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Fungal biopesticide for cattle tick control

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Abstract

Cattle ticks (*Rhipicephalus (Boophilus) microplus*) are a serious external parasite of cattle in Northern Australia. Current control strategies for ticks rely heavily on extensive chemical treatments (acaricides) which are beset with the problems of tick resistance and residues in meat and milk. Fungal biopesticides have emerged as realistic non-chemical control options for a range of pests in agriculture. Therefore one option for tick control is the use of a fungal biopesticide. The aim of this project was to refine then test a fungal biopesticide formulation against the parasitic stages of the cattle tick. A fungal biopesticide for tick control would constitute an important addition to current tick control options in the Northern cattle regions; helping to counter the problems associated with residues in meat and milk as well as the issues experienced in some areas due to tick populations resistant to most classes of acaricides.

Executive summary

Cattle ticks (*Rhipicephalus (Boophilus) microplus*) are a serious pest of cattle in northern Australia. Important control options include chemical treatments (acaricides) applied in strategic control programs along with breeding of tick resistant cattle genotypes and pasture management. However there is scope for more control options to effectively manage this serious parasite.

Agri-Science Queensland investigations found that some Australian isolates of *Metarhizium anisopliae* are extremely effective at killing ticks in the laboratory, with death occurring in 100% of ticks within two days (Leemon & Jonsson, 2007). These results compare favorably with overseas studies where 100% mortality after four days laboratory incubation is considered to be a good result. Such promising results with the Australian *Metarhizium* isolates led to a series of three field studies with a *Metarhizium*-based biopesticide conducted on cattle in Queensland in 2003-2004 (Leemon et al, 2008). These outdoor pen trials yielded positive, though variable results. It was shown that, while lethal doses of the fungal biopesticide can be applied to ticks on cattle, high temperature on the skin surface during mid summer may limit the efficacy. The third trial, conducted in extreme summer heat, was aborted when tick numbers on all animals, including the untreated controls, declined drastically. One trial in which the product appeared to be quite effective took place under cooler ambient temperatures.

It was clear that more studies were required to confirm that a *Metarhizium* formulation will kill different parasitic stages of ticks during the moderate climatic conditions experienced in either the Spring rise or Autumn. The aims of this study were twofold: first, to refine the fungal biopesticide formulation; second, to assess the efficacy of the improved fungal biopesticide against the cattle tick *Rhipicephalus* (*Boophilus) microplus* on animals housed individually in pens. Thus the objectives of this project were to:

1. Refine the previous fungal biopesticide formulation with respect to isolate, dose and carrier
2. Conduct *in vitro* evaluations of the formulation against larval and engorged female ticks, at different temperatures and against different tick strains; and
3. Carry out a pen trial to assess the efficacy of the fungal biopesticide in controlling all stages of ticks on cattle using two different doses of spores and an untreated negative control.

Extensive laboratory studies resulted in *Metarhizium* isolates ARI-M52 and ARI-M63 being selected as the best isolates for a *Metarhizium* based fungal biopesticide to be applied to cattle for tick control. Temperature assays were first conducted to determine which isolates grew best over 30°C. Virulence assays were then carried out to distinguish which isolates caused the highest mortalities in female engorged and larval ticks. Standard testing protocols were followed. Female engorged ticks were immersed in suspensions of fungal spores of the different isolates and larval ticks were challenged through packet tests in which a suspension of fungal spores was applied to the surface of the packet then allowed to dry.

Further virulence studies were also conducted using formulated spores of the best isolates against larval ticks at constant and varying temperatures. These studies showed that even at temperatures alternating up to 38°C for 12 hours per day the formulation was extremely potent towards larval ticks.

Dose titration assays were conducted to provide a basis for choosing appropriate spore concentrations to use in test formulations. It was found that larval ticks were susceptible to

much lower concentrations of spores than the female engorgeds. Additional studies showed that all the main acaricide resistant tick strains are equally susceptible to fungal attack.

Two formulations using a mixture of spores of *M. anisopliae* isolates ARI-M52 and ARI-M63 were developed for testing on animals. Two different dose levels of spores were suspended in codacide oil which was later diluted with tap water in a spray tank before application to animals. It was anticipated that such formulations would provide reasonable tick control on animals provided the surface temperatures on the trial animals dipped below 34°C for significant time periods each day.

The pen studies showed that both dose levels of the fungal biopesticide tested were highly effective when applied directly to engorged female engorged ticks that were incubated in the laboratory or when engorging female ticks exposed to the biopesticide while feeding on cattle were removed from the cattle and incubated in the laboratory. However the capacity for the fungal biopesticide at either dose level to kill ticks on animals was much lower than expected with several factors appearing to confound the outcomes from this study. **Nonetheless, despite demonstrated non-ideal conditions with respect to surface temperature on the animals, the treatment efficacy ranged between 40% and 60% for much of the time in the 14 days post treatment rising to 80% 18 days post treatment although there was no obvious dose effect.** In addition it was shown that formulated spores stayed viable in the animal coat for up to 11 days after treatment even when exposed to high levels of heat and spore damaging UV light on some animals which were kept in outside paddocks for a few days later in the trial.

When considering the growth temperatures of the *Metarhizium* isolates achieved in the laboratory studies it was clear the surface temperatures on the penned animals in the first 5 days post-treatment were infrequently in the optimal range for the *Metarhizium* isolates (<32°C). An efficacy study for a tick control product conducted in moated pens has the advantage of providing an accurate assessment of the total tick drop from treated and untreated animals. However, it appears that the design and structure of the pens used in this study may have influenced the surface temperatures of the animals in a way that impacted negatively on the fungal biopesticide.

Engorged ticks collected from treated animals and incubated in the laboratory showed both an increased mortality and subsequent decreased egg production in comparison to ticks collected from non-treated control animals. However it is speculated that the method of collecting ticks by hosing pens and tick baskets with high pressure hoses could have affected the fungus on the tick exterior before the ticks were incubated.

An additional factor that had a negative impact on the trial results was the wide disparity in tick resistance between trial animals relating to an abnormal range in the numbers and size of ticks dropping from these animals throughout the trial.

A fungal biopesticide for tick control will be an important addition to current tick control options in the Northern cattle regions that will help counter the problems associated with residues in meat and milk as well as the issues experienced in some areas due to tick populations resistant to most classes of acaricides. A fungal biopesticide would serve as a critical tool for an integrated pest management system for tick control, a tool highly compatible with a tick vaccine.

Based on the positive outcomes of the laboratory studies and the influence of the pen facility on our ability to test the fungal biopesticide under natural temperature conditions it is recommended that a field study be undertaken in the future to evaluate efficacy. Ideally this trial should be carried out either during the Spring rise or Autumn.

Contents

Page

1 Background ... 8
2 Project objectives ... 8
3 Methodology ... 9
3.1 Laboratory studies ... 9
3.1.1 Fungal isolates ... 9
3.1.2 Temperature characterisation ... 9
3.1.3 Tick assays ... 9
3.1.4 Isolate selection ... 10
3.1.5 Dose titrations ... 10
3.1.6 Tick strain comparison ... 11
3.1.7 Formulation carrier ... 11
3.1.8 Statistical analyses ... 11
3.2 Pen study ... 12
3.2.1 Animals ... 12
3.2.2 Treatment formulations ... 13
3.2.3 Treatment application ... 13
3.2.4 Assessment of treatment efficacy ... 13
3.2.5 Formulation check ... 14
3.2.6 Application check ... 14
3.2.7 Temperatures ... 14
3.2.8 Protocol variation and re-treatment ... 14
4 Results ... 15
4.1 Laboratory studies ... 15
4.1.1 Temperature characterisation ... 15
4.1.2 Isolate selection ... 16
4.1.3 Dose titrations ... 17
4.1.4 Tick strain comparison ... 20
4.1.5 Formulation carrier ... 20
4.2 Pen studies ... 20
4.2.1 Formulation check ... 20
4.2.2 Application check ... 21
4.2.3 Temperature ... 22
4.2.4 Assessment of treatment efficacy ... 25
4.2.4.1 Engorged tick mortality ... 25

4.2.4.2 Egg production *in vitro* ... 26
4.2.4.3 Engorged tick drop ... 26
4.2.5 Larval infestation ... 27
4.2.6 Spore viability in cattle coats ... 28
5 Discussion ... 31
5.1 Laboratory studies ... 31
5.2 Pen studies ... 32
6 Success in achieving objectives ... 34
7 Impact on meat and livestock industry – Now and in five years time ... 34
8 Conclusions and recommendations ... 34
9 Bibliography ... 34

List of Tables

Table 1. Source of *Metarhizium anisopliae* isolates, and date isolated from source ... 9
Table 2. Chemical resistance status of tick strains held in the DEEDI tick reference collection ... 10
Table 3. Components of formulations and spray treatments ... 13
Table 4. Average colony diameter (mm) after 14 days growth from an inoculated 8 mm disc in the temperature range of 15°C to 27°C ... 15
Table 5. Average colony diameter (mm) after 14 days growth from an inoculated 8 mm disc in the temperature range of 24°C to 36°C ... 15
Table 6. Average colony diameter (mm) after 14 days growth from an inoculated 8 mm disc at alternating temperatures ... 16
Table 7. Effective doses of *M. anisopliae* isolates ARI-M52 and ARI-M63 required to kill 50% (ED_{50}) and 90% (ED_{90}) of female engorged ticks when formulated in 0.1% T80 ... 19
Table 8. Effective doses of *M. anisopliae* isolates M52 and M63 required to kill 50% (ED_{50}) and 90% (ED_{90}) of female engorged ticks when formulated in 5% Codacide oil (first assay) 19
Table 9. Effective doses of *M. anisopliae* isolates ARI-M12 and ARI-M16 required to kill 50% (ED_{50}) and 90% (ED_{90}) of female engorged ticks when formulated in 5% Codacide oil (second assay) ... 19
Table 10. Effect of oil concentration in formulations of ARI-M52 spores on engorged female tick mortality after 2 days incubation at either high or low moisture ... 20
Table 11. Average mortality of semi-engorged female ticks sampled from different animals after spray application, the weight of the animals as well as the length of time the animals were sprayed are also given ... 21
Table 12. Number of hours surface temperatures recorded on penned animals after treatment (day 0) were within specified ranges ... 22
Table 13. Number of hours surface temperatures recorded on animals outside in paddocks after re-treatment (day 0) were within specified ranges. Note that logger 3 stopped working after day 3 and loggers 8 and 5 only recorded for part of day 4 ... 23
Table 14. Ambient temperatures recorded from three data loggers in the five days after animals were treated ... 23
Table 15. Ambient temperatures recorded from three data loggers in the five days after animals were retreated ... 24

Table 16. Schedule of larval infestation of cattle and numbers of engorged females dropping from control animals 21 days post application 28
Table 17. Number of spores washed from samples of hair taken from cattle immediately after treatment and then at two later dates as well as the resulting colonies growing from viable spores 28

List of Figures

Figure 1. Average percent mortality of engorged female ticks incubated at 27°C for 3 days after immersion in spore suspensions of different *M. anisopliae* isolates (Different letters indicate a significant difference P<0.05) 16
Figure 2. Average percent mortality of larval ticks incubated at 27°C for 4 days in packets treated with spore suspensions of the 7 different *M. anisopliae* isolates (Different letters indicate a significant difference P<0.05) 17
Figure 3. Average percent mortality of larval ticks incubated at 27°C for 3 days in packets treated with spore suspensions of the 7 different *M. anisopliae* isolates (Different letters indicate a significant difference P<0.05) 17
Figure 4. Average percent larval tick mortality when incubated for 12:12 hr at 28°C and 38°C in packets treated with either 5×10^6 or 5×10^7 spores/ml of the *M. anisopliae* isolates M52 and M63 18
Figure 5. Average percent larval tick mortality when incubated for 12:12 hr at 28°C and 38°C in packets treated with either 5×10^6 or 5×10^7 spores/ml of the *M. anisopliae* isolates M12 and M16 18
Figure 6. Average percent mortality of different strains of engorged female ticks incubated at 27°C for 3 days after immersion in spore suspension of *M. anisopliae* isolate ARI-M52..... 20
Figure 7. Average mortality, after two days, of groups of engorged female ticks immersed in samples of the low and high dose formulations collected during spraying 21
Figure 8. Average mortality in semi-engorged female ticks (4.0–7.5 mm) sampled from animals immediately after treatment 22
Figure 9. The number of hours the ambient temperature was below 22°C per day compared to the number of hours the surface temperature on animals fell below 32°C when animals were under field conditions 24
Figure 10. The number of hours the ambient temperature was below 22°C per day compared to the number of hours the surface temperature on animals fell below 32°C when animals were penned inside 25
Figure 11. Mortality of samples of engorged female ticks collected in the first four days post treatment and incubated for egg production; mortality was assessed after 7 days 25
Figure 12. Average weight of eggs produced by samples of 20 engorged ticks incubated for 7 days at 28°C 26
Figure 13. Percent efficacy of the 2 dose levels of the fungal biopesticide against ticks in the 14 days following spray treatment 26
Figure 14. Percent efficacy of the fungal biopesticide against ticks on cattle 18 to 25 days after the initial treatment and on cattle re-treated 14 days after the initial treatment. The efficacy is affected by very low and inexplicable numbers of ticks on all control animals between 19 and 23 days 27
Figure 15. *Metarhizium* colonies from spores washed out of cattle hair samples taken after spray treatment of animals from left to right: Control, Low dose; High dose 30
Figure 16. *Metarhizium* colonies from spores washed out of cattle hair samples taken 9 days after spray. Top plates (x2) are low dose treatments, bottom plates (3) are high dose treatments 30
Figure 17. *Metarhizium* colonies from spores washed out of cattle hair sample taken 11 days after cattle were retreated with fungal biopesticide. Treated cattle spent the first 3 days after treatment outside 31

1 Background

Cattle ticks are an important external parasite of cattle. Important control options include chemical treatments (acaricides) applied in strategic control programs along with breeding of tick resistant cattle genotypes and pasture management. However there is scope for more control options to effectively manage this serious parasite.

DEEDI investigations found that some Australian isolates of *Metarhizium anisopliae* are extremely effective at killing ticks in the laboratory, death occurring in 100% of ticks within two days (Leemon & Jonsson, 2007). These results compare very favorably with those from overseas laboratories, in which 100% mortality in four days would be considered to be a good result. Previous field studies with a *Metarhizium* based biopesticide conducted on cattle in Queensland yielded positive, though variable results. A series of three outdoor pen trials conducted in 2003-2004 (Leemon et al, 2008) showed that, while lethal doses of the fungal biopesticide can be applied to ticks on cattle, high temperature on the skin surface during mid summer may be a limitation on the efficacy. The third trial, conducted in extreme summer heat, was aborted when tick numbers on all animals, including the untreated controls, declined drastically. One trial in which the product appeared to be quite effective took place under cooler ambient temperatures.

More studies are required to confirm that a *Metarhizium* formulation will kill different parasitic stages of *R. microplus* under ambient conditions that result in surface temperatures on animals dipping below 32°C for periods of time. To prepare for such a study the formulation needs to be optimised by selecting strains of *M. anisopliae* that are highly virulent to both female engorged and larval ticks and grow at temperatures over 30°C. In addition dose studies need to be conducted to estimate a suitable concentration for the formulation. Earlier investigations with *M. anisopliae* identified seven candidate strains based on virulence to ticks and capacity for spore yield.

The aims of this study were twofold: first, to conduct laboratory investigations to refine the fungal biopesticide formulation; second, to investigate the efficacy of a fungal biopesticide against the cattle tick *Rhipicephalus* (*Boophilus) microplus* in a pen situation. The efficacy was assessed on animals that were individually housed in pens. The efficacy was also compared between formulations with two different doses of spores and an untreated negative control.

2 Project objectives

The primary aim of this project was to test a refined fungal biopesticide formulation against the parasitic stages of the cattle tick. Thus the work involved laboratory studies on the formulation followed by pen studies with animals to achieve the following objectives:

1. Refine fungal biopesticide formulation with respect to isolate, dose and carrier
2. Conduct *in vitro* evaluation of formulation against all larval and female engorged ticks, at different temperatures and against different tick strains
3. Carry out a pen trial to evaluate the efficacy of the fungal biopesticide in controlling all stages of ticks on cattle.

3 Methodology

3.1 Laboratory studies

3.1.1 Fungal isolates

The seven *Metarhizium anisopliae* isolates used in this study were isolated from either soil or dead insects, and two arose as laboratory variants. The isolates are distinguished by source and morphological characteristics. All isolates except ARI M81, a more recent isolate, had previously undergone molecular characterisation through sequencing of the ITS and D3 regions of the 28S ribosome. Table 1 gives the isolate, source of isolate, region in Queensland where the source was collected and year of collection. The fungal isolates were maintained on potato dextrose agar (PDA) (difco) slopes held at 4°C and -20°C. Fresh cultures initiated from these were maintained on both PDA and Sabouraud Dextrose Agar with 1% malt extract (SDAM) plates at 25°C (Goettel and Inglis, 1997).

Spores for the bioassays were produced on solid rice media as described by Gretel, (1984). The rice was dried at 20°C then harvested by shaking through 300 µm and 150 µm Endicott sieves. Harvested spores were stored at 4°C.

Table 1. Source of *Metarhizium anisopliae* isolates, and date isolated from source

Isolate	Source		Year Isolated
	Substrate	Location	
ARI-M10	Soil	Sth Johnston, Qld	1999
ARI-M12	Soil	Utchee Creek, Qld	1999
ARI-M16	Soil	Aratula, Qld	1999
ARI-M52	*Musca domestica*	Dalby, Qld	2002
ARI-M61	Lab variant	Yeerongpilly, Qld	2003
ARI-M63	Lab variant	Yeerongpilly, Qld	2003
ARI-M80	*Aethina tumida*	Bellbowrie, Qld	2006

3.1.2 Temperature characterisation

Plates of Sabouraud's Dextrose Agar (SDA) in 90 mm Petri dishes were prepared by marking X and Y axes on the underside. Spore solutions of 1×10^8 spores/ml in sterile 0.1% Tween 80 were made up for the 7 different isolates of *Metarhizium anisopliae* from the ARI fungal culture collection. Sterile 6 mm disks of filter paper dipped in a spore solution were placed on the agar above the intersection of the axes. Four replicate plates of each isolate were incubated in the dark at different temperatures for 14 days. Surface radial growth was recorded using two cardinal diameters, through the X and Y axes on days 7 and 14. This assay was carried out first with the plates incubated at 15°C; 18°C; 21°C; 24°C and 27°C. Then it was carried out with the plates incubated at 24°C; 27°C; 30°C; 33°C and 36°C. The assay was later carried out with alternating temperatures on a 12:12 hr alternation at either 25-35ºC; 30-35ºC or 28-38ºC.

3.1.3 Tick assays

All *in vitro* assays with female engorged ticks were conducted by placing fully engorged female ticks in 24 well microtitre trays (Sarstedt) that were stacked inside larger sealed plastic containers for incubation. In some assays 1.5 ml of water agar with 0.5% chloramphenicol was added to the bottom of the wells. The agar increased the relative humidity, hence promoting fungal growth on the ticks. The chloramphenicol was added to

the agar to inhibit bacteria. In other assays ticks were simply placed in the empty well. Moist sponges were added to the large plastic containers to provide some humidity in these assays. *In vitro* assays with larval ticks were conducted using a modified larval packet test. Treatments were added to gridded "larval packet test" papers which were dried and folded in half before adding larval ticks. The papers were secured on three sides with bulldog clips to make a "packet". Packets were suspended in large plastic containers lined with wet sponge to maintain a high humidity and sprayed lightly with water before the large containers were sealed and incubated. Mortality was assessed as cessation of Malpighian tubule movement for engorged female ticks (Leemon *et al*, 2008) and lack of movement in larval ticks. All ticks (both fully engorged female ticks and larval ticks) were obtained, courtesy of Dr Louise Jackson, from the tick reference strain collection maintained at the Centre for Advanced Animal Studies at Gatton. A range of tick strains, with varying resistance to different classes of acaricides actives (Table 2), were available for testing. The NRFS strain was used for all larval tick work. For work with engorged female ticks the NRFS strain was used when available, but the numbers had to be supplemented with other strains for some assays.

Table 2. Chemical resistance status of tick strains held in the DEEDI tick reference collection

Tick strain	Resistance to acaricide active
NRFS (non resistant field strain)	Susceptible to all acaricide actives
Dieldrin	Dieldrin, some organo-phosphate resistance
Mt Alford	Organo-phosphate resistance
Parkhurst	Synthetic pyrethroid resistance
Ultimo	Synthetic pyrethroid and Amidine (Amitraz) resistance)
Tiaro	Synthetic pyrethroid, Amidine and Fluazuron resistance
Broadhurst	Fluazuron and small amount Organo-phosphate resistance

3.1.4 Isolate selection

Spore solutions were prepared by suspending approximately 0.05 g of dry spore powder of each of the 7 *M. anisopliae* isolates in 20 ml of an aqueous solution with 0.1% of the surfactant Tween 80 (T80) giving an approximate spore concentration of 1×10^8 sp/ml.

Screening assays for female engorged ticks were conducted by immersing groups of 20 engorged female ticks in the solutions for 1.5 minutes, blotting on absorbent paper, then placing ticks individually in wells of prepared microtitre trays. Three replicate trays were incubated at 27°C and observed daily to record tick mortality. The assays were conducted three times.

Screening assays with larval ticks were conducted by applying 2 ml of each spore solution to the gridded packet test papers, then adding approximately 100 larvae after the papers had dried. Three replicate packets per treatment were incubated at 27°C for 4 days before the number of dead and live larvae was recorded. This assay was conducted three times; however the third time the larvae were only incubated for three days before the mortality was recorded.

3.1.5 Dose titrations

A series of dose assays were conducted with larval ticks. The first two assays used 6 different spore concentrations of isolates ARI-M52 and ARI-M63 suspended in 0.1% T80 (5×10^6; 1×10^7; 5×10^7; 1×10^8; 5×10^8; 1×10^9 spores/ml). Larvae were incubated for 4 days

before mortality was assessed. Further dose assays were conducted with only two spore concentrations of M52 and M63 (5×10^6; 5×10^7) formulated in 5% codacide. These assays were conducted at a constant temperature of 27°C, and then repeated with alternating temperatures (12:12; 38°C and 28°C). The dose assay at a constant 27°C was carried out 3 times, while the assay at alternating temperature was carried out twice. A dose assay using two other isolates (ARI-M12 and ARI-M16 at 5×10^6 and 5×10^7 sp/ml) was also carried out at the alternating temperatures twice for comparison.

Dose assays were conducted with fully engorged female ticks using isolates ARI-M52 and ARI-M63 formulated in either 0.1% T80 or 5% codacide oil to give two spore concentrations (5×10^6 and 5×10^7 spores/ml). Ticks immersed in the spore solutions made up with 0.1% T80 were incubated in microtitre trays with water agar added to the wells. Ticks immersed in solutions made up with 5% codacide did not have water agar in the microtitre trays. The assay with spores formulated in codacide oil was conducted twice.

3.1.6 Tick strain comparison

Fully engorged ticks of the acaricide resistant strains Dieldrin, Mt Alford, Parkhurst, Ultimo, Tiaro and Broadsound were treated with a suspension of spores of isolate ARI-M52 (Approximately 1×10^8 sp/ml in 0.1% T80). Groups of 12 ticks from each strain were immersed in the spore suspension for 1.5 min, blotted then placed individually into wells of microtitre trays with water agar for incubation at 27°C. Three replicate trays were used for each strain. Tick mortality was recorded daily for three days.

3.1.7 Formulation carrier

The effect of different concentrations of the oil based carrier on tick mortality was investigated under conditions of high and low moisture. Test formulations were prepared using spores of isolate ARI-M52 suspended in different amounts of codacide oil to give a final concentration of 1×10^8 sp/ml. The levels of oil tested in the formulations were: 0%; 2.5%; 5.0%; 7.5% 10% and 15%. Groups of 20 engorged females ticks were immersed in the test formulations for 1.5 minutes, blotted on absorbent paper, and then placed individually in wells of microtitre trays. Three replicates were used for each formulation. This experiment was repeated twice at high moisture and twice at low moisture. For high moisture the trays were placed inside large sealed plastic containers with moistened sponges lining the base. For low moisture the sponges were omitted. Ticks were incubated at 28°C for up to 7 days and tick mortality was recorded.

3.1.8 Statistical analyses

All analyses were conducted in Genstat (2009).

Female engorged and larval tick mortality data were subjected to binary analyses via a generalised linear regression with binomial distribution and logit link (McCullagh and Nelder. 1989). The full model was assessed and non-significant terms were progressively removed form the model to arrive at the final model.

Probit analyses were conducted to determine the effective dose of different *M. anisopliae* strains required to kill either 50% (ED_{50}) or 90% (ED_{90}) of tick larvae within a set timeframe. Data were prepared for probit analysis using Abbot's formula to give effective number treated (ENT) and corrected mortality (CM) for each dose level by isolate combination in each trial (Abbott, 1925). The data were transformed into a pivot table in a spreadsheet then formatted for GenStat. A number of analyses were performed using probit, logit and complementary log-log models for mortalities with log transformed (base e) and non-transformed concentrations to first establish the best model to suit all of the mortality data. The goodness of fit of each of these models to the data was determined by examining the

fitted curve, the residual deviance and the width of the LT_{50} fiducial limits, especially in the middle portion of the curve as this is the main area of interest. Based on these criteria the complementary log-log model was selected for analyses.

By examining the significance of the accumulated analysis of deviance for the effect of time, dose and the interaction between time and dose, the differences between the fitted dose response lines can be determined. If time, dose and the interaction between time and dose are all significantly different ($P<0.05$), different time-response lines are needed for the doses; i.e. the response to each dose is different. If the interaction between time and dose is not significant but time and dose are, it suggests the time-response lines are parallel, meaning that there is a similar response to the different doses, only the strength of the response is different. If neither dose nor the interaction between time and dose differs significantly it suggests that the time response lines are the same. There is no difference between the doses.

The efficacy of the tick treatments on animals was assessed following the method recommended by the World Association for the Advancement of Veterinary Parasitology (WAAVP) and outlined in Holdsworth *et al* (2006). This method calculates to percent efficacy of the treatment against tick parasitic stages based on the daily tick survival.

3.2 Pen study

This study was conducted at the moated cattle pens within the University of Queensland Vet Farm, 2436 Moggill Road, Pinjarra Hills, Queensland from 9 September until the end of October. The study was conducted under the APVMA Small-scale Trial Permit PER 7250 and with Animal Ethics approval from the University of Qld animal ethics committee – UQ animal ethics permit SVS/240/10MLA. The pens are situated within a fully-roofed, bird-proofed structure, open to ambient temperature with access to a common cattle crush facility. Pens have either corrugated tin walls on two sides or concrete brick walls on two sides, metal rails either end to open air, concrete flooring, with feed bins and water troughs positioned internally. A row of 12 contiguous moated pens, each measuring 1.58 m x 2.35 m (3.71 m^2), are perpendicularly adjacent to a second contiguous row of six pens, each measuring 1.70 m x 2.42 m (4.11 m^2).

3.2.1 Animals

Eighteen Hereford steers were obtained from a tick free region of northern New South Wales. The ages of the animals ranged from 8-10 months and their weights ranged from 120 to 160 kg (average 146 kg) at the start of the trial (Table 11). All steers were vaccinated against clostridial diseases (Coopers 5 in 1 cattle vaccine) and tick fever (Trivalent vaccine) and drenched for worms (oxfendazole, Coopers Systamex). Throughout the study the cattle were maintained on a standard 3% liveweight 'ZARI 64' cattle pellet ration per day supplied by Riverina Stock Feeds Pty Limited. Staff monitored and regulated food intake and any food intake abnormalities were noted. Water was available *ad libitum* throughout the course of the study.

Approximately 5,000 tick larvae (0.25 g of eggs allowed to hatch) were applied along the back of each heifer using a small paint brush three times a week for three weeks (Table 16). This approach ensured that all tick stages were present at the time of treatment. To assess the prophylactic (persistent) efficacy of treatment, three (Monday, Tuesday and Friday) infestations of 5000 NRFS larvae per animal were conducted post treatment.

A stratified randomized complete block design method was used to allocate animals to treatment groups. On day -1, cattle were ranked from highest to lowest on the basis of total individual day -3, -2, -1 pre-treatment tick counts and sequentially blocked into groups of

three. The three highest ranked animals formed Block 1, the next three highest animals formed Block 2 continuing to the lowest three animals which formed Block 6. From each block, individuals were randomly allocated to a pen from one of six pen groups (3 animals per group). Treatments were randomly allocated to the three pens within each of the six pen groups.

Animals were carefully observed daily during the trial for any adverse effects from the tick infestation and fungal treatments.

3.2.2 Treatment formulations

Dried spore powder of *Metarhizium anisopliae* isolates ARIM52 and ARIM63 was produced and supplied by Becker Underwood Australia and New Zealand. All spores were stored at 4°C before use. The spores were formulated in Codacide, an emulsifiable canola oil made by Microcide in the United Kingdom and supplied by Kendon, Melbourne, Australia. Equal amounts of the two isolates were mixed into the codacide to give two different dose rates. These formulations were later diluted to give the spray treatments as summarised in the table below.

Table 3. Components of formulations and spray treatments

	Codacide oil (L)	Weight spores (g)	Concentration of spores in formulation (g/L)	Final volume (L) of spray treatment (codacide %)	Concentration of spores in spray treatment (sp/ml)
Low Dose	3	75	25	60 (5%)	$\sim 5 \times 10^7$
High Dose	3	750	250	60 (5)	$\sim 5 \times 10^8$

3.2.3 Treatment application

During the afternoon of day 0 approximately 6-8 liters of fungal formulation was sprayed onto each of 6 animals in each treatment group. One group received the low dose ($\sim 5 \times 10^7$ spores/ml suspended in codacide oil (5%) and water). The second treatment group was sprayed with the high dose ($\sim 5 \times 10^8$ spores/ml suspended in codacide oil (5%) and water). The control group did not receive any treatment. The formulations were diluted with tap water in the spray tank at the trial site and the spray treatments were applied to cattle held in a crush using a tractor mounted Hardi PTO drive 95/10 spray unit. The spray unit operated at 6 bar, 1700 rev/min to deliver approximately 1.0 litre of formulation per minute through a Hardi – ISO Injet-01 fan nozzle. The time taken for the spray to saturate the animals was recorded. The spray technique followed that recommended by the FAO (1984) standard operating procedure for hand spraying cattle for tick control. Spraying started with the rear underparts moving to the flank, back and belly then front legs and axillae for both sides before finishing with the head and ears.

The control cattle were introduced to the crush first to conduct tick infestations, visual tick assessments and the collection of hair samples from each animal. The cattle to be treated were then taken through the crush for the application of treatments, infestation with larval ticks, visual assessment and the collection of hair samples. Once the last group had gone through the yards, both the race and cattle crush were thoroughly washed with detergent and the crush head bale was wiped over with 70% alcohol.

3.2.4 Assessment of treatment efficacy

Engorged female ticks drop off the cattle and are prevented from leaving the pen by a moat (water) surrounding the pen. The concrete floors of the pens are tapered towards a drain

that contained a wire mesh basket for collecting ticks when the individual pens were hosed out. The number of female engorged ticks collected was recorded from day -3 to day 0 and then daily from day 0 onwards for the treated and untreated groups until day 25.

In the morning of each designated day of tick collection, individual pens were hosed through and ticks that had dropped within the previous 24 hours collected into wire mesh baskets positioned under the effluent outlet of each pen. Ticks collected from the baskets were washed, dried, counted and weighed and a representative sample of 20 ticks (where available) from each animal were weighed and incubated at approximately 28°C and 85% RH for a period of 7 days. The weight of eggs produced at the end of 7 days was determined. A visual examination of the ticks for mortality and egg production was made and observations noted.

Percentage efficacy of the treatments on parasitic tick stages was calculated as outlined in the WAAVP guidelines for evaluating the efficacy of acaricides against ticks (Holdsworth *et al*, 2006) which follows:

Daily percentage (%) Efficacy = 100 – $\left(\frac{TickCountTreatedGroup}{ADEQ} \times 100\right)$

Where ADEQ is the number of ticks expected in the treated group if left untreated.

ADEQ = $\frac{Total\,Pre-TreatmentCountsTreatedGroup}{Total\,Pre-TreatmentCountsControlGroup} \times$ Daily control count

Comparisons of tick mortality after incubation as well as viable eggs produced by ticks collected from treated and control animals were made to assist in measuring efficacy.

3.2.5 Formulation check

Female engorged female ticks (20 per sample) supplied from the Biosecurity Queensland tick cultures were immersed for 1.5 min in samples of the formulation that were collected immediately before and during application to animals. Ticks were blotted on absorbent paper, then added to 24-well micro titre trays with water agar in the wells for incubation at 28°C. Tick mortality was assessed each day for three days.

3.2.6 Application check

Twenty semi-engorged female engorged ticks between 4.5 and 8 mm were removed from each animal immediately after treatment for laboratory incubation at 28°C in microtitre trays with water agar in the wells. Tick mortality was assessed daily for three days.

3.2.7 Temperatures

The ambient temperatures and the surface temperatures of selected animals were recorded. Three data loggers (Tinytag View®, Hastings data loggers) to record the relative humidity and ambient temperature every 20 minutes were placed in different locations in the pen facility. Remote sensor data loggers (Tinytag Talk®, Hastings data loggers) were attached to the base of the tail of nine animals (three / treatment group) using sticky plaster. The sensors were secured in the coat along the backline of each animal. The data loggers recorded the surface temperature of these animals every 20 minutes.

3.2.8 Protocol variation and re-treatment

When the temperature loggers on animals showed that pen temperatures had been too high for optimal fungal growth the protocol was varied. Nine animals (5 high dose and 4 low dose)

were re-treated with the fungal formulation, two low dose and one high dose animal were left untreated to gauge the effect of the original treatment. Data loggers were attached to four of the re-treated animals. After spraying, all animals were kept out of the pens so as to be exposed to the natural conditions of breeze and lowered night temperatures for up to four nights. Animals were then returned to their pens so that engorged tick collections could recommence.

4 Results

4.1 Laboratory studies

4.1.1 Temperature characterisation

All seven *M. anisopliae* isolates grew well at all temperatures between 15°C and 33°C, but no isolates were able to grow at 36°C (Tables 4 and 5). All isolates grew better at 33°C than at 15°C and the greatest colony diameters, representing the growth optima, were achieved at 24°C and 27°C. The isolates that grew best at 33°C were ARI-M63 and ARI-M12. When temperatures were alternated between a temperature outside the growth range of the isolates and one within the growth range, all isolates grew, although more slowly. Most colony diameters were approximately half those achieved at 30°C (Table 6) in the same time period.

Table 4. Average colony diameter (mm) after 14 days growth from an inoculated 8 mm disc in the temperature range of 15°C to 27°C

Isolate	15ºC	18ºC	21ºC	24ºC	27ºC
ARI-M10	30.0	47.0	54.8	58.5	71.7
ARI-M12	25.6	42.9	40.0	68.3	68
ARI-M16	25.9	45.6	45.9	63.0	62
ARI-M52	25.3	38.9	51.3	59.5	69.7
ARI-M61	24.6	40.4	45.5	48.4	61.7
ARI-M63	25.7	45.7	45.8	58.3	68.4
ARI-M80	32.6	51.8	57.0	71.8	73.9

Table 5. Average colony diameter (mm) after 14 days growth from an inoculated 8 mm disc in the temperature range of 24°C to 36°C.

Isolate	24ºC	27ºC	30ºC	33ºC	36ºC
ARI-M10	67.3	72.8	56.6	35.0	8
ARI-M12	60.3	72.4	63.3	40.0	8
ARI-M16	61.4	70.6	65.1	36.2	8
ARI-M52	66.5	68.3	50.9	33.6	8
ARI-M61	65.6	69.4	64.4	36.7	8
ARI-M63	61.0	69.5	65.1	43.4	8
ARI-M80	74.0	75.3	64.3	33.2	8

Table 6. Average colony diameter (mm) after 14 days growth from an inoculated 8 mm disc at alternating temperatures

Isolate	25 & 35ºC	30 & 35º	28 & 38ºC
ARI-M10	34.6	36.0	22.4
ARI-M12	35.4	38.7	23.9
ARI-M16	34.6	39.5	24.4
ARI-M52	30.4	31.6	16.5
ARI-M61	35.0	35.5	23.2
ARI-M63	36.5	36.1	19.5
ARI-M80	28.8	33.5	10.3

4.1.2 Isolate selection

Across the female engorged screening assays there was quite a lot of variation in the tick mortality caused by the different isolates. Isolates ARI-M52, ARI-M10, ARI-M63 and ARI-M80 appeared to be the most virulent causing average tick mortalities from 95% to 99% after 3 days incubation (Figure 1).

Due to the limits on tick availability, different strains were used for screening. However the statistical analysis of the tick mortality did not identify any effect due to tick strain on day 3.

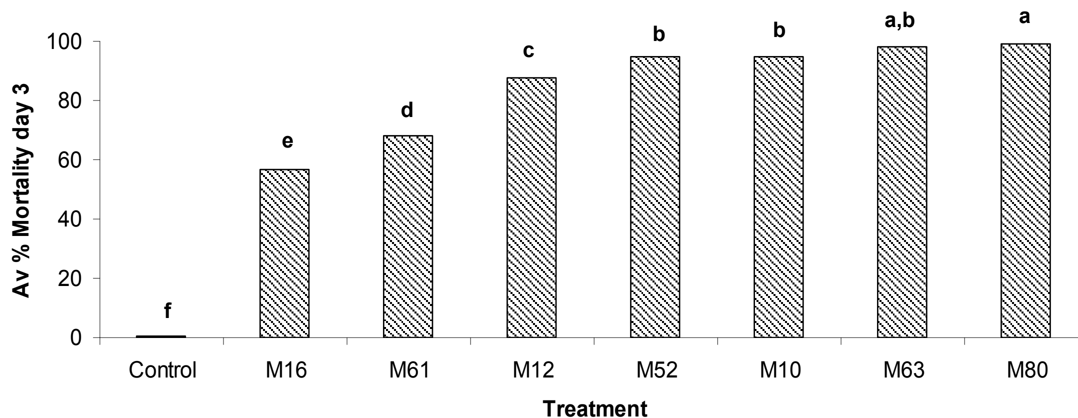

Figure 1. Average percent mortality of engorged female ticks incubated at 27°C for 3 days after immersion in spore suspensions of different *M. anisopliae* isolates (Different letters indicate a significant difference P<0.05)

When the fungal isolates were screened against larval ticks there was no significant difference in the mortality caused by 5 of the isolates after 4 days incubation (Figure 2).

However when the larval mortality was assessed after 3 days two isolates, ARI-M52 and ARI-M80 caused significantly higher mortalities (96% and 77% respectively) than the other 5 isolates (Figure 3). There was no significant difference between the other 5 isolates that caused between 49% and 30% mortality after 3 days.

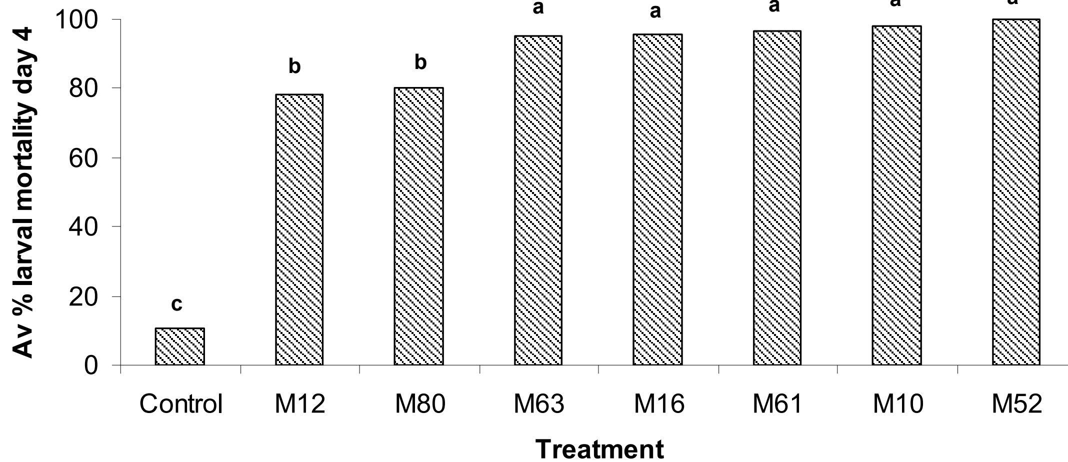

Figure 2. Average percent mortality of larval ticks incubated at 27°C for 4 days in packets treated with spore suspensions of the 7 different *M. anisopliae* isolates (Different letters indicate a significant difference P<0.05)

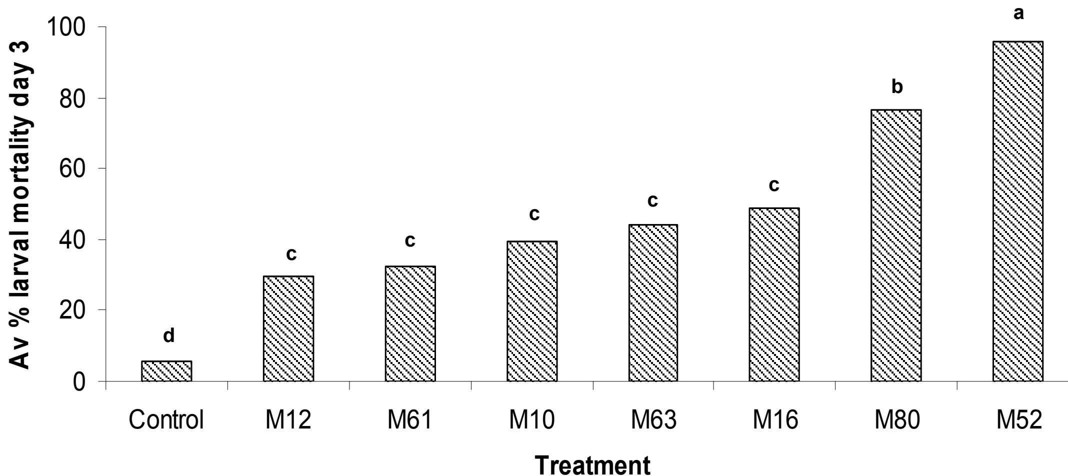

Figure 3. Average percent mortality of larval ticks incubated at 27°C for 3 days in packets treated with spore suspensions of the 7 different *M. anisopliae* isolates (Different letters indicate a significant difference P<0.05)

4.1.3 Dose titrations

When a range of spore concentrations of isolates ARI-M63 and ARI-M52 were tested against tick larvae all doses from 5×10^6 to 1×10^9 spores/ ml killed all larvae after 4 days incubation. When the dose assays were conducted with only the lower doses of 5×10^6 and 1×10^7 spores/ml all larvae were again killed after 4 days incubation at 27°C.

When these assays were repeated with the temperature alternating between 38°C and 28°C there was no significant difference between the larval mortality caused by the two isolates at either 5×10^6 or 5×10^7 spores/ml (Figure 4). Although there was a decrease in larval mortality due to the higher temperature from 100 % mortality for both concentrations to 92% for ARI-M52 and 81% for ARI-M63 at the higher spore concentration of 5×10^7 spore/ml.

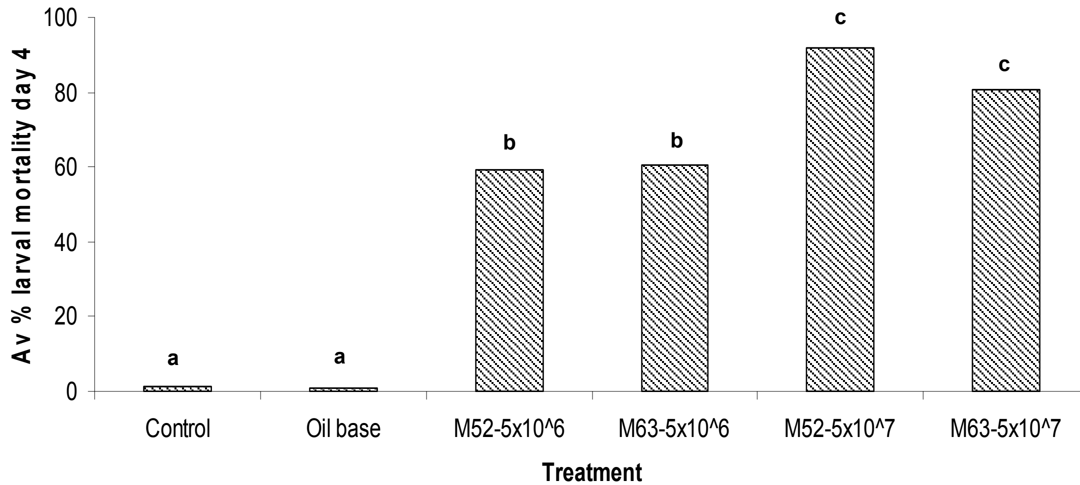

Figure 4. Average percent larval tick mortality when incubated for 12:12 hr at 28°C and 38°C in packets treated with either 5 × 10^6 or 5 × 10^7 spores/ml of the *M. anisopliae* isolates M52 and M63

As a comparison the incubation of larval ticks in treated packets at alternating temperatures was repeated with the *M. anisopliae* isolates ARI-M12 and ARI-M16 (Figure 5). Each dose of each isolate was significantly different, with ARI-M16 killing more larval ticks than ARI-M12. However ARI-M16 was not as virulent to larval ticks at alternating temperatures as either of the isolates ARI-M52 and ARI-M63.

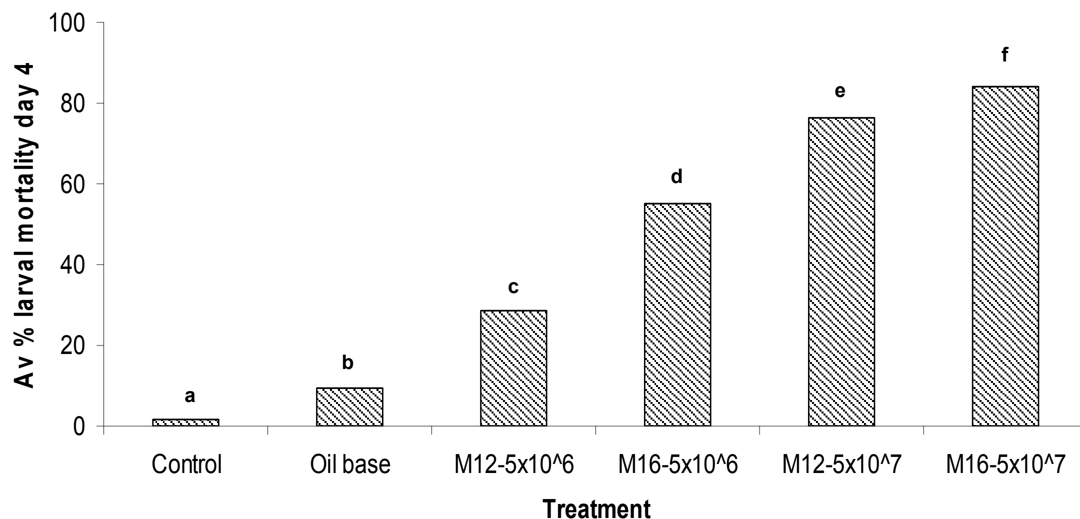

Figure 5. Average percent larval tick mortality when incubated for 12:12 hr at 28°C and 38°C in packets treated with either 5 × 10^6 or 5 × 10^7 spores/ml of the *M. anisopliae* isolates M12 and M16

Female engorged tick dose assays with the isolates ARI-M52 and ARI-M63 formulated in 0.1% T80 demonstrated no significant difference between the tick mortality caused by either isolate after 2 and 3 days. The effective dose of these isolates to kill 90% of female engorged ticks in three days was estimated to be 1.1 × 10^8 spores/ml (Table 7). The estimate for 90% mortality in two days was 9.3 × 10^9 spores/ml.

Table 7. Effective doses of *M. anisopliae* isolates ARI-M52 and ARI-M63 required to kill 50% (ED_{50}) and 90% (ED_{90}) of female engorged ticks when formulated in 0.1% T80

Isolate	Day	ED_{50} (95% CI)	ED_{90} (95% CI)
ARI-M52/M63	2	1.3×10^9 (5.3×10^9 - 1.9×10^{10})	9.3×10^9 (2.1×10^9 - 3.2×10^{12})
ARI-M52/M63	3	4.1×10^7 (3.2×10^7 - 5.1×10^7)	1.1×10^8 (8.9×10^7 - 1.62×10^8)

When the spores of these isolates were formulated in 5% codacide the concentration of spores required to kill 90% of engorged female ticks decreased (Tables 8 and 9). In the first dose assay there was no significant difference between the concentrations of ARI-M52 or ARI-M63 spores required to kill ticks after two days (Table 8). However, after three days it was estimated that a lower dose of ARI-M52 spores (1.7×10^8 sp/ml) than ARI-M63 spores (2.4×10^8 sp/ml) was required to kill up to 90% of ticks. The predicted dose for both isolates to kill up to 90% of larval ticks in 2 days is 3.55×10^8 spores/ml.

Table 8. Effective doses of *M. anisopliae* isolates M52 and M63 required to kill 50% (ED_{50}) and 90% (ED_{90}) of female engorged ticks when formulated in 5% Codacide oil (first assay)

Isolate	Day	ED_{50} (95% CI)	ED_{90} (95% CI)
ARI-M52/M63	2	1.34×10^8 (1.0×10^8 - 1.7×10^8)	3.6×10^8 (2.7×10^8 - 5.0×10^8)
ARI-M52	3	4.9×10^7 (4.0×10^7 - 6.0×10^7)	1.7×10^8 (1.3×10^8 - 2.2×10^8)
ARI-M63	3	7.1×10^7 (5.7×10^7 - 8.7×10^7)	2.4×10^8 (1.9×10^8 - 3.2×10^8)

In the second assay with spores of isolates ARI-M52 and ARI-M63 formulated in oil the effective doses of both required to kill ticks was lower than in the first assay (Table 9). The concentration of ARI-M63 spores required to kill up to 90% of ticks on both days 2 and 3 was significantly lower than that required for isolate ARI-M52 (Table 9). The predicted dose to kill 90% of larval ticks in 2 days is 1.5×10^8 spores/ml for isolate ARI-M52 and 5.7×10^7 spores/ml for isolate ARI-M63.

Table 9. Effective doses of *M. anisopliae* isolates ARI-M12 and ARI-M16 required to kill 50% (ED_{50}) and 90% (ED_{90}) of female engorged ticks when formulated in 5% Codacide oil (second assay)

Isolate	Day	ED_{50} (95% CI)	ED_{90} (95% CI)
ARI-M52	2	6.1×10^7 (4.4×10^7 - 8.3×10^7)	1.5×10^8 (1.0×10^8 – 2.3×10^8
ARI-M63	2	2.4×10^7 (1.6×10^7 - 3.2×10^7)	5.7×10^7 (4.2×10^7 – 8.5×10^7)
ARI-M52	3	1.6×10^7 (1.1×10^7 – 2.4×10^7)	3.2×10^7 (2.2×10^7 – 5.8×10^7)
ARI-M63	3	8.3×10^6 (6.2×10^6 - 1.1×10^7)	1.7×10^7 (1.2×10^7 - 2.8×10^7)

4.1.4 Tick strain comparison

There was no significant difference in the tick mortality for the different tick strains when treated with *M. anisopliae* isolate ARI-M52. When the six different tick strains were immersed in a formulation of ARI-M52 spores between 100% and 97% of ticks were dead by day 3 (Figure 6).

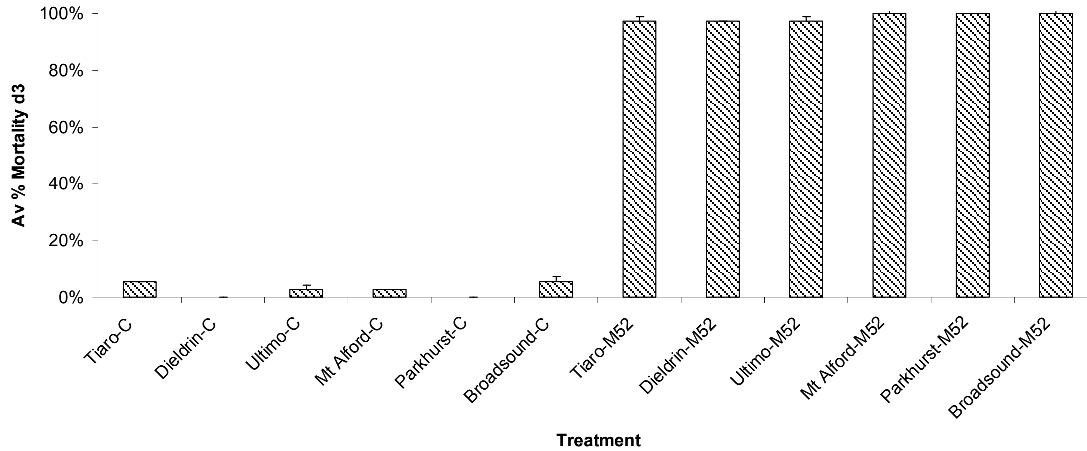

Figure 6. Average percent mortality of different strains of engorged female ticks incubated at 27°C for 3 days after immersion in spore suspension of *M. anisopliae* isolate ARI-M52

4.1.5 Formulation carrier

Increasing the level of oil in the formulation did not have any effect on engorged female tick mortality at either high moisture or low moisture (Table 10). However the level of moisture in the environment of the infected ticks had a highly significant effect on tick mortality ($P \leq 0.001$).

Table 10. Effect of oil concentration in formulations of ARI-M52 spores on engorged female tick mortality after 2 days incubation at either high or low moisture

Treatment	Average % mortality day2 at High Moisture	Average % mortality day 2 at Low Moisture
Control	0(0)	0(0)
0% Oil	59(13)	1(1)
2.5% Oil	79(11)	0(0)
5.0 % Oil	72(10)	1(1)
7.5% Oil	66(9)	1(1)
10% Oil	81(5)	0(0)
15% Oil	68(7)	2(2)

4.2 Pen studies

4.2.1 Formulation check

The virulence of the formulation was tested after it had passed through the pressurised spray unit. All engorged female ticks treated with both the low dose and high dose formulations were dead after 48 hours of laboratory incubation (Figure 7). The spray unit did not have any effect on the virulence of the formulation.

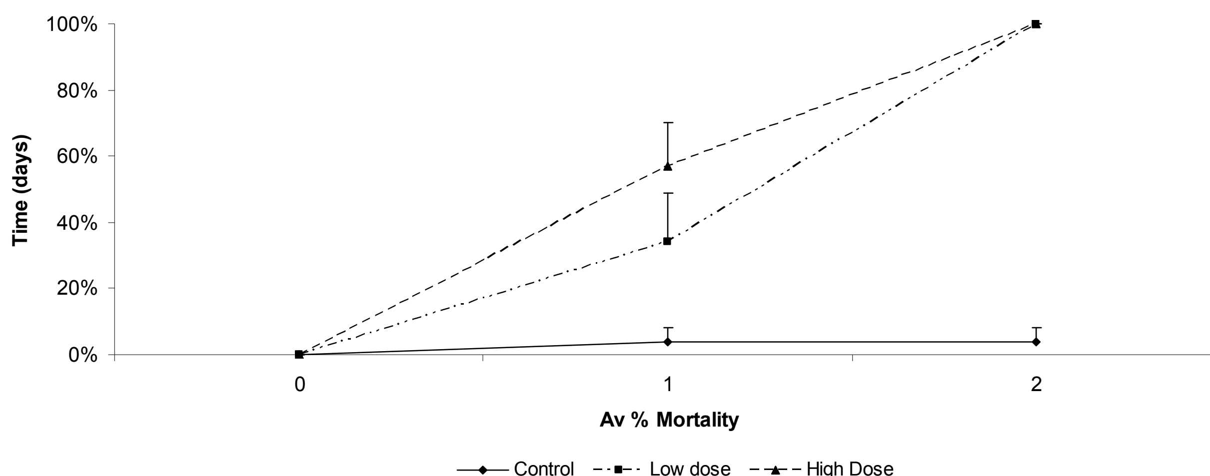

Figure 7. Average mortality, after two days, of groups of engorged female ticks immersed in samples of the low and high dose formulations collected during spraying

4.2.2 Application check

Semi-engorged female ticks were randomly sampled from treated animals immediately after spraying for laboratory incubation to check that the spray application had delivered enough spores to kill ticks under optimal conditions of temperature and moisture.

Table 11. Average mortality of semi-engorged female ticks sampled from different animals after spray application, the weight of the animals as well as the length of time the animals were sprayed are also given

Treatment	Animal	Initial weight (kg)	Spray time (min)	Total ticks sampled	Dead d1	Dead d2	Dead d3
Control	890	145	NA	20	2	2	3
Control	908	145	NA	20	0	0	0
Control	899	155	NA	20	0	0	0
Control	909	130	NA	20	1	4	4
Control	891	160	NA	20	1	2	2
Control	894	160	NA	20	0	0	1
Low Dose	892	145	7	9	0	4	6
Low Dose	897	155	8	20	1	18	20
Low Dose	905	140	6.5	20	7	15	18
Low Dose	907	160	5.5	20	2	12	19
Low Dose	906	120	5.5	20	4	16	20
Low Dose	903	140	5	20	4	17	20
High Dose	896	130	7.5	20	16	20	20
High Dose	898	150	5.5	20	19	20	20
High Dose	893	150	6	20	16	20	20
High Dose	900	160	5.5	20	18	20	20
High Dose	904	130	4.5	16	15	16	16
High Dose	889	150	5.5	20	3	20	20

All animals were sprayed until thoroughly wetted. The spray time, thus amount of formulation delivered to the animal coat, varied with animal but it did not seem to affect the rate at which the sampled ticks died in the laboratory. There did not appear to be a relationship between the length of time animals were sprayed and the weight of the animal (Table 11).

All ticks removed from animals receiving the high dose were dead after 2 days of incubation, whereas only 72% of ticks sampled from the low dose animals had died (Figure 8). By three

days 92% of ticks sampled from low dose animals were dead as compared to 23% of ticks sampled from control animals. The high control mortality may represent the damage sustained by semi-engorged female ticks when removed from cattle.

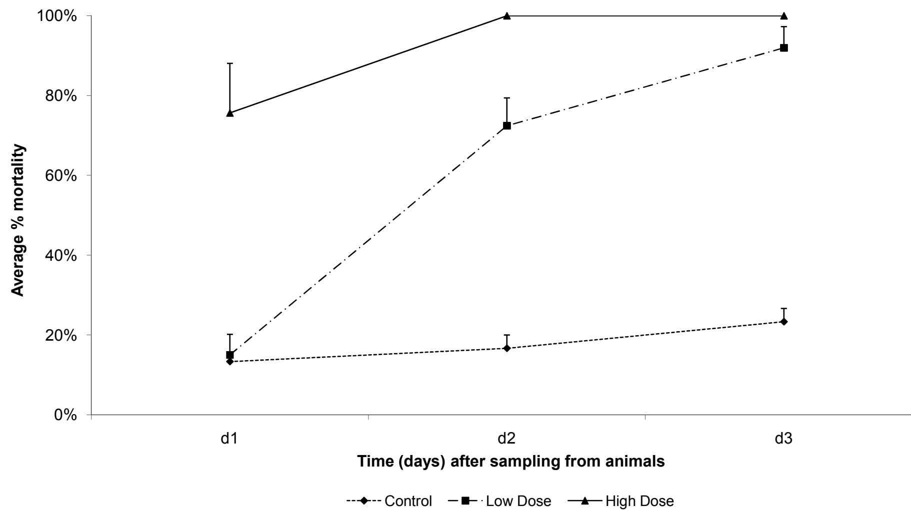

Figure 8. Average mortality in semi-engorged female ticks (4.0–7.5 mm) sampled from animals immediately after treatment

4.2.3 Temperature

The surface temperatures recorded on penned animals in the five days after treatment showed that they were rarely below 32°C, but were mostly in the range of 34-36°C (Table 12). When the animals were released out of the pens into paddocks, returning to the pens during the fourth day the surface temperatures were on average much lower (Table 13). While temperatures spiked over 36°C for periods of time during the middle of the day these temperatures were balanced by longer periods below 32°C during the night. Unfortunately some of the loggers on the animals in the paddocks ceased working after a few days, most likely due to the activity of those animals. Therefore there were fewer data logger readings after 4 days.

Table 12. Number of hours surface temperatures recorded on penned animals after treatment (day 0) were within specified ranges

Temp.	Logger	Day 0 Oct 4**	Day 1 Oct 5	Day 2 Oct 6	Day 3 Oct 7	Day 4 Oct 8	Day 5 Oct 9
>36°C	3	0	0	5	0	0	0
	5	0	3	4	17	0	
	7	6	6	8	6	0	0
	8	0	2	7	0	0	0
34 - 36°C	3	12	14	4	9	0	0
	5	0	12	8	4	15	
	7	6	12	12	17	18	0
	8	3	11	7	12	0	0
32 - 34°C	3	0	10	15	15	3	0
	5	6	9	11	9	9	
	7	0	6	4	1	6	20
	8	9	3	0	12	24	9
<32°C	3	0	0	0	0	21	24
	5	0	0	0	0	0	
	7	0	0	0	0	0	4
	8	0	8	0	0	0	15
Av hrs <32°C		**0**	**2**	**0**	**0**	**5**	**14.3**

(Loggers applied at noon so only 12 hours recorded for that day)**

Table 13. Number of hours surface temperatures recorded on animals outside in paddocks after re-treatment (day 0) were within specified ranges. Note that logger 3 stopped working after day 3 and loggers 8 and 5 only recorded for part of day 4

Temp.	Logger	Day 0 Oct 18**	Day 1 Oct 19	Day 2 Oct 20	Day 3 Oct 21	Day 4 Oct 22
>36°C	3		6	0	NR	NR
	5		5.5	3	2	0.5
	7	0	6	1	0.5	0
	8	0.5	9	1	7.5	2
34 - 36°C	3		1.5	2	NR	NR
	5	1.5	1.5	1	5	0.5
	7	0.5	1	2	0	3
	8	0	2	4	3.5	
32 - 34°C	3		3	1.5	NR	NR
	5		3	4	2	2
	7	0.5	1	1	1.5	6
	8	1	6	16	7	
<32°C	3	7	13.5	20.5	NR	NR
	5	6.5	14	16	15	7
	7	8	14	20	16	15
	8	7	7	3	6	6
Av hrs <32°C		**7**	**12**	**15**	**11**	**9**

(Loggers applied in afternoon so <12 hours recorded for that day)**

The ambient data loggers recorded similar daily temperature ranges when the animals were penned or were released to the paddock (Tables 14 & 15). Table 14 shows the ambient temperatures during the first five days after the initial treatment. During these days the temperature ranged from 16°C to 30.5°C and the average number of hours that the temperature dropped below 22°C varied between 9 and 22.3 hours per day.

While the animals were in the paddock the temperature ranged from 11°C to 34°C and the average number of hours in which the temperature dropped below 22°C varied from 6.7 to 23 hours per day (Table 15).

Table 14. Ambient temperatures recorded from three data loggers in the five days after animals were treated

Amb Logger	Temp range	Day 0 Oct 4	Day 1 Oct 5	Day 2 Oct 6	Day 3 Oct 7	Day 4 Oct 8	Day 5 Oct 9
3	Max (°C)	22	30	27	30.5	30	22.5
	Min (°C)	19	17.5	17.5	18.5	20.5	18.5
	Hrs ≤ 22°C	9	15	12	15	8	24
2	Max (°C)	22	29	27	30	30	22
	Min (°C)	20	17	16	17	19.5	17
	Hrs ≤ 22°C	9	15	14	14	9	24
1	Max(°C)	22	31.5	29	33.5	31.5	22.5
	Min (°C)	19	17	16.5	17	19.5	17.4
	Hrs ≤ 22°C	9	14	13	12	11	19
	Av hrs ≤ 22°C	**9**	**14.7**	**13**	**13.7**	**9.3**	**22.3**

Table 15. Ambient temperatures recorded from three data loggers in the five days after animals were retreated

Amb Logger	Temp range	Day 0 Oct 18	Day 1 Oct 19	Day 2 Oct 20	Day 3 Oct 21	Day 4 Oct 22	Day 5 Oct 23
Inside 1	Max (°C)	33	30	22	23.5	29	31
	Min (°C)	14	12	15	14	12	13.5
	Hrs ≤ 22°C	6	15	24	14	15	14
Inside 2	Max (°C)	34	27.5	22	23.5	26.5	29
	Min (°C)	13	11	14.5	13	11	13
	Hrs ≤ 22°C	7	15	24	21	15	14
Outside	Max (°C)	29.5	30	23.5	24.5	28	30.5
	Min (°C)	13	11	15	13	11	13
	Hrs ≤ 22°C	7	12	21	14	12	12
	Av hrs ≤ 22°C	**6.7**	**14**	**23**	**16.3**	**14**	**13.3**

When the average daily hours that the surface temperature on the animals kept in paddocks drops below 32°C is plotted with the average daily hours the ambient temperature drops below 22°C there appears to be a trend (Figure 9). This trend suggests that the surface temperature on the animals is influenced by the lower ambient temperatures. However when the same data are plotted together for animals kept in pens the trend does not appear to be as strong (Figure 10). One possible explanation for this is that the pens insulated the surface of the animals from the cooling effects of breezes and night temperatures.

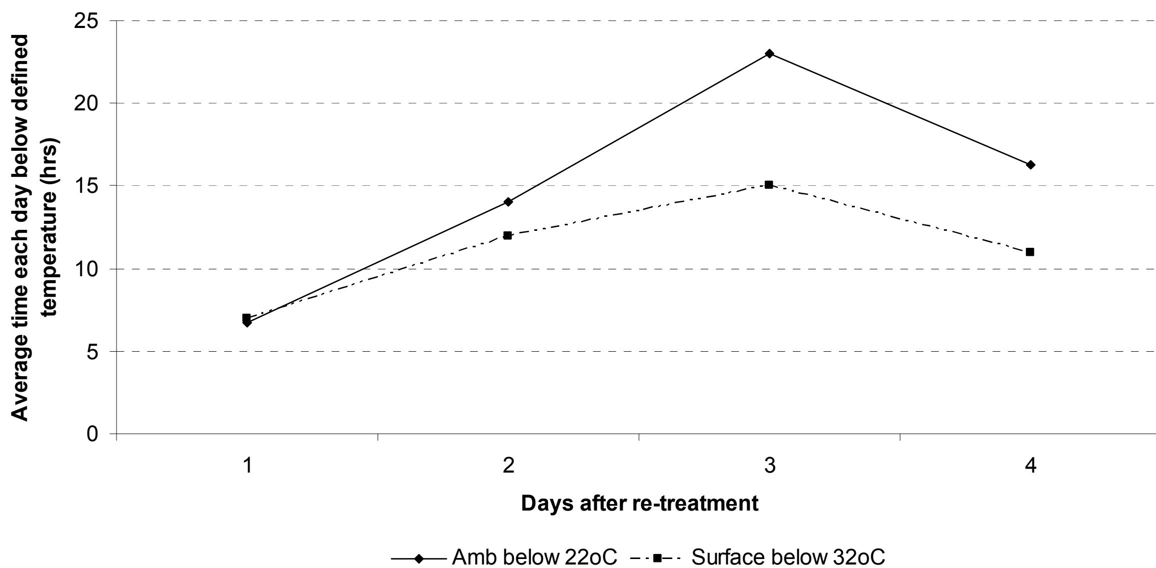

Figure 9. The number of hours the ambient temperature was below 22°C per day compared to the number of hours the surface temperature on animals fell below 32°C when animals were under field conditions

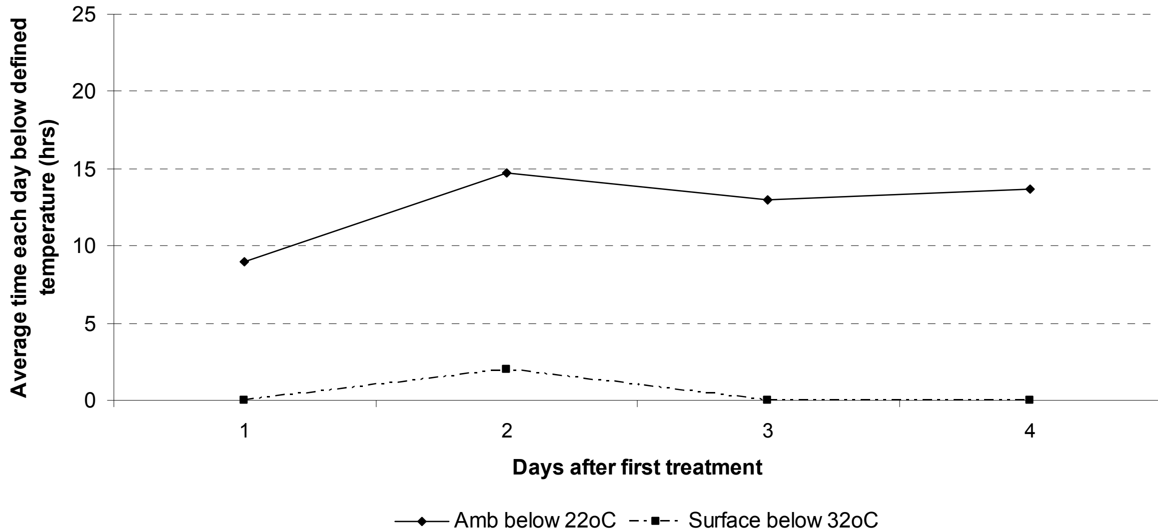

Figure 10. The number of hours the ambient temperature was below 22°C per day compared to the number of hours the surface temperature on animals fell below 32°C when animals were penned inside

4.2.4 Assessment of treatment efficacy

4.2.4.1 Engorged female tick mortality

The mortalities (%) of samples of engorged ticks incubated for 7 days at 28°C and 75% RH after collection from animals in the four days after treatment are shown in Figure 11. Ticks collected from animals treated with the high dose treatment had consistently higher mortalities than ticks collected from control or low dose animals for all days. Ticks collected from the animals treated with the low dose also had higher mortalities than ticks from the control animals every day except for day 2.

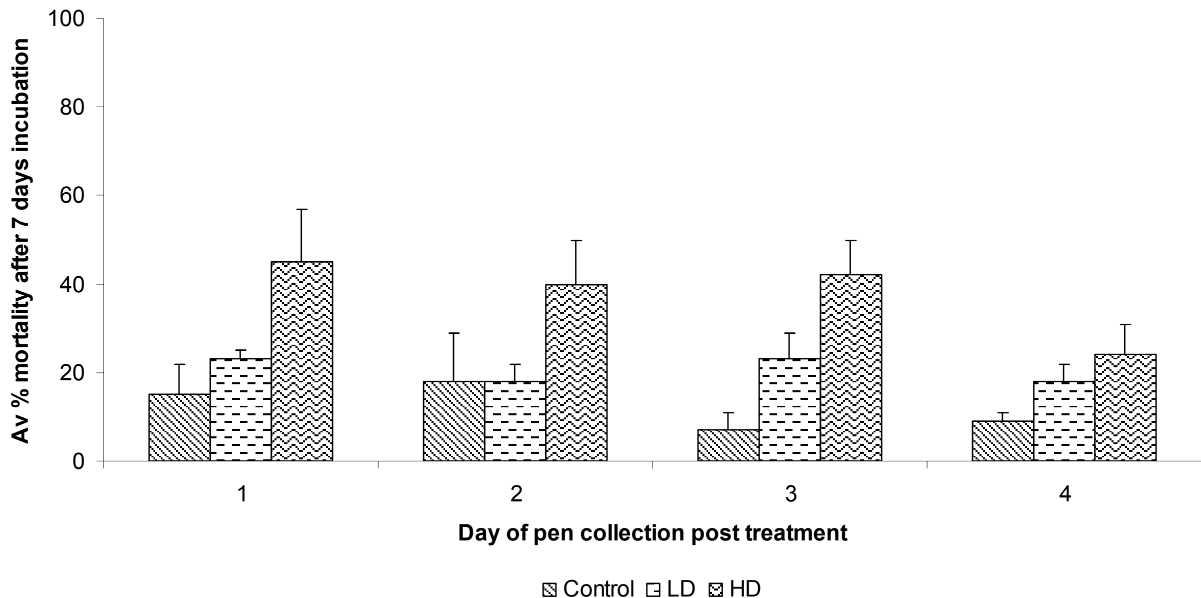

Figure 11. Mortality of samples of engorged female ticks collected in the first four days post treatment and incubated for egg production; mortality was assessed after 7 days

4.2.4.2 Egg production *in vitro*

The egg production from samples of engorged ticks collected in the first four days after treatment is shown in the figures below. Figure 12 gives the average weight of eggs (g) produced by 20 ticks sampled from each animal in a treatment group. There appears to be a slight reduction in egg production from ticks collected from treated animals in the first 3 days post treatment.

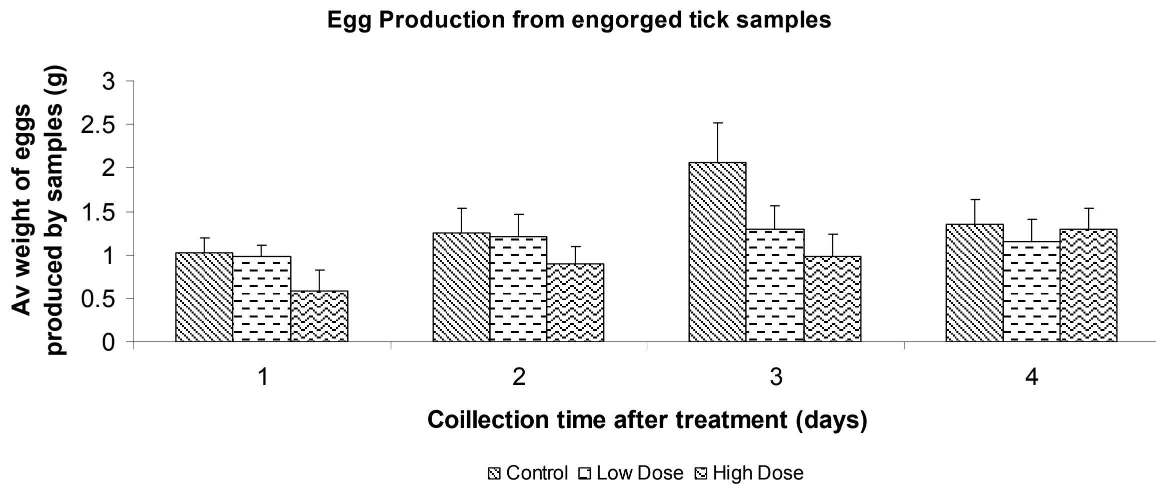

Figure 12. Average weight of eggs produced by samples of 20 engorged ticks incubated for 7 days at 28°C

4.2.4.3 Engorged tick drop

There was a very large variation in the number of engorged ticks dropping from some of the animals in each treatment group, but more so in the untreated control group. In addition a number of animals consistently dropped semi-engorged ticks (4.5 – 8 mm) until the last week of the trial when they began dropping normal engorged ticks (10 – 12 mm). The calculation of the daily percent efficacy of treatment does allow for such variation in the untreated controls. Figure 13 shows that the efficacy achieved in the first 14 days post treatment was mostly 40% and 50% rising to 60% for the high dose day 11 post treatment.

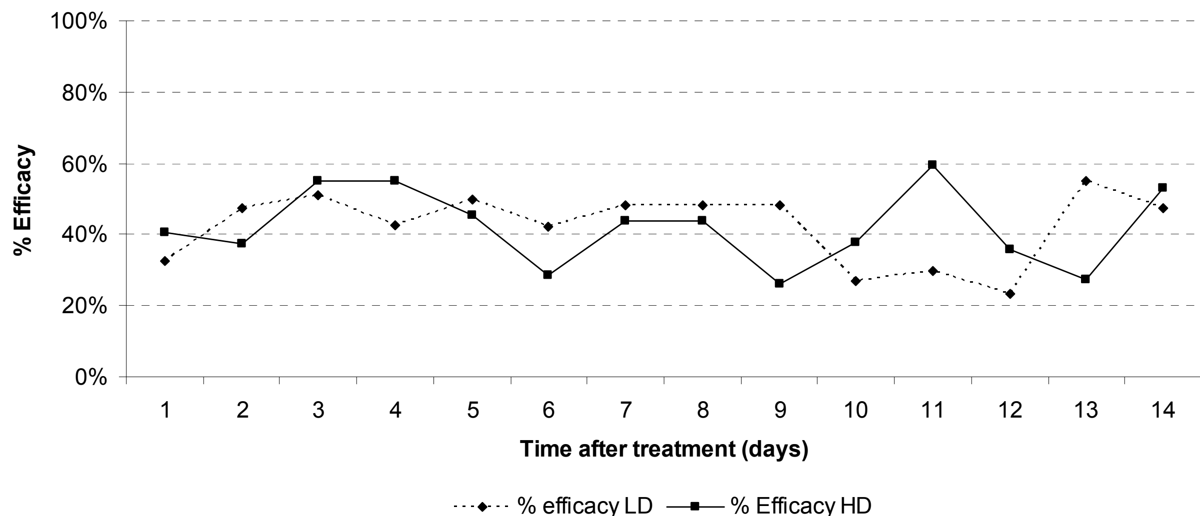

Figure 13. Percent efficacy of the 2 dose levels of the fungal biopesticide against ticks in the 14 days following spray treatment

Figure 14 shows the percent efficacy achieved after re-treatment once the animals had been returned to their pens at day 18. One group of the original treated animals were not re-treated so that the effect of the original treatment on larvae applied after treatment could be seen. In this group the efficacy was mostly over 60%, ranging from 48% up to 84%. The efficacy in the re-treated group while climbing to 70% was overall lower. The efficacy in both treatment groups dropped between days 19 and 23. During this time tick numbers on all of the untreated control animals decreased to unusually low levels increasing back to the expected levels for untreated controls in the last few days of the trial. The calculation of daily percent efficacy can't account for such unusual counts in the untreated control group, hence the efficacy for both treated groups decreased during this time.

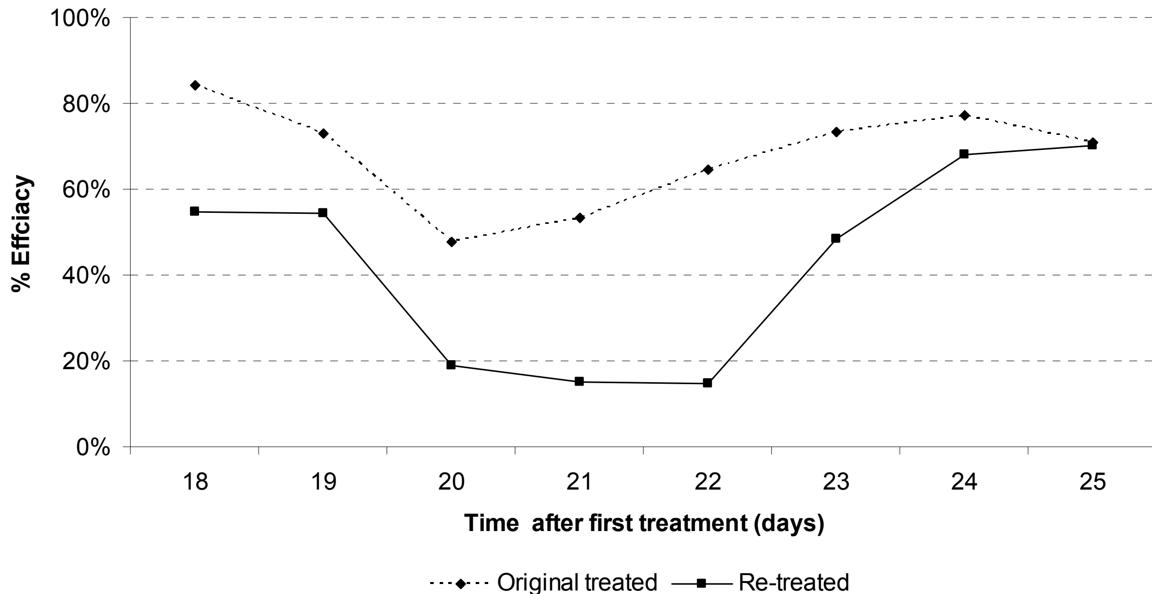

Figure 14. Percent efficacy of the fungal biopesticide against ticks on cattle 18 to 25 days after the initial treatment and on cattle re-treated 14 days after the initial treatment. The efficacy is affected by very low and inexplicable numbers of ticks on all control animals between 19 and 23 days

4.2.5 Larval infestation

The schedule for larval batch infestation is shown in Table 16. This schedule was complied with the approximate engorgement dates and average numbers of engorged ticks collected from control animals to gain an insight into why the numbers of ticks dropping from the control animals decreased to abnormally low levels 19 to 23 days post treatment. Unfortunately there does not seem to be any relationship between batch and the low numbers that can easily explain these low tick numbers.

Table 16. Schedule of larval infestation of cattle and numbers of engorged females dropping from control animals 21 days post application

Date larvae applied	Larval batch	~ drop date (21d)	Trial day	Av Control number	graph trend
10-Sep	3394	2-Oct	-2	56	low
13-Sep	3394	4-Oct	0	169	med
15-Sep	3394	6-Oct	2	281	high
17-Sep	3395	8-Oct	4	345	v. high
20-Sep	3398 (11 aug)	11-Oct	7	298	v.high
22-Sep	3398 (11 aug)	13-Oct	9	239	dec trend
24-Sep	3398 (11 aug)	15-Oct	11	247	same
27-Sep	3399 (18 aug)	18-Oct	14	172	decreasing
29-Sep	3399 (18 aug)	20-Oct	16	??	animals outside
1-Oct	3399 (18 aug)	22-Oct	18	64	v. low
4-Oct	3399 (18 aug)	25-Oct	21	28	v.v. low
6-Oct	3399 (18 aug)	27-Oct	23	94	rising
8-Oct	3399 (18 aug)	29-Oct	25	161	med -rising

4.2.6 Spore viability in cattle coats

Samples of hair taken from animals after treatment with the fungal biopesticide showed that the spores remained viable for up to 11 days. The presence of *Metarhizium* colonies growing on selective media when spore solutions from coat washings were plated gave and estimate of spore viability Table 17). The washings from hair samples taken immediately after treatment yielded a lawn of colonies too numerous to count for both doses. This confirms that the spores were viable once sprayed into the coat. Figure 15 shows *Metarhizium* colonies growing from these coat washings. Some of the samples taken nine days after treatment yielded viable spores which gave rise to many robust *Metarhizium* colonies (Figure 16). Viable spores were recovered from hair sampled from one animal 11 days post treatment (Figure 17). The 11 day period post treatment for this sample group included 4 days outside exposed to the effects of UV light and high midday temperatures on spores in the coat.

Table 17. Number of spores washed from samples of hair taken from cattle immediately after treatment and then at two later dates as well as the resulting colonies growing from viable spores

Treatment	Animal Number	Sample date	Hair washing Spores/ml	*Metarhizium* colonies on plates
Control	890	T0 (Oct 4)	0	None
	894	T0 (Oct 4)	0	None
	899	T0 (Oct 4)	0	None
	908	T0 (Oct 4)	0	None
	909	T0 (Oct 4)	0	None
	891	T0 (Oct 4)	0	None

Treatment	Animal Number	Sample date	Hair washing Spores/ml	*Metarhizium* colonies on plates
Low Dose	892	T0 (Oct 4)	9.17x10^4	lots
	897	T0 (Oct 4)	3.33x10^5	lots
	903	T0 (Oct 4)	1.05x10^6	small amount
	905	T0 (Oct 4)	3.3x10^4	lots
	906	T0 (Oct 4)	1.51x10^6	lots
	907	T0 (Oct 4)	3.23x10^5	lots
High Dose	889	T0 (Oct 4)	3.45x10^6	covered
	893	T0 (Oct 4)	2.23x10^7	lots
	896	T0 (Oct 4)	2.03x10^7	covered
	898	T0 (Oct 4)	4.44x10^7	covered
	900	T0 (Oct 4)	1.64x10^7	covered
	904	T0 (Oct 4)	1.02x10^7	covered
Control	890	T9 (Oct 13)	0	None
	894	T9 (Oct 13)	0	None
	899	T9 (Oct 13)	0	None
	908	T9 (Oct 13)	0	None
	909	T9 (Oct 13)	0	None
	891	T9 (Oct 13)	0	None
Low Dose	892	T9 (Oct 13)	7.5x10^4	Some
	897	T9 (Oct 13)	0	None
	903	T9 (Oct 13)	2.5x10^4	Some
	905	T9 (Oct 13)	0	None
	906	T9 (Oct 13)	0	None
	907	T9 (Oct 13)	0	None
High Dose	889	T9 (Oct 13)	1.3x10^5	Some
	893	T9 (Oct 13)	0	None
	896	T9 (Oct 13)	1.5x10^5	Some-low
	898	T9 (Oct 13)	0	None
	900	T9 (Oct 13)	3.45x10^5	Some-low
	904	T9 (Oct 13)	1.75x10^5	Some
Control	890	T11 (Oct 29)	0	None
	894	T11 (Oct 29)	0	None
	899	T11 (Oct 29)	0	None
	908	T11 (Oct 29)	0	None
	909	T11 (Oct 29)	0	None
	891	T11 (Oct 29)	0	None
Low Dose	892	T11 (Oct 29)	0	None
	897	T11 (Oct 29)	0	None
	903	T11 (Oct 29)	0	None
	905	T11 (Oct 29)	0	None
	906	T11 (Oct 29)	0	None
	907	T11 (Oct 29)	0	None
High Dose	889	T11 (Oct 29)	0	None
	893	T11 (Oct 29)	0	None
	896	T11 (Oct 29)	0	None
	898	T11 (Oct 29)	0	None
	900	T11 (Oct 29)	0	None
	904	T11 (Oct 29)	6.67x10^4	Some

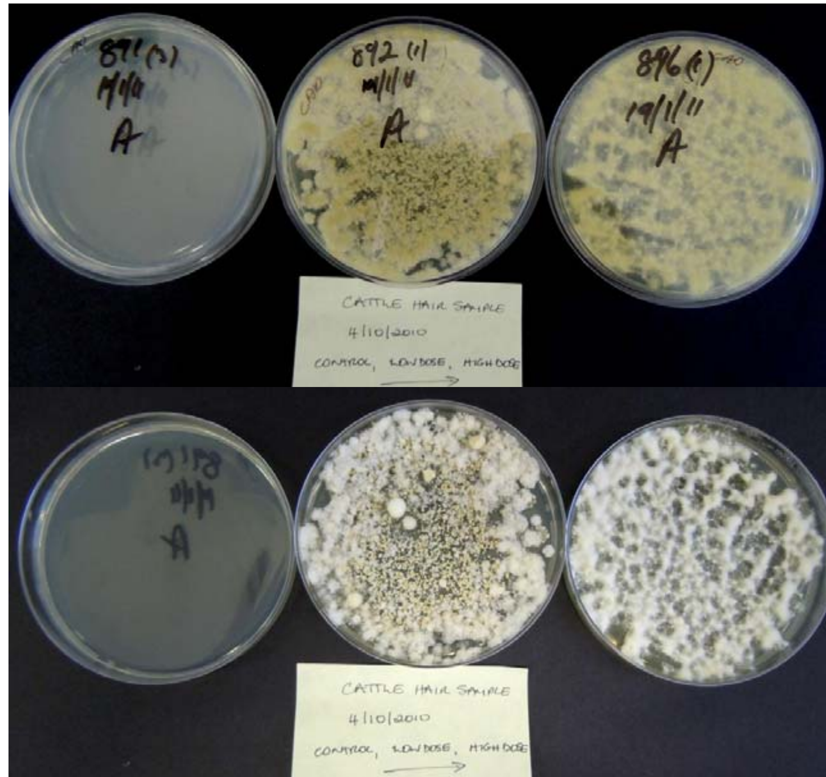

Figure 15. ***Metarhizium*** **colonies from spores washed out of cattle hair samples taken after spray treatment of animals from left to right: Control, Low dose; High dose**

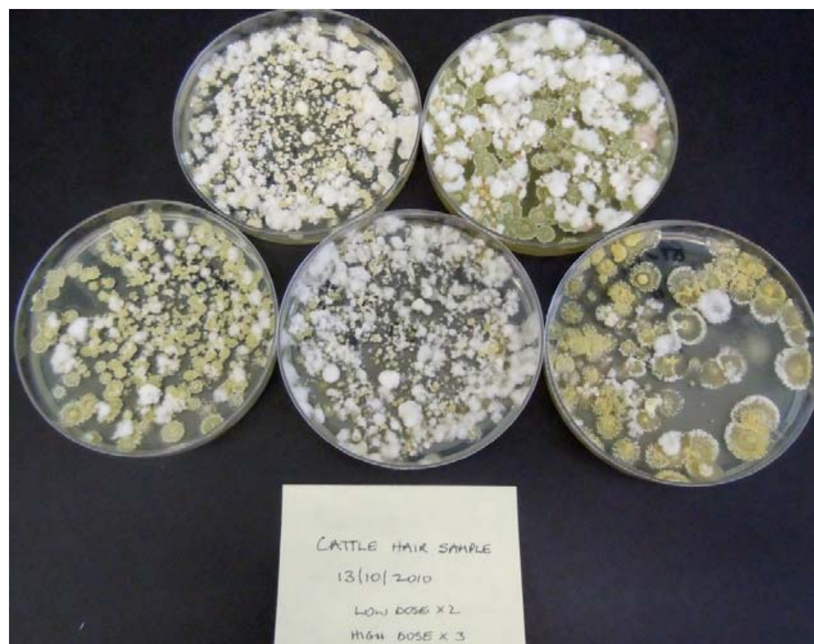

Figure 16. ***Metarhizium*** **colonies from spores washed out of cattle hair samples taken 9 days after spray. Top plates (x2) are low dose treatments, bottom plates (3) are high dose treatments**

Figure 17. *Metarhizium* colonies from spores washed out of cattle hair sample taken 11 days after cattle were retreated with fungal biopesticide. Treated cattle spent the first 3 days after treatment outside

5 Discussion

5.1 Laboratory studies

Laboratory investigations were carried out to select the best isolates of *Metarhizium anisopliae* and the best concentration of spores to use in formulations for efficacy testing in animal trials. The results of the temperature assays and virulence assays as well as observations on the spore production by all isolates were considered in the choice of isolates ARI-M52 and ARI-M63 for further investigations.

While isolate ARI-M52 did not have the largest colony diameter after 14 days at either 33°C or when the temperature was alternated during growth, it showed very high virulence to both larval and female engorged ticks. Isolate ARI-M63 had both a high growth optima, as shown by a large colony diameter, at 33°C and high virulence to larval and female engorged ticks. Both of these isolates produced good yields of spore powder when grown on the spore production media. Isolate ARI-M80 showed high virulence to ticks, but was rejected because it had the smallest colony diameter, relating to the lowest growth optima, at 33°C and when grown at alternating temperatures. While isolates AR-M12 and ARI-M16 were very promising due to their high growth optima, they did not appear to be as virulent to ticks as other isolates. In previous research isolate ARI M16 had always shown a high virulence to ticks, so the lower virulence shown in this work was surprising. These isolates were tested against larval ticks at alternating temperatures as an extra comparison. Both of these isolates killed fewer larvae at the same spore concentrations than isolates ARI-M52 and ARI-M63; thus adding further supporting the choice of isolates ARI-M52 and ARI-M63.

It was not possible to use the same strain of ticks in the screening assays with female engorged ticks because of the large numbers required for replication (480 per assay). Therefore the different tick strains were blocked within each assay so that a statistical analysis could be carried out to check for any differences due to strain. In addition one assay was conducted to see if different tick strains had differing susceptibility to the fungal spores. There were no statistically significant differences in fungal susceptibility by ticks of varying acaricide resistance.

Assays with female engorged ticks were conducted by immersing the ticks in formulated spores, to ensure that they were thoroughly wetted. This method was chosen as it is envisaged that female engorged ticks on animals will be thoroughly wetted by a formulation sprayed onto the animals. Larval tick assays were conducted using a modification of the standard larval packet test, in which larval ticks picked up spores applied to the surface of the packets. It is envisaged that on treated animals larvae will move about the animal and hence pick up spores deposited in the animal coat.

Dose assays were conducted to provide a basis for choosing appropriate spore concentrations to use in test formulations. When female engorged ticks were immersed in different doses of isolates of ARI-M52 and ARI-M63 it was shown that by formulating the lipophilic spores in 5% codacide oil rather than in water with 0.1% of the surfactant Tween 80 the effective dose required to kill ticks was markedly reduced. There was some difference between the two assays using spores formulated in oil, but the highest spore concentration to kill 90% of female engorged ticks within 2 days was estimated at 3.5×10^8 spores/ml.

Dose assays with larval ticks showed that larval ticks are susceptible to much lower concentrations of spores than female engorged ticks as long as the larvae are exposed to the spores in a moist environment. Dose assays showed that the lowest dose tested (5×10^6 spores/ml) for both ARI-M52 and ARI-M63 could kill all tick larvae within 4 days at 27°C. The assays were repeated with alternating temperatures to get a more realistic estimate of the effect of the spores on larval mortality in the coat of an animal under natural conditions. Therefore the temperatures were alternated between 38°C, which is too high for *M. anisopliae* growth, and 28°C which is close to the optimal growth temperature for this fungus. The latter temperature is also a reasonable estimate of the surface temperatures expected on the surface of cattle at night time in open paddocks during spring or autumn. Under these alternating temperatures the selected isolates still killed up to 92% of larvae with 5×10^7 spores /ml after 4 days.

5.2 Pen studies

Subsequent to the laboratory studies the pen trial was in effect both a proof of concept and dose determination trial. For such trials Holdsworth *et al* (2006) recommends that doses of 0.5, 1.0 & 2 times the estimated dose be used. From laboratory studies the most effective dose for both female engorged and larval ticks was estimated at 1×10^8 sp/ml. It was decided to use only two spore concentrations of 0.5 and 5 times this concentration to take the top dose a bit higher than that recommended by Holdsworth *et al* (2008). Therefore oil based formulations of spores of a mixture of *M. anisopliae* isolates ARI-M52 and ARI-M63 at concentrations of 5×10^7 spores/ml and 5×10^8 spores/ml were tested on animals for tick control. It was believed that such formulations should be able to provide reasonable tick control on animals provided the surface temperatures on the trial animals dipped below 34°C for periods of time each day.

The pen studies showed that both dose levels of the fungal biopesticide tested were highly effective when applied directly to engorged female ticks that were incubated in the laboratory (Figure 7) or when ticks exposed to the biopesticide while feeding on cattle were removed from the cattle and incubated in the laboratory (Figure 8). For the later, the higher spore dose appeared to kill ticks faster. The capacity of the fungal biopesticide at either dose level to kill ticks on animals was much lower than expected with several factors appearing to confound the outcomes from this study. Nevertheless, despite demonstrated non-ideal conditions with respect to surface temperature on the animals, the treatment efficacy ranged between 40% and 60% for much of the time in the 14 days post treatment (Figure 13) rising to 80% 18 days post treatment although there was no obvious dose effect (Figure 14).

When considering the growth temperatures of the *Metarhizium* isolates achieved in the laboratory studies (Tables 5 and 6) it is obvious the surface temperatures on the penned animals in the first 5 days post-treatment (Table 12) were infrequently in the optimal range for the *Metarhizium* isolates (<32°C). As the temperature increases above 32°C fungal growth will slow until it ceases at about 36°C. Growth will recommence as the temperatures fall back towards the optimal range, with the rate increasing the closer the temperature comes to the optimal temperature for the fungus. Death from thermal stress not does usually occur until the fungus has been exposed to temperatures in excess of 50°C. Once animals in this trial were left outside the true effect of cooling night temperatures were evident with the surface temperature on the animals dropping below 32°C for a considerable time each day (Table 13). The ambient temperatures during these two periods were quite similar (Tables 14 and 15). An efficacy study for a tick control product conducted in moated pens has the advantage of providing an accurate assessment of the total tick drop from treated and untreated animals. However, it appears that the design and structure of the pens used in this study may have influenced the surface temperatures of the animals in a way that impacted negatively on the efficacy of the fungal biopesticide. The pens used in this study were relatively small and had a wall of corrugated tin about 1.2 m high around the lower section that may have acted to insulate the animals from most breezes. In addition the cattle were noted to lie down in the evening so that they were surrounded by solid walls; this would further insulate the cattle from cool night temperatures.

Engorged ticks collected from treated animals and incubated in the laboratory showed both an increased mortality and subsequent decreased egg production in comparison to ticks collected from non-treated control animals (Figures 11, 12). Unfortunately the increase in mortality and decrease in egg production were less than achieved in previous studies (Leemon *et al*, 2008). Two factors may have impacted upon this. The pens were hosed daily with high pressure hoses to remove the accumulated manure and ticks that had dropped from the animals. Runoff was collected in large wire baskets that were subjected to further hosing to separate the ticks from the manure. This high pressure hosing may have affected or removed some of the fungal spores and surface growth on the ticks. Research studies (Leemon, 2009) revealed that the mechanism for rapid tick death from *Metarhizium* infection involves the surface growth of the fungus associated with tick cuticle destruction. Removal of some of the fungal hyphae before laboratory incubation may have resulted in lower mortality. The second factor impacting on the poor results might be attributable to the conditions under which the ticks were incubated. The relative humidity the engorged ticks were incubated at turned out to be too low to support good fungal growth. In the previous studies (Leemon *et al*, 2008) engorged ticks were collected directly off animals and incubated at a higher humidity which was closer to that expected in the moist soil under vegetation in paddocks.

An additional factor that had a negative impact on the trial results was the wide disparity in tick resistance between trial animals relating to an abnormal range in tick numbers dropping from these animals. Some animals dropped very low numbers of ticks all through the trial. Other animals dropped small “stunted” ticks similar in size to semi-engorged female engorged ticks, through most of the trial. It was suggested that these ticks looked similar to those which drop from cattle treated with macrocyclic lactones. The size of the ticks dropping from these animals only achieved a normal size (10–12 mm) in the last 3 days of the trial. The number of engorged ticks dropping from animals in the control group decreased to extremely low levels after day 14 for 7 days then began to rebound after day 22. The reasons for the large difference in the size and number of ticks dropping from animals during the trial can only be guessed at. It is possible that despite the specifications in the purchase request for the trial animals some animals may have had prior exposure to macrocyclic lactones. But this would only explain some of the variation observed.

6 Success in achieving objectives

The three objectives of this project have been met. Extensive laboratory studies were undertaken to find the best isolates and dose for a *Metarhizium* based fungal biopesticide to be applied to cattle for tick control. These studies included many *in vitro* assays to evaluate the formulation against female engorged and larval ticks at constant and varying temperatures that showed that even at temperatures alternating up to 38°C for 12 hours per day the formulation was extremely potent towards larval ticks. It was also shown that all main acaricide resistant tick strains are susceptible to fungal attack. The pen trial conducted to evaluate the capacity of the fungal biopesticide to kill all stages of ticks on animals gave disappointing results which we believe were influenced by artificial factors related to the design of the pen facility. However the trial was still able to demonstrate that even under non-ideal conditions the fungal biopesticide provided some efficacy against ticks on animals as well as infecting and increasing the mortality in engorged ticks which dropped from the animals in the first days after treatment. In addition it was shown that formulated spores stayed viable in the animal coat for up to 11 days after treatment even when exposed to high levels of heat and spore damaging UV light on animals there were kept outside in paddocks.

7 Impact on meat and livestock industry – Now and in five years time

A fungal biopesticide for tick control will be an important addition to current tick control options in the Northern beef regions that will help counter the problems associated with residues in meat and milk as well as the issues experienced in some areas due to tick populations resistant to most classes of acaricides.

8 Conclusions and recommendations

Based on the positive outcomes of the laboratory studies and the influence of the pen facility on our ability to test the fungal biopesticide under natural temperature conditions it is recommended that a field study be undertaken to evaluate efficacy. Ideally this trial should be carried out either during the Spring rise or Autumn.

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