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Investigating *Metarhizium* for *Culicoides brevitarsis* control

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Abstract

The biting midge *Culicoides brevitarsis* is the principal vector of bluetongue and Akabane viruses in cattle in Australia. The aim of this project was to investigate *Metarhizium anisopliae,* a fungal pathogen that infects and kills insects, as a control option for *C. brevitarsis*. Four different isolates of *Metarhizium* were used in the testing. Adult *C. brevitarsis* were killed, dying 3-8 days after walking on a substrate treated with *M. anisopliae* indicating that it has potential as a surface treatment or topical application control strategy. Incorporating *Metarhizium* into freshly excreted cattle dung, where the midge develops for up to 14 days, reduced the emergence of adult midge to very low levels indicating that *Metarhizium* has the potential to control *C. brevitarsis* at the larval stage if fed to cattle. Dung beetles play an important role in pasture management so the potential of *Metarhizium* to affect dung beetle populations was also investigated. *Metarhizium* was found to infect dung beetles with infection and mortality rate varying between isolates. However, one isolate capable of controlling *C. brevitarsis* was found to have a minimal affect on dung beetles.

Executive Summary

The biting midge *Culicoides brevitarsis* is the principal and most widely distributed vector of the livestock viruses bluetongue and Akabane in Australia. Bluetongue virus and Akabane virus have important export market and animal health consequences for the Australian sheep and cattle industries. There is no evidence of Bluetongue virus causing clinical illness in Australia however infected cattle are prevented from entering disease sensitive countries, such as China, the US and European Union countries, and this limits the industry's potential livestock export market. Cows that become infected with Akabane virus for the first time when pregnant will abort or give birth to a calf with congenital deformities. Controlling *C. brevitarsis* will reduce the number of blood meals taken and the likelihood that the bluetongue or Akabane virus will be acquired and transmitted to susceptible animals. A trial carried out by NSW Department of Primary Industries over the summer of 2009-10 compared three strains of the insect killing fungus *Metarhizium anisopliae* for their efficacy against *C. brevitarsis*. This preliminary trial showed all three strains suppressed the emergence of adult *C. brevitarsis* when mixed into freshly excreted cattle dung (Adrian Nicholas unpublished data). The data were sufficiently robust to provide evidence that the *Metarhizium* killed the early life stages of *C. brevitarsis* and that a quantifiable level of control could be achieved.

In addition to *C. brevitarsis, Metarhizium anisopliae* is known to infect over 200 other insect species. As a consequence any control strategy for *C. brevitarsis* that utilises *M. anisopliae* has the potential to disrupt dung beetle populations and this non-target effect could negate its use. Dung beetles play an important role in pasture management. By burying dung they not only reduce the breeding sites for flies, including *C. brevitarsis,* but they reduce the infective stages of gastrointestinal parasites of livestock, aerate the soil, replace nutrients and facilitate soil moisture penetration.

This project investigated the potential of four isolates of *Metarhizium anisopliae* (M10, M16, M52 and M91) to control *Culicoides brevitarsis* and assessed their affect on dung beetles. To achieve this laboratory and field trials were carried out to test:

1. the effects of *M. anisopliae* on the survival of adult *C. brevitarsis.*
2. the effects of *M. anisopliae* on the survival of the larval stages of *C. brevitarsis* based on the emergence of adults from cattle dung.
3. the non-target effects of *M. anisopliae* on four species of dung beetle.

The project achieved its principal objectives. It demonstrated for the first time that *M. anisopliae* can infect and kill adult *C. brevitarsis* when exposed to a treated substrate and confirms that *M. anisopliae* has considerable potential as in a spray-on control strategy for *C. brevitarsis*. In experiments to control cattle ticks Leemon (2011 MLA project B.AHE.0020) found that with ultraviolet protection *Metarhizium* remained viable on cattle for up to 11 days. A similar period of control could be expected for *C. brevitarsis*.

It also demonstrated that when incorporated into freshly excreted cattle dung *M. anisopliae* can reduce the emergence of adult *C. brevitarsis* to very low levels. This shows that the *M. anisopliae* isolates tested survived any gastric fluids and organisms remaining in the excreted dung at least long enough to infect and kill the developing *C. brevitarsis* larvae. *Culicoides brevitarsis* is dependent on dung for larval development and this offers a new opportunity for a life stage and site specific control strategy.

In experiments to assess the potential of *M. anisopliae* to disrupt dung beetle populations, four species, *Onthophagus binodis, O. gazella, O. taurus* and *Euoniticellus fulvus,* were exposed to four *Metarhizium anisopliae* isolates, M10, M16, M52 and M91. *M. anisopliae* isolate M16 was the most effective against *C. brevitarsis*, providing a high level of control at all three rates (0.25,

0.5, & 1.0g *Metarhizium* / kg cattle dung; the powder contained between $2.7x10^6$ and $5.2x10^6$ spores/mg). Isolates M10 and M52 provided a high level of control at the 0.5 and 1.0 g/kg rates. Isolate M91 provided only a moderate level of control even at the higher 1.0g/kg rate and was the least effective isolate. There were significant differences in mortality rates of dung beetles between *M. anisopliae* isolates. Isolate M91, the least effective against *C. brevitarsis*, caused the highest level of mortality in dung beetles (72%). Whereas isolate M52 which caused high *C. brevitarsis* mortality at the 0.5g/kg rate caused the least mortality (12%) in dung beetles which was not significantly different from the untreated control. The isolate M52 result is consistent with it having originally been isolated from another Diptera (fly) species, *Musca domestica*.

While *Metarhizium* isolate M52 was the most suitable of those tested for *C. brevitarsis* control, because it is likely to be the least disruptive to dung beetles, it may not be the best isolate available. Hundreds of isolates of *Metarhizium* originating from a diverse range of insect species exist and it is highly likely that there will be isolates better suited to *C. brevitarsis* control and even less disruptive to dung beetles than the four tested in this study. In addition to concerns about the impact on beneficial insects, formulating a commercial product for use on animals and/or surfaces is bound to present a major obstacle, mainly because of stability problems.

The lack of a laboratory breeding colony was a major limitation to this study, as it is to all vector and disease research that involves *C. brevitarsis.* Currently to acquire sufficient *C. brevitarsis* for experimentation very large numbers of infested dung pats have to be collected and held until the insects emerge. The benefit of a breeding colony would be the availability of large numbers of insects at all life stages. It would not only facilitate the study of vector ecology, population monitoring and the development of control techniques, but would be a valuable asset for animal health pathologists undertaking virus and virus transmission research for both endemic and exotic diseases.

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1 Background

The biting midge *Culicoides brevitarsis* Kieffer (Diptera: Ceratopogonidae) is the principal and most widely distributed vector of the livestock viruses, bluetongue virus and Akabane virus, in Australia (Muller, 1995, Anon 2001). Bluetongue virus and Akabane virus have important export market and animal health consequences for the Australian cattle industries**.** There is no evidence of Bluetongue virus causing clinical illness in Australia however infected cattle are prevented from entering disease sensitive countries and this limits the industry's potential livestock export market. Cows that become infected with the Akabane virus for the first time when pregnant will abort or give birth to a calf with congenital deformities (Brightling 2006). At present the control of *C. brevitarsis* is limited to cattle pour-on applications of deltamethrin which provides 14 days protection (Anon 2007). Deltamethrin is also reported to adversely affect dung beetles. Macrocyclic lactones (e.g., ivermectin) applied orally for parasite control in cattle are also known to kill *C. brevitarsis* larvae in dung pats but at the same time affect a wide range of non-target species, including dung beetles (Wardhaugh and Longstaff 2001). Macrocyclic lactones are not registered for *Culicoides* control.

NSW Department of Primary Industries conducted a preliminary trial over the 2009-10 summer to compare three strains of *Metarhizium* for their efficacy against *C. brevitarsis*. *Metarhizium anisopliae* is an insect killing fungi and the different strains are, in general, specific or limited in the species of insect they kill. In the preliminary trial all three strains suppressed the emergence of adult *C. brevitarsis* when mixed into freshly excreted cattle dung (Adrian Nicholas unpublished data). The data was sufficiently robust to provide evidence that *Metarhizium* killed the early life stages of *C. brevitarsis* and that a quantifiable level of control may be achievable.

Overseas researchers have recently demonstrated that, when applied to surfaces such as manure, leaf litter and livestock, *Metarhizium anisopliae* kills the larval and adult stages of *Culicoides nubeculous* (Ansari et al, 2010, 2011). This species is closely related to the species that vector bluetongue in Australia, namely *C. brevitarsis, C. wadai, C. actoni* and *C. fulvus*. However there are two important differences between the species of *Culicoides* in Europe and *C. brevitarsis* in Australia. Firstly, *C. brevitarsis* cannot be reared under laboratory conditions. For the most part, this limits the study of *C. brevitarsis* to field work, and means that the ability to identify which life stages (adult, egg or larval instar) are affected by *Metarhizium* is limited by the need to isolate specimens from field collected dung. Secondly, unlike most species of *Culicoides,* where the larval stages develop in dirty or dung contaminated water, *C. brevitarsis* (and *C. wadai*) are obligated to develop in dung, preferentially in fresh cattle dung but also sheep dung.

It is this dependency on dung that offers the potential for a new control strategy for *C. brevitarsis.* By incorporating *Metarhizium* into the dung it may be possible to directly target the larval stages thus preventing adult emergence. One method of incorporating *Metarhizium* into the dung is by directly feeding it to the animal. *Metarhizium* loses its pathogenicity while in the gut and to address this Fravel et al (1985) developed a method of encapsulating biocontrol in an alginate-clay matrix. Using a similar encapsulation technique described by Graminha et al. (2009), Alves, et al. (2009) demonstrated that encapsulated *Metarhizium* fed to cattle survived passing through the animal and significantly reduced the emergence of horn fly from excreted dung. While the different isolates of *Metarhizium anisopliae* are in general narrow in the insect species they kill, they are not strictly limited and other target and non-target effects are possible. For example, strains that kill *Culicoides* may also affect dung beetles, Buffalo fly or both. *Metarhizium* is not toxic to animals (or humans) and has the potential to be a safe, environmentally friendly biological control agent for *Culicoides* species in Australia.

Dung beetles that utilise cattle and sheep dung have been introduced into Australia since 1968 and they now play an important role in pasture management (Edwards 2007, Spence 2007). By burying dung they reduce the infective stages of gastrointestinal parasites of livestock and reduce the breeding sites of flies and therefore fly numbers, including *C. brevitarsis* (Doube and Dalton 2003, Spence 2007). In addition, dung beetle activity aerates the soil, replaces soil nutrients and facilitates soil moisture penetration (Doube and Dalton 2003). *Metarhizium anisopliae* is known to infect over 200 insect species across seven orders including Coleoptera (beetles) (Zimmermann 2007). *Metarhizium anisopliae* is known to adversely affect the survival of larvae of some species of dung beetle if parental care is removed (Halffter et al. 1996). Any control strategy for *C. brevitarsis* that utilises *Metarhizium anisopliae* has the potential to disrupt dung beetle populations and this non-target effect could negate its use.

2 Project Objectives

The aim of this project was to investigate the potential of *Metarhizium anisopliae* to control *Culicoides brevitarsis* and the possible effect on dung beetles. To achieve these aims laboratory and field trials were carried out to test:

1. the effects of *Metarhizium anisopliae* against adult *Culicoides brevitarsis.*
2. the effects of *Metarhizium anisopliae* on the control of the *C. brevitarsis* larval stages based on the emergence of adults from cattle dung *(under semi-field conditions).*
3. the non-target effects of *Metarhizium anisopliae* on dung beetles *(under semi-field conditions).*

The effects on Buffalo fly were also recorded by monitoring adult emergence from the above experimentation.

3 Methodology

3.1 Pre-trial operations

3.1.1 *Metarhizium* production

Eleven isolates of *Metarhizium anisopliae* were sourced from NSW Department of Primary Industries Scientific Collections Unit (Orange) and Agri-Science Queensland (Brisbane). Four isolates were chosen for evaluation in this project based on their source of isolation. Isolates ARI-M10 and ARI-M16 were from soil, ARI-M52 from *Musca domestica* (Diptera a cosmopolitan order that includes *Culicoides* spp.) and ARI-M91 was isolated from the small hive beetle *Aethina tumida* (Table 1).

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

Isolate	Source		Year isolated
	Substrate	Location	
ARI-M10	Soil	Sth Johnston, Qld	1999
ARI-M16	Soil	Aratula, Qld	1999
ARI-M52	*Musca domestica*	Dalby, Qld	2002
ARI-M91	*Aethina tumida*	?	?

Conidia (asexual spores) were produced using an established technique based on nutrient broth and rice (SunRice® Long Grain) substrates (Goettel & Inglis 1997, Dr D. Leemon QDPI, personal communication).

3.1.2 Conidia viability and number per gram

The viability and number of conidia per gram for each isolate of *Metarhizium anisopliae* was calculated and tested using standard techniques (Goettel & Inglis 1997). The spore counts were: M10: 0.48825 $x10^{10}$/g, M16: 0.52325 $x10^{10}$/g, M52: 0.275 $x10^{10}$/g and M91: 0.47325 $x10^{10}$/g. After the standard 18 hour incubation period, the germination rate of isolates M10, M16 and M91 conidia was greater than 99%. Isolate M52 developed more slowly with only 16.6% germination after 18 hours increasing to 78% after 42 hours incubation.

3.1.3 Adult *Culicoides brevitarsis*

Adult *C. brevitarsis* emerging from field collected infested dung were captured using darkened development / emergence chambers. Emergence chambers consisted of two modified buckets, one bucket inverted on top of the other. Both buckets were lined with black card to absorb condensation to prevent emerging insects from becoming trapped. The inverted (top) bucket has a hole in the top over which an inverted funnel is attached which leads to a ventilated catching chamber. Once the infested dung pat is placed inside a chamber the two halves are sealed together with tape and held under glasshouse conditions with a maximum temperature of 27°C (Figure 1). The midges were fed a 10% sugar / water solution (w/v) via a cotton wool pad placed on top of the observation chamber. The sugar solution pads were changed daily. Newly emerged midges were collected daily at 11am and held for a further 24 hours before being used for experimentation. The number of midges emerging each day varied but these were evenly divided between experimental treatments.

Figure 1 Emergence chambers (with catching chamber attached).

3.1.4 Dung beetles

Four species of dung beetle were collected (trapped), *Onthophagus binodis*, *O. taurus*, *O. gazella* and *Euoniticellus fulvus*. Dung beetle traps were constructed using a 50cm x 50cm plastic box with a wood framed chicken mesh lid. The trap contained 20mm of soil. The lid was covered with paper towelling and fresh dung (approx 1.5kg) was placed on top as bait. The traps were set just before dusk at locations of known beetle activity. Beetles burrowing into the dung and through the paper are trapped in the box below. Beetles were collected from sites in NSW and in South Australia by the company Dung Beetle Solutions – Australia.

3.2 Susceptibility of adult *Culicoides brevitarsis* to *Metarhizium anisopliae*

To expose adult *C. brevitarsis* to *Metarhizium,* treatment/observation chambers were constructed from 250mL sample jars. The chambers were lined with tissue paper and the lids replaced by insect proof netting. The tissue paper surface was dusted with dry conidia of *M. anisopliae* isolates M10, M16, M52 or M91, applied at the rate of 0.01g / chamber (equivalent to 0.7g / m^2). The conidia were applied using a small paint brush. The experiment included untreated controls. Adult midges were then placed into clear plastic treatment observation chambers. Roughly even numbers of midges were placed in each chamber, in each replicate, depending upon the number available, which varied between 20 and 80 per chamber. The midges were fed a 10% sugar / water solution via a cotton wool pad placed on top of the observation chamber. The sugar solution pads were changed daily. The midge survival rate was recorded daily for 8 days. Dead insects were removed, surface sterilised with ethanol, placed on damp filter paper and sealed into sterile petri dishes with parafilm™. The subsequent growth of *Metarhizium* on the dead insect was recorded and taken as confirming the cause of death (Figure 2a & b). The experiment was fully replicated five times, once per day for five days.

Figure 2a & b *Culicoides brevitarsis* uninfected (a) and infected with *Metarhizium anisopliae (b)*.

Data analysis

Probit analysis incorporating Abbott's correction for natural mortalities has been applied, using ASREML, to compare the survival of *C. brevitarsis* exposed to the 4 isolates of *Metarhizium*.

3.3 Emergence of *Culicoides brevitarsis* from *Metarhizium anisopliae* treated cattle dung

Trial 1: 10-19 January 2012

Freshly excreted cattle dung was collected and weighed into stainless steel bowls in 1kg amounts. *Metarhizium* conidia were added and incorporated into the dung using a domestic food mixer. The treatments were 0g (control), 0.5g, and 1g conidia /kg dung for each of the isolates M10, M16, M52 and M91. This was repeated twice a day (am and pm replicates) for

four days (4 isolates x 3 rates x 2 replicates / day x 4 days = 96 dung pats). Each day the 24 dung pats making up the two replicates, were arranged in a 4x6 grid of a randomised complete block design. Each dung pat was separated from its neighbour by a minimum of 25cm. The dung pats were placed on green shade cloth to prevent burial by dung beetles (Figure 3).

Culicoides brevitarsis is known to locate suitable ovipositioning sites (i.e., dung pats) visually (Campbell 1974) so to ensure all pats were equally attractive they were formed by inverting the dung filled stainless steel bowls which resulted in a dome shaped pat with a base diameter of 19.5cm and a thickness of 6cm. The bowls held 1kg of dung (1.2L water). The January field trial was carried out in paddock 15 at Duck Creek Research Station, 143 Pimlico Road, Ballina NSW (Lat: 28.874933, Long: 153.504825). The dung pats remained in the paddock for 6 days, the period that *C. brevitarsis* is known to oviposit in cattle dung following excretion (Campbell 1974). The paddock contained 57 Angus and Brahman cattle which, to prevent trampling, were kept at least 1.5m away from the trial plots by a portable electric fence.

On completion of the six day in-field ovipositioning period the dung pats were transferred into darkened development-emergence chambers (as in section 3.1.3) and held under glasshouse conditions with a maximum temperature of 27°C. The emerging insects were removed and the adult *C. brevitarsis* and Buffalo fly identified and counted.

Figure 3 Trial site layout (Duck Creek, Ballina, NSW January 2012)

Trial 2: 14-23 February 2012

Field Trial 2 used the same four isolates of *Metarhizium* and was conducted using the same method as Trial 1 but with an additional treatment rate. The trial 2 treatment rates were 0g (control), 0.25g, 0.5g and 1g / kg of cattle dung. Each day the 32 dung pats making up the two replicates, were arranged in a 4x8 grid of a randomised complete block design. Trial 2 was conducted in paddock 14 at the Duck Creek Research Station. The cattle were relocated from paddock 15 to paddock 14 on 26 January, allowing 20 days for the population of *C. brevitarsis* to migrate from the neighbouring paddock (15) and become established before Trial 2 commenced.

Note that there was no direct contact between *Metarhizium* and cattle during these field trials.

Data analysis

Data were analysed to evaluate differences between the control (no *Metarhizium*) and *Metarhizium* treatments, and between the 4 *Metarhizium* isolates and their 3 rates of application. The two trials have been analysed independently because of confounding effects between trials and seasonal factors. Generalised linear models (GLM's) with AR1 spatial correlations were used to accommodate spatial effects and identify isolate and treatment effects.

3.4 Susceptibility of adult dung beetles to *Metarhizium anisopliae*

Four species of dung beetle, *Onthophagus binodis, O. gazella, O. taurus* and *Euoniticellus fulvus* were exposed to four isolates of *M. anisopliae* (M10, M16, M52 and M91) to investigate infectivity and mortality rate. The *Metarhizium* was incorporated into freshly excreted cattle dung at the rate of 1g/kg, as described in Section 3.3.

Onthophagus gazella

Six adult beetles were individually placed into 500mL plastic containers with 750g of treated or untreated freshly excreted cattle dung (Figure 4). The treatments were 0.75g of *Metarhizium* in 750g of dung for each of the four isolates and an untreated control (6 beetles x 5 treatments = 30 samples). The beetles remained in the treatment containers for a one week infection period. After the infection period the beetles from all treatments were transferred to new plastic containers containing 50g of untreated freshly excreted cattle dung and 150g of pasteurised soil. The dung and soil were replaced weekly at which time the beetles were counted and any dead beetles removed. Dead beetles were surface sterilised with ethanol, placed on damp filter paper and sealed inside sterile petri dishes using parafilm™ (Figure 4). The subsequent growth of *Metarhizium* emerging from within the dead insect was recorded and taken as confirming the cause of death (Figures 5a &b). The experiment was repeated three times.

Figure 4 Dung beetle infection chamber (bottom) and infection development chamber (top).

Data analysis

A GLM was used to determine *Metarhizium* effects on *O. gazella* mortality over the 8 week experimental period. The *O. gazella* data was converted to a form that identified the number of dung beetles that died each week from *Metarhizium* as a proportion of the dung beetles in each treatment and replicate group.

Figures 5a & b
Dung beetles (*Onthophagus gazella*) uninfected (a) and infected with *Metarhizium anisopliae* (b)

Onthophagus binodis, O. taurus and *Euoniticellus fulvus*

The efficacy of *Metarhizium* against the dung beetle species, *Onthophagus binodis, O. taurus* and *Euoniticellus fulvus* was tested using the method described for *Onthophagus gazella* but with minor modification. The number of dung beetles per 500ml container was increased from one to ten and the number of containers reduced to one / replicate. Each combination of the 4 *Metarhizium* isolates and 3 dung beetle species was replicated six times over 4 days. Mortality was measured weekly for 8 weeks.

Data analysis

A GLM was used to look for differences in the mortality response from the 3 species of dung beetle to the four isolates of *Metarhizium* over an 8-week period.

3.5 The sub-lethal effects of *Metarhizium anisopliae* on dung beetles

Two species of dung beetle, *O. binodis* and *O. taurus* were exposed to two isolates of *Metarhizium (M10 and M16)* to look for any sub-lethal effects such as reduced fecundity, larval development or survival. Eighteen breeding pairs of dung beetles of each species were placed individually into breeding chambers (Figure 6a). Each breeding chamber contained 8L of pasteurised friable soil. The two isolates of *Metarhizium* were incorporated into freshly excreted cattle dung at the rate of 1g/kg (6 chambers/isolate). For comparison the experiment included six untreated controls. A total of 6 breeding pairs x 2 species x 3 treatments. The treated dung was placed on the soil surface for a period of one week to facilitate infection. The breeding chambers were held under glasshouse conditions with a maximum temperature of 27°C. After the infection period the treated dung in all breeding chambers was replaced with untreated freshly excreted dung. The dung was then changed weekly. After six weeks the chambers were opened and the presence and condition of surviving adult beetles and larvae recorded (Figure 6b). The growth of *Metarhizium* through the insect's integument (body covering cuticle) is recorded to confirm the cause of death.

Data analysis

No analysis was required because data were limited.

Figures 6a & b Dung beetle larval development chamber and larvae

3.6 Buffalo fly

Buffalo fly emerging from the dung pats in all the *Metarhizium* / *C. brevitarsis* trial treatments (see section 3.3) were collected, identified and recorded.

4 Results

4.1 Susceptibility of adult *Culicoides brevitarsis*

Adult *C. brevitarsis* exposed to *M. anisopliae* isolates M10, M16, M52 and M91 were killed over an 8 day period (Figure 7). For the first two days of the exposure period no significant difference (*P = 0.05*) in mortality was detectable between any of the treatments, including the untreated controls. A significant difference between the *Metarhizium* treatments and the untreated control became clear on day three of the exposure period and continued for the remaining five days. The results show that all isolates infect adult *C. brevitarsis* resulting in significantly greater mortality than the untreated controls. On completion of the eight day exposure period isolates M10, M16 and M91 at 60%, 60% and 55% respectively, had caused a significantly higher level of mortality than isolate M52 at 40%, based on the growth of *Metarhizium* from within the dead insect (Figure 8). The untreated control had a mortality rate from *Metarhizium* of 6%. There was no significant difference in mortality between isolates M10, M16 and M91. It was observed that *Metarhizium* conidia became attached to the insect's body, particularly the legs, which restricted its mobility prior to death.

Figure 7 The mortality rate of adult *Culicoides brevitarsis* exposed to *Metarhizium anisopliae* over 8 days of exposure

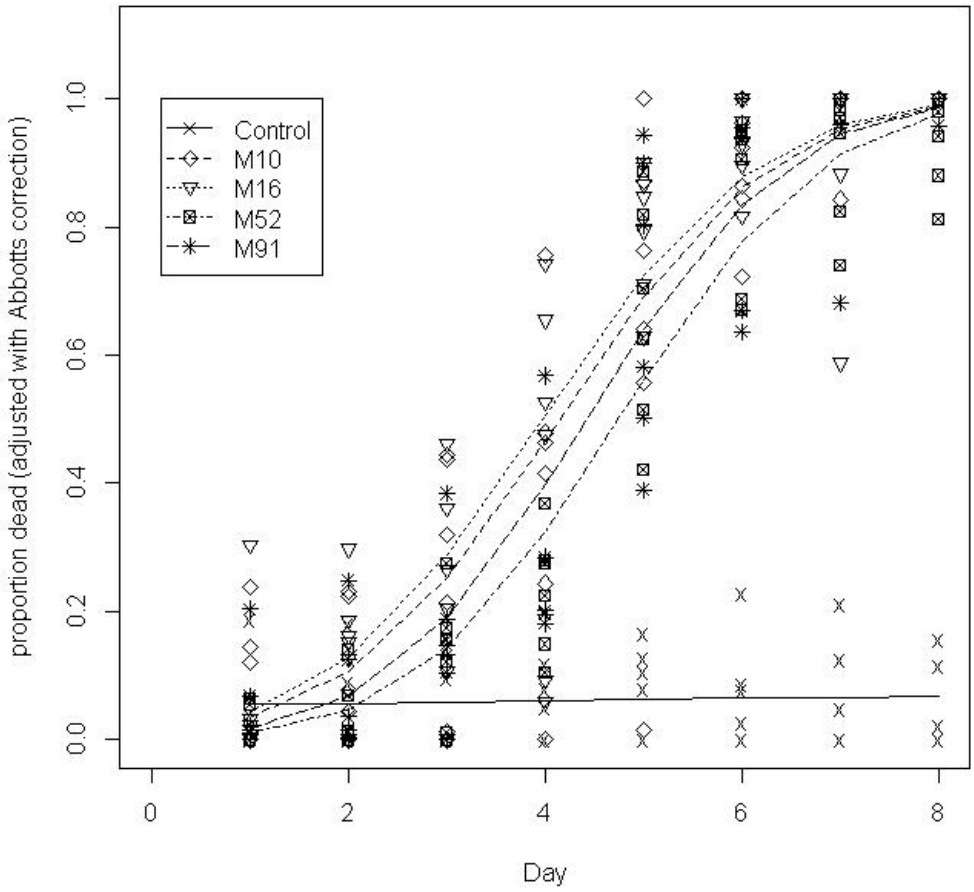
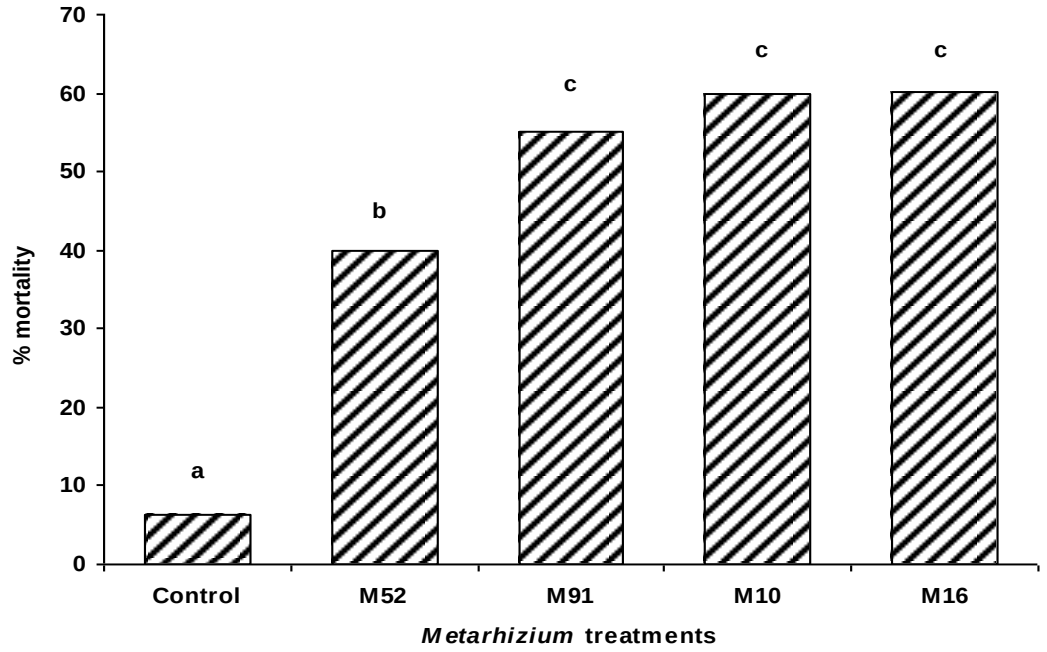


Figure 8 Percentage of adult *Culicoides brevitarsis* mortality after 8 days of exposure to isolates of *Metarhizium anisopliae* on a paper substrate (different individual letters indicate a significant difference P<0.05).

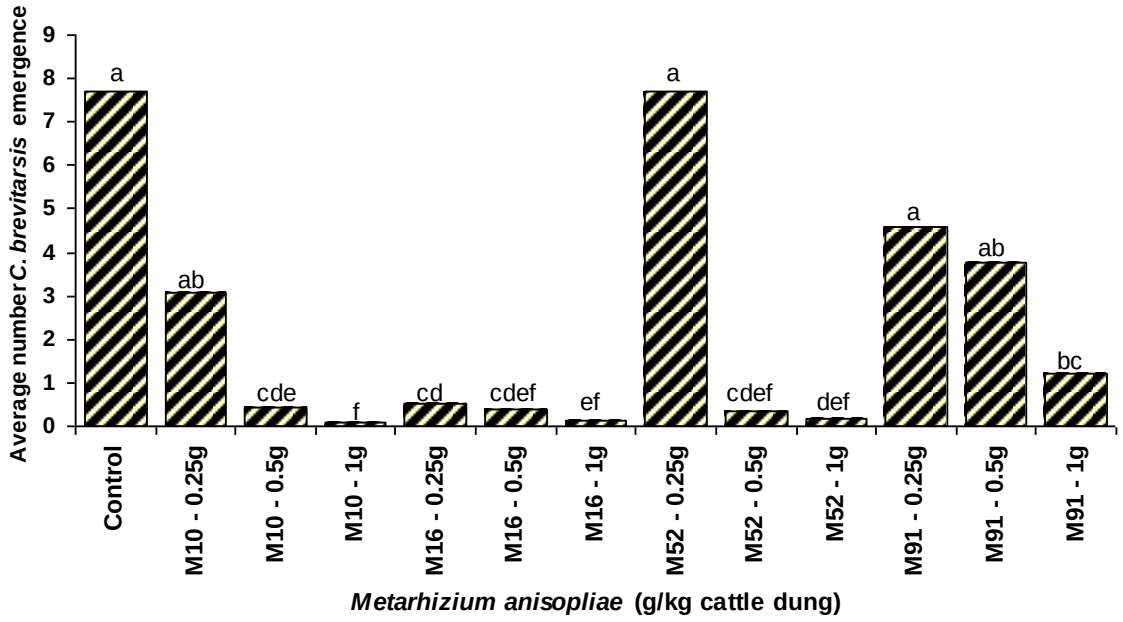


4.2 Emergence of *Culicoides brevitarsis* from *Metarhizium* treated cattle dung

An extreme rainfall event occurred in January during the six day in-field ovipositioning period. This rainfall event resulted in localised flooding at the site and while the actual trial area was not affected, the surrounding area was inundated to a depth of up to 10cm. At 0.5mm in length *C. brevitarsis* is a very small insect and conditions such as persistent rainfall, low and high temperatures and wind speeds in excess of 2.47m/s are known to prevent flight, ovipositioning site detection and ovipositioning (Campbell 1974). Very few adult *Culicoides* emerged from the dung in Trial 1 conducted in January 2012, and the variation within treatments was so large that no significant differences between treatments could be determined. These data have been excluded from the analysis. All significant differences referred to in this report are at the 95% confidence level (*P< 0.05*).

Weather patterns for the February Trial 2 ovipositioning period were favourable for *Culicoides* swarming and ovipositioning with 0-2m/s winds and temperatures (24-27°C). The February results show that all isolates of *Metarhizium* tested suppressed the emergence of adult *C. brevitarsis* from the dung with isolate M16 providing significantly better or equivalent control to isolates M10, M52 and M91 (Figure 9). At the 0.25g/kg rate there was no significant difference between isolates M10, M52, M91 and the untreated control in the level of *Culicoides* emergence. Isolate M16 reduced emergence to very low levels at the 0.25g/kg rate compared to the other isolates at the same rate and the untreated control.

Figure 9 Average number of adult *Culicoides brevitarsis* emerging from cattle dung pats treated with different isolates and rates of *Metarhizium anisopliae conidia* (Trial 2) (different individual letters indicate a significant difference P<0.05).



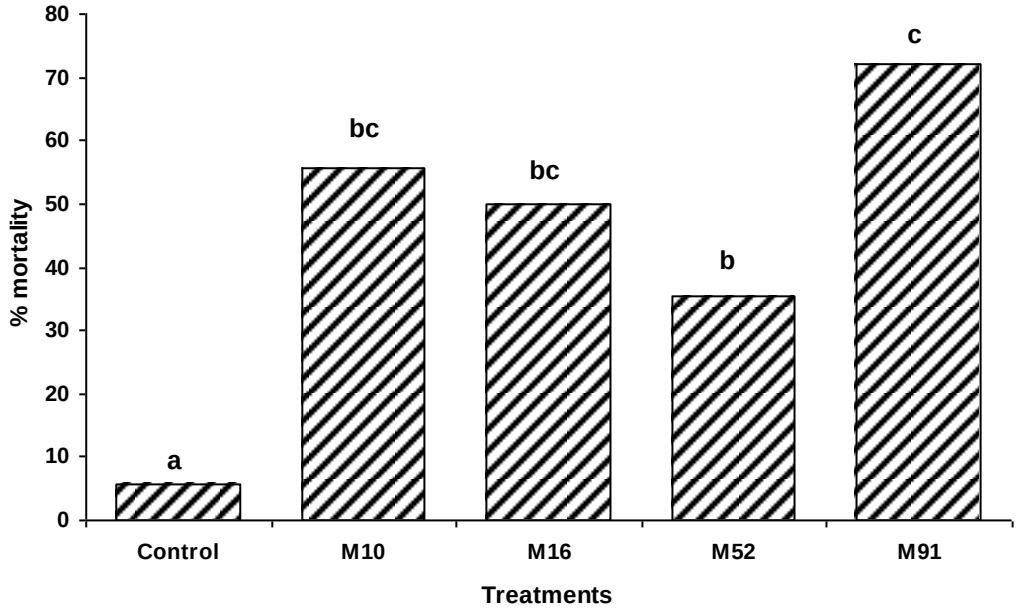
At the 0.5g/kg rate treatments M16, M10 and M52 had significantly lower levels of emergence than M91 and the untreated control. At the 1.0g/kg rate there was no significant difference between isolates M10, M16 and M52, which all had significantly lower levels of emergence than isolate M91 and the untreated control. Overall the results found that isolate M16 significantly reduced emergence of adult *C. brevitarsis* at all application rates (0.25, 0.5, and 1.0g/kg dung).

4.3 Susceptibility of adult dung beetles to *Metarhizium anisopliae*

Onthophagus gazella

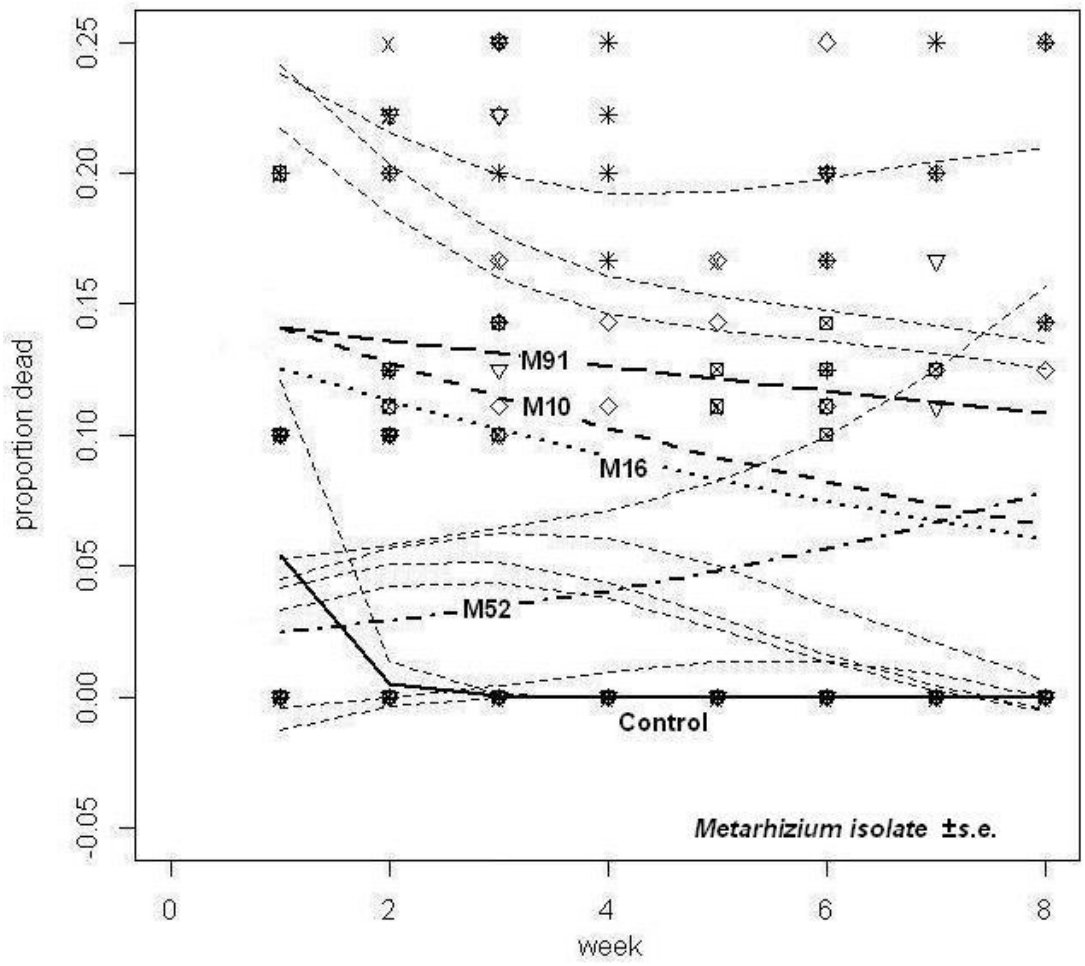
After 8 weeks, that is one week's exposure to *Metarhizium* and a seven week development period, all isolates of *Metarhizium* tested resulted in significant levels of dung beetle mortality (M10 - 55%, M16 – 50%, M52 – 35.5% and M91- 72%). The untreated control had a mortality rate of 5.5% due to inadvertent *Metarhizium* infection (Figure 10).

Figure 10 Percentage mortality of adult dung beetle *Onthophagus gazella* after one week exposure to *Metarhizium anisopliae* and 7 weeks development time (different individual letters indicate a significant difference P<0.05).



Analysis of the data, on a weekly basis over the 8 week experimental period, shows a high level of variation and significant differences between isolates in *O. gazella* mortality were not clearly defined. After some initial mortality in weeks one and two the untreated control remained mortality free for the remaining six weeks. All isolates of *Metarhizium* caused significantly higher levels of weekly mortality than the untreated control (Figure 11). The results also show that over the eight week period for *O. gazella* exposed to isolates M10, M16 and M91 mortality rates declined with time, whereas for those exposed to isolate M52 mortality rates increased with time (Figure 11).

Figure 11 The predicted mortality of dung beetle *Onthophagus gazella* over eight weeks following exposure to isolates of *Metarhizium anisopliae*.



SED: Min 0.1357 Mean 3.0319 Max 14.4750

Onthophagus binodis, O. taurus and *Euoniticellus fulvus*

The average weekly mortality rates for *Onthophagus binodis, O. taurus* and *Euoniticellus fulvus* over the eight week trial period were 3.2% 2.7% and 7% respectively. These results show that *E. fulvus* is significantly more susceptible to *Metarhizium* than *O. binodis* and *O. taurus* (Figure 12). There was no significant difference in mortality between *O. binodis* and *O. taurus*. The average weekly mortality rate caused by isolates M10, M16, M52 and M91 were 6.9%, 6.7%, 1.48% and 9.6% respectively. The untreated control had a mortality rate from *Metarhizium* infection of 1.2% (Figure 13). After the eight week trial period isolates M10 (55%), M16 (53.6%) and M91 (77%) had caused significantly more mortality in dung beetles than isolate M52 (12%) and the untreated control (9%). Dung beetle mortality with isolate M52 was not significantly different to the untreated control.

Figure 12 **Mean percentage mortality of adult dung beetles *Onthophagus binodis (Ob), O. taurus (Ot)* and *Euoniticellus fulvus (Ef)* after 8 weeks following exposure to *Metarhizium anisopliae* (different individual letters indicate a significant difference P<0.05)**

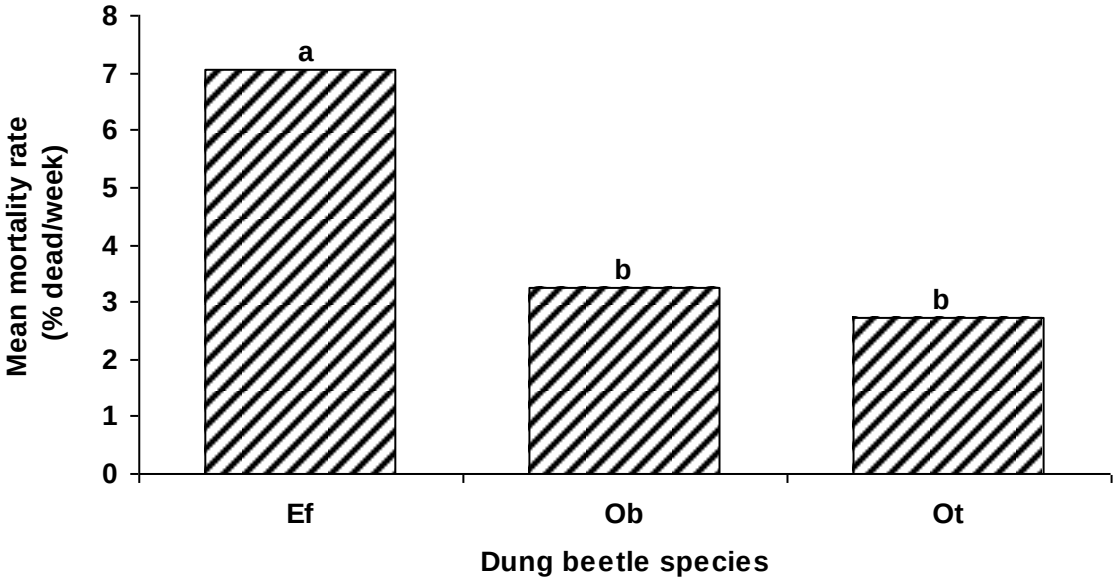
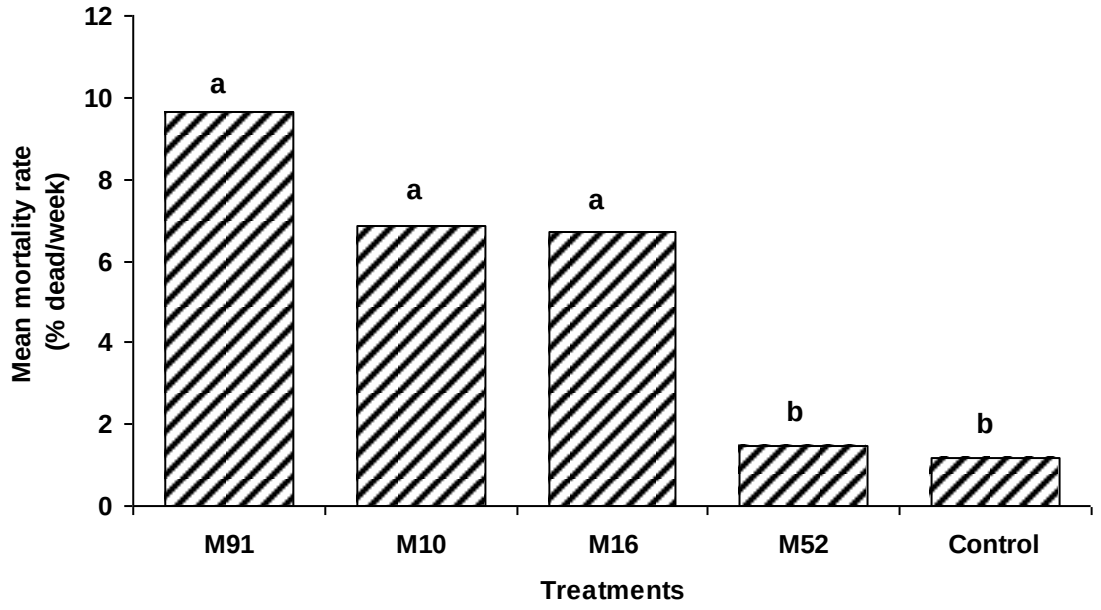


Figure 13 **Mean percentage mortality of adult dung beetles, *Onthophagus binodis, O. taurus* and *Euoniticellus fulvus,* after 8 weeks following exposure to *Metarhizium anisopliae* (different individual letters indicate a significant difference, P<0.05).**



4.4 The sub-lethal effects of *Metarhizium anisopliae* dung beetles

The level of mortality amongst breeding pairs was extremely high across treatments including the untreated controls and is consistent with the results targeting adults (Section 4.3). The results are presented in Table 2. Of the 72 adult dung beetles (36 breeding pairs) at the start only 26 survived the 6 week experimental period with only three breeding pairs surviving. Forty eight individual adult dung beetles (24 pairs) were exposed to *Metarhizium* and of these only seven were found to have been infected; six by isolate M10 and one by isolate M16. The reason for this high level of mortality amongst adults in the untreated controls is unknown but it is suspected that glasshouse conditions, e.g., temperature, may not have been suited to dung beetle survival.

Table 2 The effect of *Metarhizium anisopliae* isolates M10 and M16 on adult and immature stage of dung beetles.

Species & Treatment	No. of breeding pairs	Adult survival	Breeding pair survival	*Metarhizium* infected adult	Larvae	*Metarhizium* infected larvae
O.b Control	6	4	0	0	20[a]	0
O.b M10	6	5	2	3	15[b]	2
O.b M16	6	6	1	3	5[c]	4
O.t Control	6	5	0	0	3[d]	0
O.t M10	6	2	0	0	0	-
O.t M16	6	4	0	1	1	1

O.b – Onthophagus binodis, O.t – Onthophagus taurus
a - 17 larvae in breeding chamber 2, 3 larvae in breeding chamber 5.
b - 14 larvae in breeding chamber 1, 1 larvae in breeding chamber 3.
c - 2 larvae in breeding chamber 1, 3 larvae in breeding chamber 5.
d - 3 larvae in breeding chamber 1.

The breeding experiments resulted in very few progeny and this prevented any meaningful analysis. Of those larvae exposed to *Metarhizium,* isolate M16 infected a greater proportion than isolate M10.

4.5 Buffalo fly

Only 4 individual Buffalo fly emerged from the trial dung pats and no assessment on the effects of *M. anisopliae* on Buffalo fly emergence was therefore possible. All 4 specimens were recovered from untreated dung.

5 Discussion

This study demonstrates for the first time that the entomopathogenic fungi *Metarhizium anisopliae* can infect and kill adult *C. brevitarsis,* and when incorporated into freshly excreted cattle dung can reduce adult emergence to very low levels. Controlling *C. brevitarsis* will reduce the number of blood meals taken from cattle and the likelihood that the bluetongue or Akabane viruses will be acquired and transmitted to susceptible animals.

All the *M. anisopliae* isolates tested infected and killed the adult midge over an 8 day continuous exposure period. There was no difference in the level of mortality between isolates M10, M16 and M91. The mortality rate for isolate M52 was slightly slower and this is consistent with the slower growth rate of isolate M52 recorded during spore viability testing. In ultraviolet radiation exposed situations where the *Metarhizium* may be killed or rendered ineffective, for example when applied directly to cattle, the faster growth rate may be important and result in isolates

M10, M16 and M91 being more effective. In experiments to control cattle ticks Leemon (2011) found that with ultraviolet protection *Metarhizium* remained viable on cattle for up to 11 days. A similar period of control could possibly be expected for *C. brevitarsis*.

The dependency of *Culicoides brevitarsis* on dung for its development offers a new opportunity for a life stage and site specific control strategy. This study demonstrated that when incorporated into freshly excreted cattle dung all the isolates of *Metarhizium* tested reduced adult emergence. This indicates that the *M. anisopliae* isolates survived any gastric fluids and organisms remaining in the excreted dung long enough to infect and kill the developing *C. brevitarsis* or the adults prior to or during emergence. Whether the *Metarhizium* infects the *C. brevitarsis* larval stages or the adults just prior to emergence has not been determined but it is known that *Metarhizium* infects the larval stages of *Culicoides nubeculosus* (Ansari et al 2010) so it is likely that the *Metarhizium* infected the larval stages of *C. brevitarsis* in this study. The results show significant isolate and rate effects with isolate M16 being the most effective. Isolate M16 provided a high level of control at all three application rates (0.25, 0.5, & 1.0g/kg), isolates M10 and M52 provided a high level of control at the 0.5 and 1.0g/kg rates. Isolate M91 provided only a moderate level of control even at the higher 1g/kg rate and was the least effective of the four isolates tested.

In experiments to investigate the potential of *M. anisopliae* to disrupt dung beetle populations all species of dung beetle tested, *Onthophagus binodis, O. gazella, O. taurus* and *Euoniticellus fulvus,* were infected and killed. In the *O. gazella* trial isolate M91 caused the highest mortality (72%) and M52 the least (35%). *Onthophagus gazella* mortality decreased with time for isolates M10, M16 and M91 and increased with time for isolate M52. This indicates that isolates M10, M16 and M91 infected and killed *O. gazella* more quickly than isolate M52. Again this is consistent with the slow growth of isolate M52 as observed in the spore viability tests. The age of the dung beetles at the time of capture is not known and the increasing mortality over the eight week trial period may be the result of the slow rate of infection in an aging population, rather than an increasing mortality rate. Combing the *Onthophagus binodis, O. taurus* and *Euoniticellus fulvus* mortality data showed that isolates M10, M16 and M91 caused the most mortality and are likely to cause the most disruption to dung beetles under field conditions. In both adult dung beetle trials the results indicate that isolate M52 is likely to cause the least amount of disruption to dung beetle populations.

Very few buffalo fly were observed on the cattle at the Ballina trial site, and only 4 individual flies emerged from the trial dung pats. No assessment on the effects of *M. anisopliae* on Buffalo fly emergence has been possible but it is worth noting that all 4 specimens were recovered from untreated dung and further investigation is warranted. In the course of the project an additional seven isolates of *M. anisopliae* were acquired and these offer the potential for future testing for increased efficacy and target organism specificity.

6 Conclusion and recommendations

This project has achieved its principal objectives. It has demonstrated that *M. anisopliae* applied as a surface spray can infect and kill adult *C. brevitarsis* and when incorporated in cattle dung it can kill the larval stages of *C. brevitarsis*. It has also provided useful information on the infectivity of *Metarhizium* to dung beetles. The results would indicate that incorporated in cattle dung some *Metarhizium* isolates are likely to be highly disruptive to the local dung beetle population while others are may to be relatively benign.

This study relied on recovering *C. brevitarsis* from field collect infested cattle dung. Hundreds of dung pats were collected and held in emergence chambers in order to collect enough specimens to carry out the required experiments. This is time consuming and expensive and a major impediment to the study of this important insect vector. A laboratory breeding colony would be beneficial to the study of *Culicoides* monitoring, management and control.

In addition to concerns about the impact of this fungus on beneficial insects such as dung beetles, a potential commercial product will face the practical difficulty of developing a suitable formulation for use on animals or surfaces. No further R&D investment is recommended at this stage.

7 Acknowledgements

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