20	10.65	6.25	0.00	1.00
Feedlot D Control	3	100	2.00	21.99	2.27	0.00	0.78
Feedlot A Pre Spray	3	108	2.03	12.41	0.00	0.00	0.00
Feedlot A Post Spray	3	26167	4.42	29.22	72.16	4.76	32.00
Feedlot C Pre Spray	3	83	1.92	14.94	6.67	0.00	1.73
Feedlot C Post Spray	3	38417	4.58	45.59	67.58	1.19	34.93
Feedlot B Control	4	16	1.20	10.02	0.00	0.00	0.00
Feedlot D Control	4	0	0.00	38.41	0.00	0.00	0.00
Feedlot A Pre Spray	4	90	1.95	13.52	0.00	0.00	0.00
Feedlot A Post Spray	4	42706	4.63	39.13	93.09	9.52	49.74
Feedlot C Pre Spray	4	373	2.57	30.60	1.67	0.00	0.69
Feedlot C Post Spray	4	26902	4.43	70.45	82.18	3.59	58.73
Feedlot B Control	5	8	0.90	13.42	0.00	0.00	0.00
Feedlot D Control	5	90	1.95	30.92	1.39	0.00	0.64
Feedlot A Pre Spray	5	16	1.20	13.46	0.00	0.00	0.00
Feedlot A Post Spray	5	11631	4.07	55.34	83.44	0.00	45.67
Feedlot C Pre Spray	5	12	1.08	63.29	1.00	0.00	0.58
Feedlot C Post Spray	5	7655	3.88	67.29	55.33	0.00	37.18
Feedlot B Control	5 (+1)	6	0.78	8.12	0.00	0.00	0.00
Feedlot D Control	5 (+1)	0	0.00	8.90	0.00	0.00	0.00
Feedlot A Treatment	5 (+1)	1914	3.28	5.71	0.00	1.19	1.09
Feedlot C Treatment	5 (+1)	1202	3.08	10.98	20.00	0.00	3.85
Feedlot B Control	6	0	0.00	12.08	37.70	0.00	7.45
Feedlot D Control	6	0	0.00	18.08	5.51	0.00	2.17
Feedlot A Pre Spray	6	118	2.07	22.79	0.00	0.00	0.00
Feedlot A Post Spray	6	13451	4.13	43.24	83.54	4.76	43.22

Feedlot C Pre Spray	6	365	2.56	14.01	5.56	0.00	1.67
Feedlot C Post Spray	6	72235	4.86	53.76	74.06	4.76	44.22
Feedlot B Control	6 (+1)	0	0.00	10.90	0.00	0.00	0.00
Feedlot D Control	6 (+1)	0	0.00	9.52	4.17	0.00	0.93
Feedlot A Treatment	6 (+1)	3292	3.52	6.18	12.50	0.00	1.09
Feedlot C Treatment	6 (+1)	3500	3.54	18.92	1.92	0.00	0.74
Feedlot B Control	7	479	2.68	13.85	0.00	0.00	0.00
Feedlot D Control	7	142	2.15	22.18	0.00	0.00	0.00
Feedlot A Pre Spray	7	629	2.80	15.95	0.00	0.00	0.00
Feedlot A Post Spray	7	170417	5.23	61.64	78.77	11.31	52.49
Feedlot C Pre Spray	7	529	2.72	16.17	0.00	0.00	0.00
Feedlot C Post Spray	7	138750	5.14	74.79	86.00	12.25	67.19
Feedlot B Control	7 (+1)	33	1.52	4.59	0.00	0.00	0.00
Feedlot D Control	7 (+1)	17	1.23	7.71	0.00	0.00	0.00
Feedlot A Treatment	7 (+1)	4650	3.67	16.11	0.00	0.00	0.00
Feedlot C Treatment	7 (+1)	3125	3.49	11.87	0.00	0.00	0.00
Feedlot B Control	7 (+2)	8	0.90	8.46	0.00	0.00	0.00
Feedlot D Control	7 (+2)	8	0.90	5.07	0.00	0.00	0.00
Feedlot A Treatment	7 (+2)	196	2.29	19.31	0.00	0.00	0.00
Feedlot C Treatment	7 (+2)	871	2.94	8.19	0.00	0.00	0.00

Appendix IV: Predicting nuisance fly outbreaks on cattle feedlots in subtropical Australia.

R. M. Godwin[A], D.G. Mayer[B], G.W. Brown[B], D.M. Leemon[B] and P.J. James[A]

[A]Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, Brisbane Queensland.

[B]Agri-Science Queensland, Department of Agriculture and Fisheries, Ecosciences Precinct, 41 Boggo Road, Dutton Park Qld 4102

Corresponding Author

Dr Peter James

Queensland Alliance for Agriculture and Food Innovation (QAAFI)

The University of Queensland, Brisbane, Australia.

Postal address: Animal Science, Level 2A West, EcoSciences Precinct, GPO Box 267, Brisbane Qld 4001

Physical address: Animal Science, Department of Agriculture and Fisheries, Level 2A West, Ecosciences Precinct, 41 Boggo Road, Dutton Park, QLD 4102

Phone: 61 7 3255 4268

Fax: 61 7 38466258

Mob: 61 408148511

Email: p.james1@uq.edu.au

Summary text for the Table of Contents

Nuisance flies are important arthropod pests in cattle feedlots with the potential to cause production loss and health impacts on livestock, workers and surrounding communities. Population models were developed for fly abundance and showed that fly numbers could be predicted using time of year and rainfall. The models provide a useful tool for optimising the timing of fly control treatments, such as insecticide or biopesticide applications, adding to the effectiveness of integrated pest management programs for the control of nuisance flies in feedlots.

Abstract. (less than 250 words)

Flies are important arthropod pests in intensive animal facilities such as cattle feedlots with the potential to cause production loss, transmit disease and cause nuisance to surrounding communities. In this study, seasonal population dynamics of three important nuisance flies, house flies (*Musca domestica* L.), bush flies (*M. vetustissima* Walker) and stable flies (*Stomoxys calcitrans* L.) (Diptera: Muscidae) were monitored on cattle feedlots in south-eastern Queensland, Australia, over seven years. *Musca domestica* was by far the dominant species, comprising 67% of the total flies trapped. Models were developed to assess the relationship between weather parameters and fly abundance and to determine if population trends could be predicted to improve the timing of control measures. For all three species, there were two main effects – 'time-of-year' (mainly reflected by minimum temperatures and solar radiation) and rainfall. The abundance of all three species increased with increasing temperature and rainfall, reaching a peak in summer before decreasing again. Rainfall events resulted in significantly elevated numbers of *M. domestica* for up to five weeks, and for one week for *M. vetustissima.* Peak fly numbers were predicted by the model to occur in spring and summer following 85-90 mm weekly rainfall. The population dynamics of *S. calcitrans* were least influenced by rainfall and it was concluded that weather variables were of limited use for forecasting stable fly numbers in this environment and production system. The models provide a useful tool for optimising the timing of fly control measures, such as insecticide or biopesticide applications, adding to the efficiency of integrated control programs.

Keywords (3-6) : Population dynamics, *Musca domestica*, *Stomoxys calcitrans*, *Musca vetustissima*, Integrated Pest Management

Introduction

House flies (*Musca domestica* L.), bush flies (*Musca vestustissima* Walker) and stable flies (*Stomoxys calcitrans* L.) are common nuisance flies associated with intensive animal facilities such as cattle feedlots (Matthiessen 1983; Hogsette *et al.* 2012; Urech *et al.* 2012). Flies can become a significant problem in these areas because of the presence of large amounts of manure and feed in which flies can oviposit and develop. Uncontrolled fly populations constitute a significant nuisance and threaten the health and welfare of livestock, farm workers and surrounding communities through their capacity to transmit pathogens (Graczyk *et al.* 2005; Förster *et al.* 2007; Ahmad *et al*. 2007; Macovei *et al*. 2007; Förster *et al.* 2009; Baldacchino *et al.* 2013). Control measures may involve various integrated pest management (IPM) strategies including feedlot design, management and biological control, but also rely significantly on pesticide applications. However, excessive reliance on pesticide applications is undesirable because of the development of insecticide resistance, potential environmental contamination and health and safety concerns (Wang *et al.* 2012; Khan *et al.* 2013; Scott *et al.* 2013) and IPM strategies are not always optimally utilised.

Abiotic factors such as temperature, moisture and solar radiation have a direct influence on the fecundity and duration of the lifecycle of agriculturally important insects including nuisance flies (Drake 1994) and there are threshold temperatures above and below which different life stages will not develop and survive. Under suitable weather conditions, particularly when favourable temperatures, rainfall and humidity coincide, fly outbreaks can

occur in cattle feedlots even with the best preventative strategies in place. Fly populations can build rapidly to reach problem levels if control measures are left too late whereas miss-timed precautionary treatments, when fly numbers would not have reached problem levels, needlessly incur labour, treatment costs and increased selection for resistance. Accurately predicting when flies will become a problem would enable the strategic timing of control measures to maximise both effectiveness and cost efficiency of treatments.

Weather parameters have been used to develop models for predicting calyptrate fly numbers in the United Kingdom (*M. domestica* and *Calliphora* spp) (Goulson *et al.* 2005) and for stable flies in Nebraska (Taylor *et al*. 2007). The study by Goulson *et al.* (2005) examined the relationship between fly numbers and weather conditions using a four year data set of weekly fly catches and meteorological data in the southern UK. They found that fluctuations in fly populations were largely driven by the weather rather than by biotic factors. Predictive models based on rainfall, temperature and humidity were strongly correlated with observed fly numbers (R^2 = 0.52-0.84). For *M. domestica,* temperature in the week prior to trap collection was the best single predictor although other aspects of temperature in the preceding 3 weeks also contributed significant predictive power to the model. Weather factors, in particular temperature 0 to 2 weeks before fly collection and precipitation 3 to 6 weeks before collection, were also the most important determinants of stable fly populations in Nebraska (Taylor *et al.* 2007). These models were developed in temperate regions of the Northern Hemisphere and may not be applicable to the subtropical region in Australia where many cattle feedlots are located.

For this study a number of large data sets for fly numbers on feedlots located in sub-tropical south-eastern Queensland, Australia, collected over seven years, were used with accompanying weather data from the Queensland Government SILO (Scientific Information for Land Owners) database (https://www.longpaddock.qld.gov.au/silo/). We investigated the utility and accuracy of predicting periods of high fly numbers using weather records to facilitate optimal timing of fly control strategies.

Materials and methods

Adult fly monitoring was conducted in seven feedlots in south-eastern Queensland between October 2001 and April 2008. The feedlots were in three districts, namely Dalby, the Brisbane Valley and Warwick. The historical data were used in conjunction with meteorological data to test the accuracy of predicting fly numbers from different weather variables. The major climatic characteristics of these areas are shown in Table 1 and details of the location of the feedlots, monitoring period and trapping sites are given in Table 2. All seven feedlots were managed according to standard commercial practice. Integrated pest management programs for fly control, which included regular fence line and sedimentation system cleaning, the release of parasitic wasps (*Spalangia endius*), biopesticides *(Metarhizium anisopliae)* and chemical treatments, were in place on two of the feedlots while fly control procedures on the other feedlots included irregular manure removal, insecticide treatments and parasitic wasp releases.

Numbers of the three main nuisance species, house flies, bush flies and stable flies were monitored using alsynite sticky traps (Olson Products, Ohio, USA) which were supported on stakes 0.9 -1.2 m off the ground (Urech *et al.* 2012). The traps were placed within the feedlots at selected sites near manure piles, feed processing areas, cattle pens, vegetation

between pens, the cattle induction area, silage pits, sedimentation ponds and horse stables. Traps were serviced at intervals of between 1 and 17 days depending on the time of year and fly populations. Trapped flies were brought to the laboratory, identified and counted as per the procedure described in Urech *et al*. (2012).

The Queensland Government SILO database (https://www.longpaddock.qld.gov.au/silo) was used to provide daily weather data corresponding to the GPS coordinates of each feedlot and appropriate fly monitoring period. The SILO database uses historical climate records for Australia and observational records provided by the Bureau of Meteorology (http://www.bom.gov.au/) together with GPS coordinates to derive daily datasets for different locations which are both spatially and temporally complete. Climate variables used in the construction of models included maximum (Tmax) and minimum (Tmin) temperatures (°C), rainfall (Rain) (mm), evaporation (Evap) (mm), solar radiation (Radn) (MJ/m^2), vapour pressure (VP) (hPa), and relative humidity (%) at both the maximum (RHx) and minimum (RHn) temperatures.

Data processing and Modelling

Daily weather data were converted to weekly data by averaging across each 7 day period. Cumulative weekly totals were calculated for rainfall and evaporation. Fly counts were converted to weekly data by first converting trap data to average flies per trap per day for each monitoring interval and then accumulating the data by date to give weekly counts for each species. These weekly intervals corresponded to the same weekly intervals as for the weather data. The two data sets (weekly fly counts and weekly weather data) were then combined into one dataset for modelling purposes. Data for each fly species were analysed separately using Genstat for Windows® v16.1 (GenStat 2015).

The $\log_{10}$(catch+1) transformation was adopted for the dependent variables, as these were highly-skewed with heterogeneous variance. This implies a multiplicative relationship between the effects of the independent variables, as is biologically expected. The regression models were fitted using residual maximum likelihood (REML) in GenStat (2015), with an autoregressive (lag-one) error term to accommodate for the significant autocorrelations between weeks within feedlots. Step-forward, step-backward and all-subsets regressions, plus random forests (multiple regression-tree models (Elith *et al.* 2008)), were used to screen the potential predictor variables. Overall shape and degree of curvature of the regression lines were tested using smoothing-spline, nonlinear and quadratic models, with the best-fit and biologically-appropriate forms adopted for the fitted relationships.

The effects of feedlot treatments were tested using binary contrasts – firstly as 'overall' (any of the treatments, compared to no treatment), as well as testing the individual types – 'insecticide application' (only tested for the week of application, and the following week), 'parasitic wasps' (only from the second week after the first release), and 'IPM', fitted as an additional effect to the parasitoid wasp treatment, as wasps were always released as part of IPM, but a number of feedlots not using IPM also released wasps. The effect of feedlot size on fly numbers was also tested, but found to be not significant (P>0.05) and excluded from the model.

There were high degrees of correlation amongst some weather variables (see Table 3), but this is not statistically a problem in forecasting when the degrees of correlations are

expected to remain approximately similar (Dormann *et al.* 2013). All catch number results and forecasts were derived using the bias-corrected back-transformation from the log-scale (Kendall *et al.* 1983).

Results

The total number of trapped flies in this study was 1,185,581 of which 67% were *M. domestica*, 21% were *M. vetustissima* and 12% were *S. calcitrans.* Other fly species which only contributed a small proportion of the total flies trapped, do not generally breed within the feedlot and therefore were not included. Of the three fly species studied, *M. domestica* were most affected by weather variables, showing a higher degree of fit in the derived models than the other two species.

Catch data for *M. domestica* for each of the seven feedlots showed that the highest populations occurred in a broad peak during the summer months at all 7 feedlots (Fig. 1). Numbers dropped to very low levels during the winter months. All feedlots followed the same trend although fly numbers in FL 2 in the Brisbane Valley did not decrease to the same extent as FL 3 and 4 in the winters of 2005 and 2006.

The total numbers of *M. vetustissima* (data not presented) were generally a third of those for *M. domestica* except in November/ December 2007 in the Warwick district (FL5 and FL6) where numbers of *M. vetustissima* were 5 to 10 times higher than *M. domestica*. Reasons for the outbreak of *M. vetustissima* are unknown but probably reflect suitable conditions for bushfly breeding or winds favourable for bushfly immigration to these feedlots (Hughes and Nicholas 1974) at this time. Numbers of *S. calcitrans* trapped were lowest of the three main species, being only about a tenth those of *M. domestica* and half those of *M. vetustissima*. Although the population patterns of the three species were generally similar, peak numbers of *M. vetustissima* and *S. calcitrans* occurred slightly earlier in the summer (October to November) than *M. domestica* and then rapidly decreased. Overall, seasonal data showed a strong relationship between the spring/early summer rainfall and increased populations of *M. domestica* (Fig. 2) across years and locations ($R^2 = 0.96$). Higher fly numbers occurred in the Brisbane valley which received higher rainfall than Dalby and Warwick.

Investigations using weekly data showed that, for all species, there were two main climatic effects – ‘time-of-year’ (as best represented by minimum temperatures, or solar radiation), and rainfall. Interactions between the weather terms in the model were relatively minor and not significant ($P<0.05$). This is probably because the log relationship adopted implicitly accommodates the expected multiplicative relationship between month and rainfall. The ‘just weather terms’ models had notably high and biased residuals for November and December indicating that the higher catch rates found in these months were not solely explained by the weather terms. Temperatures and rainfall similar to those observed in November and December were also experienced in late summer and autumn (March to May), but fly catch rates then were markedly lower.

Alternative models for the ‘time of the year’ effect were investigated, including ‘month’ (a factor with 12 fixed levels, representing the ‘baseline catch rates’ for each month) and a Fourier curve. In combination with the rainfall effect the model with ‘month’ lifted the degree of fit (adjusted R^2) for *M. domestica* from 61% (for the model with just weather terms) to

69%, and from 31% to 59% for *M. vetustissima*. For *S. calcitrans* the alternative models provided approximately the same level of fit, but month was adopted for consistency.

The shape of the rainfall effect curve was somewhat difficult to determine, given the generally-high degree of variability in catch rates, plus the sparseness of rainfall events with greater than 100 mm per week (there were only nine of these in our data-set). Adopting log rainfall as a linear term, or rainfall as a quadratic effect, produced very similar degrees of fit. For *M. domestica* the adjusted R^2 values were 68.3% and 68.8% respectively. The quadratic relationship was adopted due to the slightly-better fit and better overall agreement with the non-parametric spline models (which indicated a slight depression in catch numbers above about 100 mm per week). Rainfall had extended effects on fly populations with significant increases in fly numbers persisting for up to five weeks after rainfall events. The fitted coefficients were quite consistent, predicting maximum fly numbers at 90, 87, 88, 89 and 99 mm rainfall per week for lags of 1 to 5 weeks respectively. The fitted relationship for a three week lag is shown in Fig.3

The effects of feedlot treatments on *M. domestica* populations were mixed. The release of parasitoid wasps had a significant ($P < 0.01$) effect, with the coefficient of –0.2131 (on the $\log_{10}$ scale) translating to a fitted 39% reduction in fly numbers following the release of wasps. IPM had no additional statistical effect on fly numbers over the effect of parasitic wasp releases, which were always part of the IPM program. There was also no significant effect of insecticide applications on house fly numbers. The non-significant treatment terms ($P>0.05$) were dropped from the final model which included only a 'parasitoid wasp releases' factor.

When months were investigated (as 'time of the year' effects) in combination with the quadratic rainfall effect for *M. vetustissima*, the degree of fit was significant for a one week lag only (adjusted R^2 of 59.1%). The effect of rainfall was greatest between October and December with little effect for the remainder of the year. Maximum fly numbers were observed after weekly rainfall of 86 mm (Fig. 4) indicating the relatively instant and short term effect of rain on *M. vetustissima* populations. Feedlot treatments did not significantly affect *M. vetustissima* numbers, although there was an average reduction in catch numbers of 36% ($P = 0.10$) following insecticide sprays.

S. calcitrans populations were least affected by climate, showing only a low degree of fit (R^2 = 24.2%) when months were investigated in combination with a quadratic rainfall effect for lags of 3, 4 and 5 weeks. There was no significant effect of feedlot treatments but there was an average reduction of 43% in catch numbers following insecticide applications ($P = 0.09$). The low degree of fit suggests that weather variables were of limited use for forecasting stable fly numbers in this environment and production system.

Discussion

South East Queensland has a subtropical climate with hot humid summers. Winters are drier, mild to warm, but with cool overnight temperatures. For all fly species, initial screening indicated two main effects on fly numbers, 'time-of-year' (which represents changes in both minimum temperatures and solar radiation) and rainfall. Between April and October, temperature was likely the main factor limiting fly numbers. Rainfall had little effect on any of the fly species during the winter months. There are always localised areas in feedlots where

moisture is present and flies can breed and as temperatures rose from October to November, the base number of flies (assuming no rainfall) increased by an average of 352%. The greatest effect of rainfall was seen in the spring and early summer when temperature had increased sufficiently for rapid reproduction and development of flies and the main factor limiting population growth was moisture. The months from November to February were the main period of fly breeding and the model predicted that rainfall of 25 mm would be expected to increase fly numbers by a further 46 %, whereas 50 mm could be expected to give an approximate 88% increase in numbers of flies and 90 mm would cause fly numbers to increase by 120%. The model indicates a multiplicative effect of season and rainfall events suggesting that highest fly numbers will occur following successive rainfall events during early summer.

In contrast to the results reported here, Goulson *et al.* (2005) found that in the UK, temperature was the best predictor of fly numbers. This is not surprising since population dynamics are governed by the 'law of the minimum' and in the temperate wet climate of the UK, temperature rather than moisture is likely to be the limiting factor for a large proportion of the year.

For the major fly species, *M. domestica*, both factors showed extended effect with significant associations between fly numbers and weather factors measured up to five weeks previously. This is not unexpected as increased moisture impacts favourably on a number of different life processes of house flies including oviposition, egg development and larval survival and development (Williams *et al.* 1985). The period of effect of rainfall events will also be determined by factors such as the amount of rain received, follow up rain and environmental influences that affect the rate of drying of the larval habitat, such as soil moisture, humidity and wind. The amount of rainfall predicted to produce maximum *M. domestica* numbers with lags of 1, 2, 3, 4 and 5 weeks was remarkably consistent at 90, 87, 88, 89 and 99 mm/week, respectively. The scarcity of higher rainfall events during the period of the study did not allow us to accurately extrapolate the effects of rainfall above 100 mm but the shape of the rainfall–fly numbers curves suggests that there is a maximal level of rain for fly production above which further increases in fly numbers do not occur. Weekly rainfall above this level, may be detrimental to fly populations by causing drowning or suffocation fly larvae, pupae and eggs (Farkas *et al.* 1998).

Higher *M. domestica* catches were recorded in the months of November and December than during months with similar rainfall and temperatures later in summer. This is likely to be due to the effects of predators and parasites. When conditions become favourable, flies build up very rapidly. However, predators and parasites that feed on or parasitise flies generally breed more slowly and take some time 'to catch up' and exert a regulating influence on fly numbers. Later in the season it is likely that there are more predators and parasites present and these have a greater effect in suppressing fly populations than earlier in the year. This hypothesis is supported by the significant effect of augmentative releases of parasitoids in suppressing fly numbers seen in this study and similar effects noted with flies breeding in poultry facilities (Peck and Anderson 1970; James *et al*. in press).

The effect of rainfall was less pronounced on *M. vestustissima* with falls of 85 mm having a relatively instant, but short term, effect on fly numbers. The short term effect is not surprising since *M. vetustissima* breeds mainly in manure pats outside the feedlot area. These dry out more quickly than the large accumulations of manure present within feedlots. Increases in *M.*

vestustissima abundance in the feedlots may have been the result of immigrant flies attracted to the feedlots by moisture, odours and potential protein sources (Hughes *et al*. 1972). The effect of spraying was more pronounced with *M. vetustissima* than house flies and this was probably because few bush flies emerge from pupae within feedlots whereas house fly populations are supplemented daily by flies emerging on site. In addition, resistance is known to be widespread in *M. domestica* populations world wide. This was not assessed in this project, but may also have contributed to the lack of effect from spraying noted in this study.

Numbers of *S. calcitrans* were less affected by weather variables than either *M. domestica* or *M. vestustissima*. A previous study in a similar location showed that although both house flies and stable flies originated mainly from within the feedlot area, *M. domestica* was found mainly in areas containing animals and feed whereas the highest catches of *S. calcitrans* were near the feed mill, silage pits and piled manure (Urech *et al.* 2012). *S. calcitrans* breeds mostly in spilled feed or mixtures of dung and decaying fibrous material (Meyer and Petersen 1983; Hogsette *et al.* 1987; Dawit *et al.* 2012) and numbers of this species are largely determined by the availability of these resources.

Seasonal patterns of *S. calcitrans* abundance observed in other studies have been highly variable depending on location, climatic conditions and management regime (Lysyk 1993; Taylor *et al.* 2007; Skovgård and Nachman 2012; Jacquiet *et al.* 2014). Temperatures above 30°C have been found to have a negative impact on *S. calcitrans*, (Lysyk 1998; Gilles *et al.* 2005; Skovgård and Nachman 2012) which may explain the reduction in fly numbers observed in this study during the hot summer months. Additionally, Urech *et al*. (2012) found *S. calcitrans* was more abundant on central New South Wales feedlots which were located 4-8° further south and had lower summer temperatures than feedlots in southern Queensland.

Taylor *et al.* (2007) developed population models for *S. calcitrans* based on temperature and precipitation and found that temperatures 0 to 2 weeks before collection and precipitation 3 to 6 weeks before collection were the most important variables influencing stable fly numbers. During midsummer, precipitation, not temperature, was the major factor limiting stable fly populations. However, the major source of stable flies in their study was from pastures and, more particularly, sites where large round hay bales were fed to cattle. Thus their model was developed in a cattle management system that was quite different to the present study. They noted that the relationship between weather variables and fly numbers would likely vary depending on larval development sites, climatic zone and cultural conditions and highlighted the need for predictive models to be substantiated under a range of conditions to determine their universality.

The models developed here will provide useful tools to assist timing the application of insecticides or biopesticides for fly control in South East Queensland feedlots. The models suggests that fly treatments will seldom be justified during months from March to October. As the models are based on seven years of data obtained from seven feedlots distributed across an area of approximately 15 000 km^2 they are likely to have application at least at regional level and in other areas of the world with a similar subtropical climate. Whether or not to treat and after what amount of rainfall treatments should be applied will depend on individual tolerances to fly numbers, management circumstances and the perceived likelihood of follow up rainfall and temperatures to sustain fly breeding. However, the ‘rules

of thumb' presented here, used within an integrated control program and adapted to individual feedlot circumstances, will enable much more targetted application of pesticide treatments, reducing cost and the undesirable effects of unneeded treatments and providing more efficient fly control.

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Figure captions

Fig. 1. *M. domestica* numbers (Log_{10} weekly fly count+1) for the seven study feedlots (FL) plotted by district (a) Dalby, (b) Brisbane Valley and (c) Warwick district.

Fig. 2. The relationship between Log_{10} average *M. domestica* catch rates and rainfall from November to January.

Fig. 3. Fitted values for *M. domestica* numbers (per trap per week) in response to rainfall 3 weeks previous to trapping for each month of the year.

Fig. 4. Fitted values for *M. vetustissima* numbers (per trap per week) in response to rainfall 1 week previous to trapping for each month of the year.

Table 1. Climate statistics for the Dalby, Brisbane Valley and Warwick districts averaged over the last 20 years

District	Elevation (m)	Annual Rainfall (mm)	Average Summer Temperature (°C)		Average Winter Temperature (°C)	
			Max	Min	Max	Min
Dalby	344	683	31.7	18.1	19.6	4.9
Brisbane Valley	113	999	30.1	18.9	20.9	7.4
Warwick	477	692	29.5	16.6	18.7	3.7

Table 2. Location and details of fly monitoring at each of the seven southern Queensland feedlots

District and dates of monitoring	Feedlot	Location (GPS)	Carrying capacity (SCU*)	Number of Trapping sites
Dalby Oct 2001 – Oct 2003	1	27° 02' S, 151° 20' E	13 000	3 - 8
Brisbane Valley Nov 2004 - Apr 2008	2	29° 24' S, 152° 21' E	3 100	2 - 4
	3	27° 03' S, 152° 18' E	700	1 - 5
	4	27° 06' S, 152° 21' E	1 000	1 - 5
Warwick Nov 2006 - Apr2008	5	28° 09' S, 152° 06' E	2 000	2 - 4
	6	28° 03' S, 151° 54' E	1 000	1 - 4
	7	28° 06' S, 151° 51' E	8 000	1 - 4

* Standard cattle units

Table 3. Correlation coefficients (r) for the relationship between climate variables included in the initial models: maximum temperature (Tmax), minimum temperature (Tmin), rainfall (rain), evaporation (Evap), radiation (Radn), vapour pressure (VP) and relative humidity at the maximum (RHx) and minimum (RHn) temperature.

Tmax	1						
Tmin	0.838	1					
Rain	-0.019	0.260	1				
Evap	0.870	0.669	-0.074	1			
Radn	0.743	0.514	-0.113	0.906	1		
VP	0.782	0.952	0.290	0.542	0.425	1	
RHx	-0.341	0.180	0.506	-0.497	-0.496	0.292	1
RHn	-0.325	-0.163	0.163	-0.538	-0.384	0.075	0.566
	Tmax	Tmin	Rain	Evap	Radn	VP	RHx

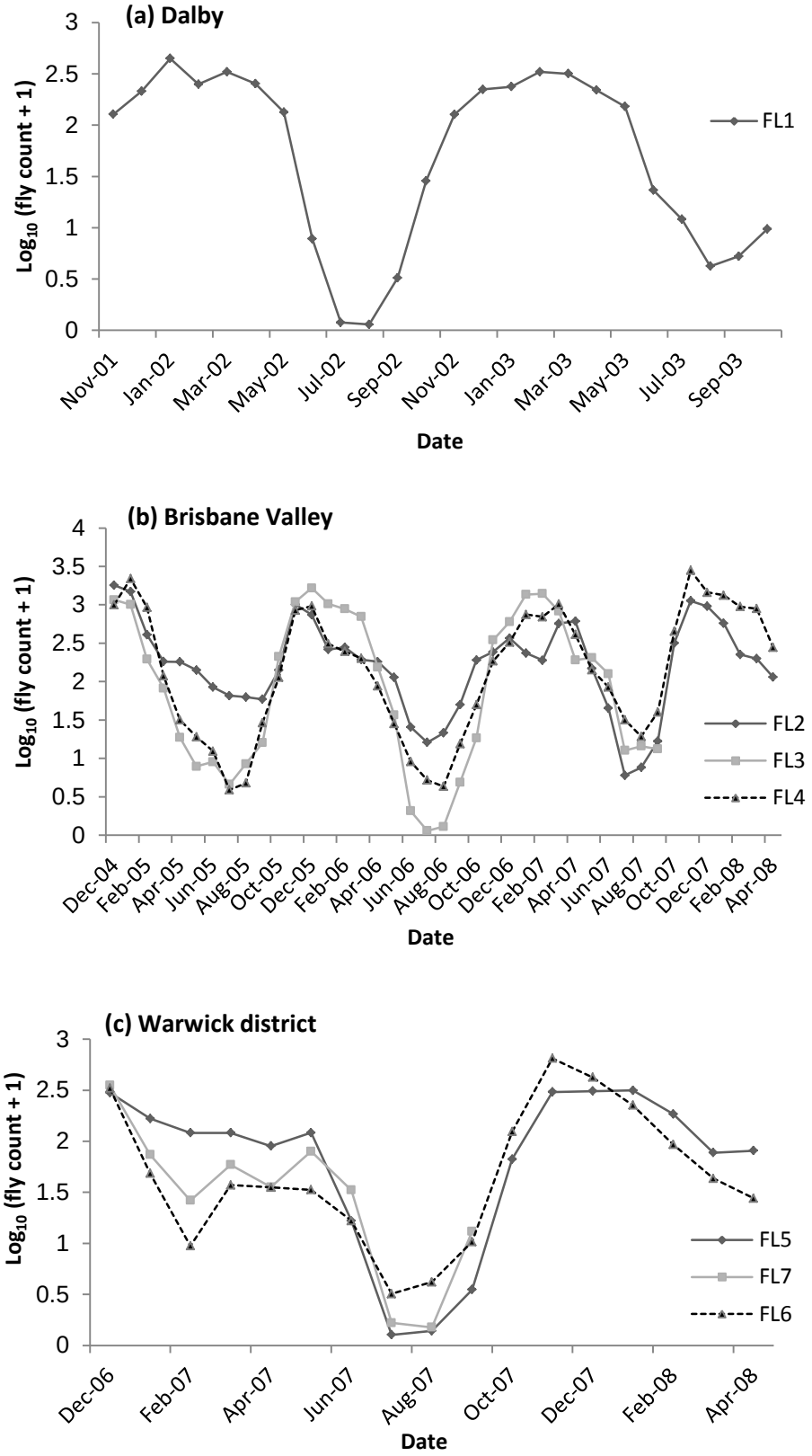


Fig. 1. *M. domestica* numbers (Log_{10} weekly fly count+1) for the seven study feedlots (FL) plotted by district (a) Dalby, (b) Brisbane Valley and (c) Warwick district.

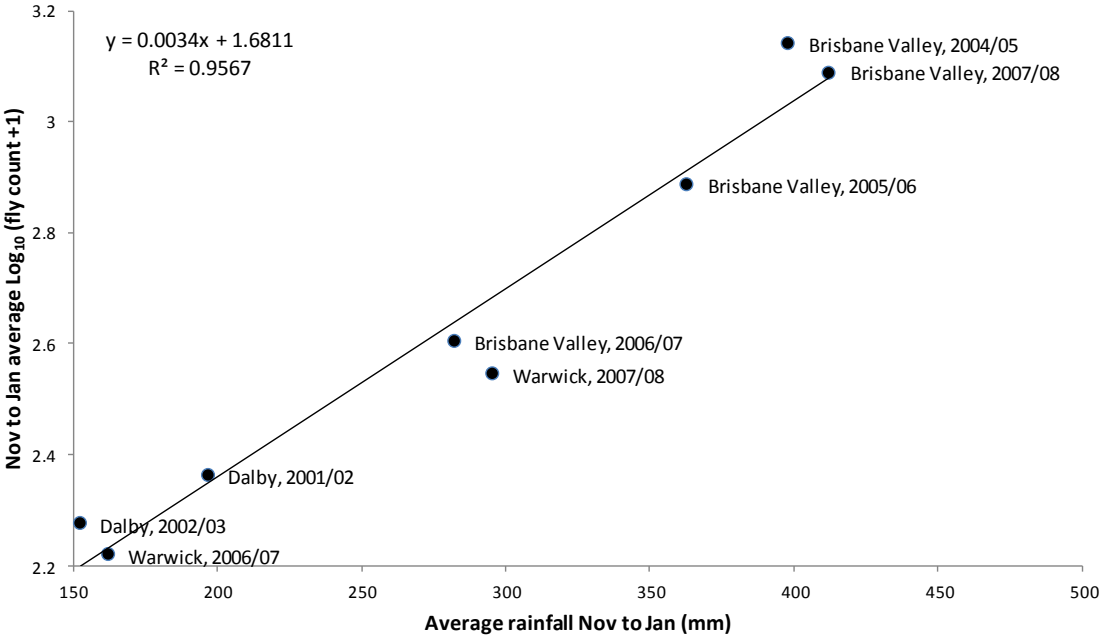
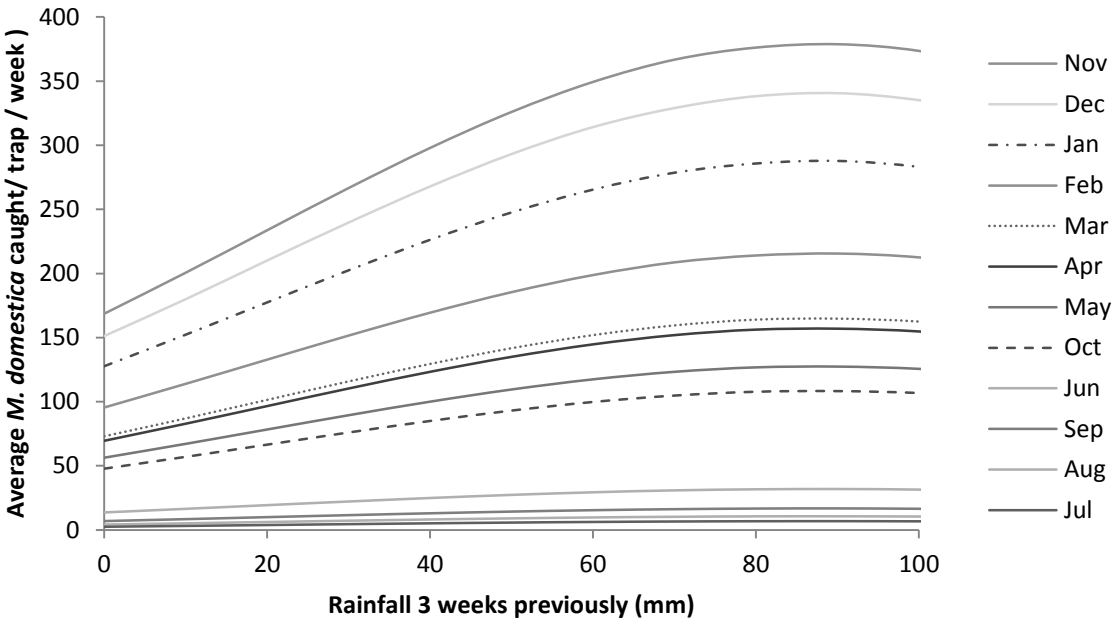


Fig. 2. The relationship between Log_{10} average *M. domestica* catch rates and rainfall from November to January



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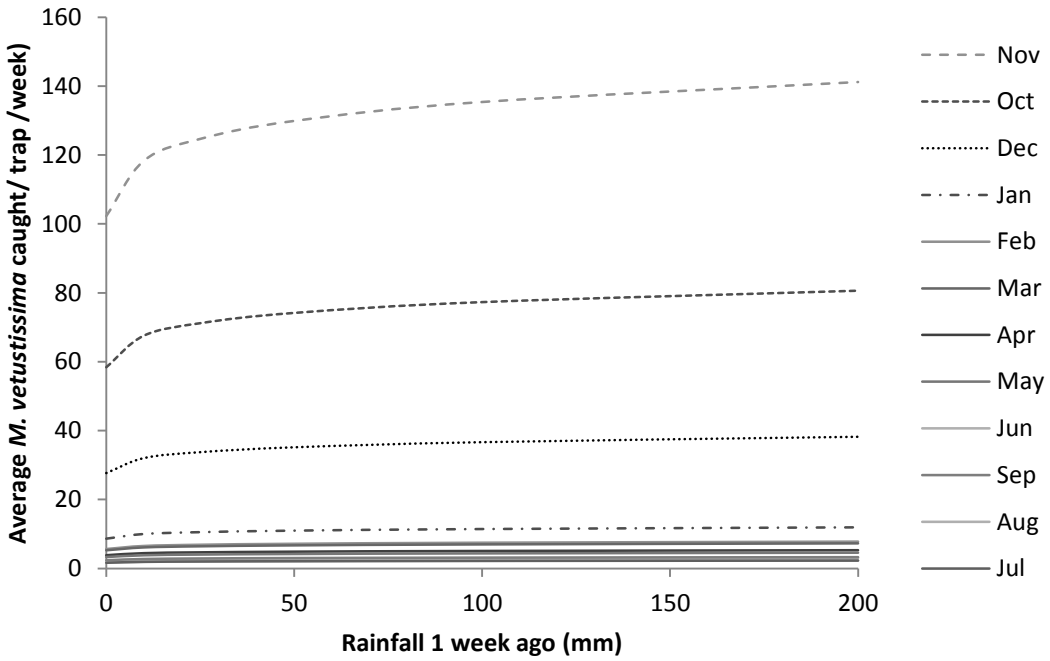

Fig. 4. Fitted values for *M. vetustissima* numbers (per trap per week) in response to rainfall 1 week previous to trapping for each month of the year.