Development of formulations and delivery systems to control economically important ticks with entomopathogenic fungi

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Declaration

The experimental material and results described in this thesis is my original work (except where the input of others is acknowledged), conducted in the Programme for Phytomedicine, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, and at the International Centre for Insect Physiology and Ecology, Nairobi, Kenya. This work has not been submitted in any other form to any other University or academic institution. I declare the above statement to be true.

Signed: .....

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Date: 28/10/2009

Dedication

To my family

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List of Abbreviations used

AAAP Attraction-Aggregation-Attachment Pheromone

CO_2 Carbon dioxide

DCM Dichloromethane

DRC Democratic Republic of Congo

ICIPE International Centre of Insect Physiology and Ecology

LC_{50} Lethal concentration needed to kill fifty percent (50%) of ticks

SP Direct inoculation and indirect substrate contamination of ticks for duration of 0-5 minutes

SS Direct inoculation of ticks and indirect substrate contamination for duration of 12 hours (SS)

SW Indirect inoculation by substrate contamination for a 12-hour duration

ULV Ultra low volume

UV Ultraviolet

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Laboratory screening of entomopathogenic fungi against *Rhipicephalus pulchellus* Gerstäcker (Acari: Ixodidae).

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SUMMARY

Due to the rapid development of tick resistance to synthetic chemical acaricides and the potential risk posed by these chemicals to non-target species, efforts are intensifying towards the development of entomopathogenic fungi as an environmentally benign alternative tick control strategy. The main objective of this study is the development of formulations and delivery systems for control of ticks with entomopathogenic fungi.

Screening of entomopathogenic fungal isolates is the first step towards the development of mycoacaricides. Twelve isolates of *Metarhizium anisopliae* (Metschnik.) Sorok. and three isolates of *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) were evaluated for pathogenicity against unfed *Rhipicephalus pulchellus* Gerstäcker (Acari: Ixodidae) adults under laboratory conditions. Out of the 15 isolates screened, 4 isolates were virulent against *R. pulchellus*, inducing significantly (F value = 11.86; DF = 14, 75; $P < 0.0001$) higher tick mortalities; i.e. ICIPE 78 (76.1 ± 5.9%), ICIPE 69 (62.6 ± 5%), ICIPE 62 (49.8 ± 5.8%) and ICIPE 60 (49.6 ± 7.7%), four weeks post-treatment compared to the others. The results of this study demonstrate that *R. pulchellus* ticks were susceptible to some *M. anisopliae* isolates.

Since the process of strain selection is an important step in the development of insect pathogens for biological control, an appropriate bioassay technique for infecting different life stages of a host is of paramount importance. Bioassays were conducted in the laboratory to evaluate the efficacy of different methods of inoculation using *R. pulchellus* as a model. In a preliminary experiment, an oil-based formulation of *M. anisopliae* titred at 10^9 conidia ml^{-1} was applied to *R. pulchellus* adults using a Burgerjon spray tower or a microapplicator. Inoculation by microapplicator yielded poor results (25.0% tick mortality) compared to Burgerjon's spray tower (52.3% tick mortality) , although the mean number of fungal conidia on *R. pulchellus* adults was lower ($1.5 \times 10^4 \pm 1.1 \times 10^3$ conidia ml^{-1}) after spraying by Burgerjon's spray tower compared to 1×10^6 conidia ml^{-1} obtained with the microapplicator. Thus, inoculation by Burgerjon's spray tower was selected for further investigations. Different modes of inoculation were tested and included direct spray of inoculum on the tick and substrate (SS), direct spray on the substrate and tick followed by transfer of the tick to clean uncontaminated Petri dish (SP) or indirect inoculation of ticks through substrate (SW). The LC_{50} values following contamination of nymphs ($LC_{50} = 1.4 \times 10^7$ conidia ml^{-1}) and adults ($LC_{50} = 6.7 \times 10^7$ conidia ml^{-1}) in SS were significantly lower compared to SP; nymphs

(LC_{50} = 5.7 x 10^8 conidia ml^{-1}) and adults (LC_{50} = 5.3 x 10^9 conidia ml^{-1}) and SW; nymphs (LC_{50} = 5 x 10^8 conidia ml^{-1}) and adults (LC_{50} > 10^{10} conidia ml^{-1}). Although the LC_{50} value in SS was the lowest, it recorded the highest tick mortality among control ticks (24.2% at two weeks post-treatment) and (23.3% at three weeks post-treatment) in nymphs and adults respectively compared to SP (2.5 and 5.8% respectively) and SW (0.0 and 0.0). Results show that among the modes of contamination tested, SP was the most appropriate for contaminating *R. pulchellus* adults. SW and SP were identified as appropriate techniques for infecting the *R. pulchellus* nymphs with conidia formulated in oil.

The integrated use of semiochemicals and entomopathogenic fungus (hypocreales) may optimise the performance of a fungus as a biocontrol agent against ticks. Initially, experiments were conducted to evaluate the attraction of the hunter tick *Amblyomma variegatum* Fabriscius (Acari: Ixodidae) to semiochemicals. In one of the experiments, the simultaneous release of 1-octen-3-ol and AAAP together with CO_2 from a trap in simulated field plots attracted up to 94 ± 6% of adult ticks from a distance of 6 m, and up to 24.0 ± 5.1% from 8 m. Formulations of *M. anisopliae* (dry powder, oil, and emulsifiable) applied within the trap baited with AAAP and 1-octen-3-ol resulted in high levels of contamination of the ticks attracted to the traps. However, 48 hr after autoinoculation, 89.1 and 33.3% of conidia were lost in dry powder and oil formulations, respectively. Emulsifiable formulation lost the lowest number of propagules (17.1%). Samples of ticks attracted to the baited traps were transferred to plastic basins containing grass and maintained for 5 weeks. The experiment was conducted in the rainy and dry seasons. The emulsifiable formulation gave the highest relative reduction in tick numbers in both seasons: 54.7 and 46.5% in the rainy and dry seasons, respectively, followed by the oil formulation (32 and 23.8%) and the powder formulation (38.0 and 24.4%).

Following the high attraction and contamination of *A. variegatum* under semi-field conditions, experiments were done to evaluate the efficacy of *M. anisopliae*-treated semiochemical-baited traps for control of *A. variegatum* under field conditions. Unfed *A. variegatum* adults (118) were seeded in each 100-m plot. An emulsifiable formulation of *M. anisopliae* (consisting of 49.5% sterile distilled water, fungal conidia, 49.5% corn oil [CHEF cooking oil, Premier Oil Mills LTD] and 1% Tween 80) titrated at 10^9 conidia ml^{-1} was applied in a semiochemical-baited traps (900 cm^2) which were placed at 5 spots within the plot. The control and fungal treatments were repeated after 14 and 28 days soon after rotating

the traps clockwise (45°) in order to cover different sections of the plot. In the control plots, traps baited with semiochemicals only were used. Six weeks after the initiation of the experiments, five semiochemical-baited traps (untreated) were deployed in each plot for 3 successive days to trap ticks in the treated and control plots. The percentage of ticks recovered in the fungus-treated plots were significantly lower (31.1 ± 5.2%) than in the control plots (85.6% ± 3%) ($P < 0.001$), which represented a relative tick reduction of 63.7%. Mortality of 93.8 ± 2.3% was observed among the ticks that were recovered from the field and maintained in the laboratory for two weeks; while only 3.3 ± 0.9% died from the control plots.

The results of this study open up the possibility of developing an environmentally friendly, low cost product to control these economically important ticks.

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CHAPTER ONE

General introduction, Background/Literature Review, Hypotheses and Objectives

1.1 General Introduction

Ticks are haematophagous ectoparasites, capable of transmitting diseases to vertebrates and therefore represent a threat to human, domestic and wildlife health (Norval, 1994). Tick and tick-borne diseases have impacted negatively on development of the livestock industry in Africa (Walker *et al.* 2003). Ixodid ticks such as *Amblyomma variegatum* Fabriscius and *Rhipicephalus appendiculatus* Neumann (Acari: Ixodidae) in particular, are among the most economically important parasites in the tropics and subtropics (Bram, 1983). Another hard tick that is gaining recognition as an important vector of tick-borne pathogens is *Rhipicephalus pulchellus* Gerstäcker (Acari: Ixodidae) (Walker *et al.*, 2003). Control of this pest largely depends on synthetic acaricides including chlorinated hydrocarbons, pyrethroids, organophosphates and formamidines (amitraz) (Davey *et al.*, 1998; Rodríguez-Vivas and Domínguez-Alpizar, 1998; George *et al.*, 2004). However, extensive use of these chemicals has favoured acaricide resistance in ticks (Baker and Shaw, 1965; Solomon *et al.*, 1979; Alonso-Díaz *et al.*, 2006) and led to heightened concerns over health and environmental impact (Dipeolu and Ndungu, 1991; Gassner *et al.*, 1997). Furthermore, synthetic acaricides are expensive to livestock farmers in Africa who mainly practice subsistence farming. These setbacks have motivated the search for alternative tick control strategies that are more environmentally benign. These strategies include the use of entomopathogenic fungi and nematodes, predators, parasitic hymenoptera, tick vaccines, plant extracts, tick pheromones and host kairomones, and integrated use of semiochemicals and acaricides (Mwangi *et al.*, 1991; Kaaya, 2000a; Samish *et al.*, 2004; Maranga *et al.*, 2006).

There is particular interest in microbial control agents, especially entomopathogenic fungi Isolates of *Metarhizium anisopliae* (Metschnik.) Sorok. and *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) are pathogenic against tropical ixodid ticks, *Amblyomma variegatum*, *Rhipicephalus appendiculatus*, *Rhipicephalus sanguineus* Latreille, *Rhipicephalus* (*Boophilus*) *microplus* Canestrini, *Rhipicephalus* (*Boophilus*) *decoloratus* Koch, the deer tick *Ixodes scapularis* Say (Acari: Ixodidae) and other hard ticks such as *Amblyomma americanum* Linnaeus and *Amblyomma maculatum* Koch in the laboratory

(Kaaya *et al.*, 1996; Frazzon *et al.*, 2000; Onofre *et al.*, 2001; Benjamin *et al.* 2002; Kirkland *et al.*, 2004a: 2004b). In field experiments, Kaaya (2000b) and Benjamin *et al.* (2002) reduced *R. appendiculatus* larvae and *Ixodes scapularis* Say (Acari: Ixodidae) unfed adults, in the vegetation by spraying the vegetation with aqueous suspensions of *M. anisopliae*.

Although these fungi are pathogenic to ticks, different fungal species as well as different fungal isolates of the same species show varying degrees of virulence against ticks (Kirkland *et al.*, 2004a: 2004b and Samish *et al.*, 2001). Therefore, screening of different fungal isolates against different tick species is necessary for the development of an effective biological control agent. So far, no literature is available documenting the susceptibility of *R. pulchellus* to entomopathogenic fungi. *Rhipicephalus pulchellus* is one of the most abundant tick species in East Africa (Walker *et al.*, 2003) and evaluation of fungal isolates for pathogenicity against this species is essential to the selection of virulent isolates for further investigation.

The process of strain selection is the first important step in the development of fungal pathogens for biological control (Soper and Ward, 1981). Diverse modes of inoculation are used to evaluate the virulence of mitosporic Hyphomycetes for strain selection. They include spraying conidia on the host organisms; exposing arthropods to treated leaves; dipping them into titrated conidial suspensions; and exposing ticks to the substrate (Hall and Papierok, 1982). The most common mode of inoculation of ticks has so far been by dipping them into titrated conidial suspensions (Frazzon *et al.*, 2000; Onofre *et al.*, 2001; Samish *et al.*, 2001; Kirkland *et al.*, 2004a). Laboratory dipping assays showed *Metarhizium anisopliae* (Metsch.) Sorok. (Ascomycota: Hypocreales) to be an excellent pathogen of members of the genus *Rhipicephalus* (Acari: Ixodidae) (Samish *et al.*, 2001; Gindin *et al.*, 2002). However, this method of inoculation is unlikely to occur in reality and can provide misleading data since ticks walk on their tarsi or pulvilli, and in situations where fungal suspensions are sprayed on the vegetation using ultra low volume application equipment, which produces small droplets. In addition, dipping in a conidial suspension can cause blockage of the spiracles of the host, resulting in high mortalities (Soarés, 1982). Optimization of a technique that replicates potential methods of exposure may improve strain selection for tick control in the field. Ultra low volume application has been used successfully to apply oil suspensions of *Metarhizium* for control of locusts over wide area of land in Africa (Kooyman *et al.*, 1997). This technique may be useful in large-scale application of entomopathogenic fungi against of ticks that quest from the vegetation.

Inundative and augmentative releases are widely used to introduce entomopathogens into ecosystems for control of arthropod pests (Lacey and Geottel, 1995). Despite demonstrated reductions in tick populations on cattle and vegetation following spray applications, the use of blanket sprays may lead to contamination of non-target species and is expensive (Hajek and Geottel, 2000; Ginsberg *et al.*, 2002; Dowd and Vega, 2003; Brownbridge and Glare, 2007). Furthermore, blanket spray may not be appropriate for *Amblyomma* ticks that spend time crawling on the soil in search of suitable hosts to attach and feed. Alternative application strategies such as autoinoculation technology are being investigated. For example, the integrated use of semiochemicals, such as attraction-aggregation-attachment pheromones (AAAP) and kairomones (CO_2), and an entomopathogenic fungus in an inoculation device (trap), can attract conspecific ticks to a fungus in pheromone-baited trap and reduce the need for on-host control of ticks (Maranga *et al.*, 2006). Semiochemicals modulate the behaviour of ticks (Sonenshine, 2006). Carbon dioxide increases the attraction of *Amblyomma* ticks to an AAAP source in the field (Maranga *et al.*, 2003). Osterkamp (1999) found that 1-octen-3-ol produced additive attractive effects on hard ticks to semiochemicals. A combination of two or more semiochemicals in an appropriate ratio may optimize attraction of host-seeking *A. variegatum*. The more ticks that are attracted to a semiochemical-baited trap containing a virulent entomopathogenic fungal isolate suspended in a suitable formulation, the more efficient the control. This PhD study focused on (i) the identification of virulent fungal isolates against for use *R. pulchellus* (ii) optimizing inoculation procedures for the screening of mitosporic entomopathogenic fungi, and (iii) the development of autoinnoculation technology to infect *A. variegatum* in the field.

1.2 Background/Literature Review

1.2.1 Project conception

In parts of East Africa, especially in protected areas it is not uncommon to encounter many tick species on grass vegetation in open areas frequented by games and livestock. Obviously, reducing tick populations in such areas is a difficult task. Environmental benign biological control agents such as entomopathogenic fungi might be important in controlling ticks in these areas. Fungal pathogens of Acari are also called "acaropathogenic fungi" (Chandler *et al.*, 2000). Several methods of application of fungus are being investigated, for examples; spraying fungal suspensions on ticks feeding on cattle; spraying of ticks in vegetation; treatment of nesting materials with fungal suspension and the use of attractants to attract ticks to fungus-treated traps (Kaaya and Hassan, 2000; Hornbostel *et al.*, 2005; Maranga *et al.*,

2006; Alonzo-Díaz *et al.*, 2007). According to Kaaya and Hassan (2000), spraying vegetation has an advantage of killing a far greater number of ticks than spraying on cattle, thus reducing the numbers of ticks that would have attached on cattle and hence the frequency of acaricide application. Spraying vegetation will also have an advantage over spraying on cattle because it will kill ticks before they attach on cattle to suck blood and transmit diseases.

Ultra low volume application technique produces small droplets and has been used for spray application of entomopathogenic fungus on a large-scale (Kooyman *et al.*, 1997). However, with ULV technique the carrier agent must be of low volatility, low viscosity, compatible with the pesticide (Hill, 1987). Pure oil is compatible with ULV application and lipophilic *Metarhizium* spp conidia. Oil formulation enhances efficacy of the fungus, but, it may also be toxic (Polar *et al.*, 2005a). Hydraulic sprayer can be used for spray-application of aqueous and emulsifiable fungal formulations. Aqueous formulation is less effective under field conditions as a carrier. On the otherhand, oil-water emulsion enhances efficacy under laboratory and field conditions (Polar *et al.*, 2005a). Hydraulic sprayer has an advantage; spores are released under increased pressure and this may increase penetration of the fungus into tick-infested vegetation (Ekesi *et al.*, 1998). However, large volumes of fungal suspensions is required if large areas are to be treated, making hydraulic sprayers less cost effective than ULV application (Bateman and Alves, 2000). The choice of spray equipment to be used for applying fungus in the field would depend on the vastness of the targeted area for treatment, application strategy (on-host or off-host) as well as the behaviours of the ticks inhabiting the targeted area for tick control. Considering the large areas of grass vegetation that may require spray-application of entomopathogen, ULV spray technique might be a more suitable technique for spraying certain ticks on vegetation. According to Bateman and Alves (2000) the environmental risks associated with biopesticides can be considered to be substantially less than conventional chemicals and they should therefore enjoy the benefits of efficient application methods using small droplets. Selecting ULV spray technique created the need to optimise the inoculation methods for fungal pathogens against ticks that quest on vegetation in bioassays.

Ticks find their hosts in several ways (Walker *et al.*, 2003). Many *Ixodes* species and argasids spend their entire life cycle in their host's nest and attach to their hosts there. This is called nidiculous or endophilic behaviour. Other ticks such as the dog tick *R. sanguineus*, have adapted to living in housing built by humans and this behaviour is called domestic behaviour.

Some ticks live in open environment and their questing behaviours can be placed into two categories; (i) "Ambushers": These are ticks that seek their host by climbing on the vegetation waiting for their host to pass by so they can attach. Examples of such ticks include some members of the genus *Rhipicephalus*. (ii) "Hunters": They actively seek their host. Members of the genera *Amblyomma* and *Hyalomma* are hunter ticks (Sonenshine, 1991; Walker *et al.*, 2003). These ticks follow chemical cues to locate their hosts. Controlling this tick under field conditions can be difficult, since the nymphs and adults of this tick mostly move on the soil or underneath leaf litters and vegetation in search of their hosts. Previously, researchers at ICIPE, Kaaya *et al.* (1996) had identified *M. anisopliae* ICIPE 7 as highly pathogenic against *Amblyomma variegatum*. The fungus was originally isolated from *A. variegatum*. Also, Maranga *et al.* (2006) had developed a fungus-treated trap baited with semiochemicals. This trap was baited with semiochemicals that attracted *A. variegatum* to the fungus and they became contaminated on contact. Despite the demonstrated success of the trap in reducing *A. variegatum* population under field conditions, there were some aspects of the trap that needed improvement: the trap was still expensive, considering the large areas of land that are expected to be treated, and the distance from which tick can be attracted to the trap may be improved if the pheromone is optimised.

It is based on this background that the current research was developed. The broader goal was to develop formulation and delivery systems to simultaneously control both ambusher and hunter ticks (for examples, members of the genera *Rhipicephalus* and *Amblyomma* respectively) that may occur in a game reserve or open grassland. Two important tick species *A. variegatum* and *R. pulchellus* representing the hunter and ambusher strategies, respectively, were selected for this study.

1.2.2 Tick biology

1.2.2.1 Tick classification

Ticks are obligate haematophagous ectoparasites of vertebrates (Mans and Neitz, 2004). They belong to the subclass Acari and sub-order Ixodida which comprises three families; Argasidae (soft ticks), Ixodidae (hard ticks) and Nutalliellidae (Sonenshine, 1991). Ixodid ticks spend several days feeding on the host while argasids feed rapidly with feeding events usually lasting less than an hour (Krantz, 1978). Ixodid ticks have only one nymphal stage while argasid ticks have at least two nymphal stages (Sonenshine, 1991). Due to the medical

and economic importance of ixodid ticks, the focus of this study was on two genera of the family Ixodidae; *Amblyomma* and *Rhipicephalus*.

1.2.2.2 Life cycle of ixodid ticks and development patterns

The life cycle of ixodids consist of four stages: an embryonated egg followed by three active stages. The three active stages include the six-legged larva, an eight-legged nymph, and a sexually mature eight-legged adult (Sonenshine, 1991). Hard ticks exhibit varied developmental patterns during their life cycles and as a result are grouped as one-host, two-host or three-host ticks (Bedford, 1934). Ticks with a one-host life pattern such as *R. microplus* need only one host to complete their life-cycle. All the stages of a one-host tick feed on a single host with only the engorged adults dropping off the host. The adult ticks feed and mate on the host. Following mating, a female tick imbibes an enormous amount of host blood to attain full engorgement and thereafter drops off from the host into vegetation to lay eggs. The eggs hatch into host-seeking larvae. In two-host ticks such as *Rhipicephalus evertsi evertsi* (Acari: Ixodidae), the larvae attach to a host, feed to repletion and moult into nymphs while on the host. The unfed nymphs reattach and feed to full engorgement on the same host. Having engorged, the nymphs drop off from the host to a sheltered microenvironment and moult into a male or female adult tick. The emerged adult ticks quest for a suitable host to attach and feed. Three-host ticks such as *A. variegatum*, need three hosts to complete their life cycle (Sonenshine, 1991).

1.2.2.3 Morphology of ixodid ticks and adaptation

The body of a tick is comprised of two main regions, i.e. the gnathosoma and the idiosoma. The gnathosoma includes the basis capituli and the mouthparts. The mouthparts of hard ticks consist of a pair of four-segmented palps, a pair of two-segmented chelicerae and a hypostome (Walker *et al.*, 2003). Ticks use the chelicerae to penetrate the epidermis of their host and insert the hypostome with retrograde teeth into the wound. The retrograde teeth on the hypostome, together with cement secreted by the tick's salivary glands, enhances attachment of a tick to its host (Sonenshine, 1991). The idiosoma bears the legs, genital pores and spiracles. The chitinous scutum in female ticks covers the anterior third of the dorsal side of the idiosoma while in males it extends over the entire dorsum of the idiosoma (Krantz, 1978).

Hard ticks have evolved and adapted successfully to a blood-feeding mode of life (Mans & Neitz, 2004). In order to maintain the flow of blood during feeding, the salivary glands of ticks secrete anticoagulants and vasodilators (Sauer *et al.* 2000). Tick salivary glands also secrete immunomodulators, which can suppress the immune system of the host (Barriga, 1999). The prolonged feeding duration of hard ticks facilitates transmission of a variety of microrganisms to humans and livestock (Sonenshine, 1991). In addition, they imbibe a large quantity of blood from their hosts.

Ticks have highly efficient sensory organs, which consist of chemosensilla, mechanosensilla, photosensilla and thermosensilla. The tick's sensory organ, the Haller's organ, is situated on the dorsal surface of tarsi of each foreleg and it has both olfactory and gustatory chemosensilla (Sonenshine, 1991). Olfactory chemoreceptors or sensilla perceive volatiles while gustatory chemoreceptors perceive stimulus on contact (McMahon *et al.*, 2003). Carbon dioxide stimulus has been shown to stimulate questing in ticks (Perritt *et al.*, 1993). Also, Carroll (1998) demonstrated that aqueous wipes of the metatarsal gland of white-tailed deer elicited an arrestant response in *I. scapularis*. Some ixodid ticks such as *Hyalomma truncatum* (Acari: Ixodidae) possess eyes equipped with photoreceptors and are capable of perceiving visual signals (Bergermann & Gothe, 1997).

1.2.3 *Rhipicephalus pulchellus* Gerstäcker, 1873 (Zebra tick)

This tick is commonly known as the Zebra tick with a three-host life cycle and can transmit the protozoan *Trypanosoma theileria* to cattle (Burgdorfer *et al.*, 1973). *R. pulchellus* (Figure 1.1) is found in savannah, steppe, and desert regions and is the most common tick in North East Africa and the Rift Valley (Figure 1.2). It feeds on a wide variety of hosts including zebra, black rhinoceros and eland (Walker *et al.*, 2003).

Figure 1.1 Adult female (left) and adult male (right) *Rhipicephalus pulchellus*

Figure 1.2 Distribution of *Rhipicephalus pulchellus* in Africa (Walker *et al.*, 2003)

1.2.4 *Ambyomma variegatum* Fabricius, 1794 (Bont tick)

Amblyomma variegatum, commonly known as the tropical bont tick (Figure 1.3) is one of the commonest and most widely distributed ticks on livestock in Africa (Figure 1.4). This tick occurs in areas with a wide variety of climates, from rainforest, to temperate (highland) regions, savanna through to steppe. *Amblyomma variegatum* is a three-host tick and all stages of this tick infest cattle, sheep, goats and other large herbivores such as buffaloes. Adults attached on the dewlap, sternum, flanks, ears, around the genitalia, and the udders (Walker *et al.*, 2003). It transmits *Ehlichia ruminantium* (Rickettsiales: Anaplasmataceae) a rickettsia that causes heartwater in ruminants, and the bacterium *Dermatophilus congolensis* van Saceghem 1915 (Actinobacteria: Dermatophilaceae), an acute bovine dermatophilosis.

Amblyomma variegatum is also a vector of *Rickettsia africae* (Rickettsiales: Rickettsiaceae)(Kelly *et al.*, 1996) sp. nov, the causative agent of African tick-bite fever (Morita *et al.*, 2004). *Amblyomma variegatum* actively search for their hosts and are sometimes referred to as the hunter tick. Host seeking behaviour is modulated by information-bearing compounds known as semiochemicals (Sonenshine, 2006).

Figure 1.3 Female (left) and male (right) *Amblyomma variegatum*

Figure 1.4 Distribution of *Amblyomma variegatum* in Africa (Walker *et al.*, 2003)

1.2.5 Economic importance of ticks

The economic impact of ticks is considerable and it has been estimated that worldwide, the cost for both the control of ticks and tick-borne diseases and direct losses occurring as a result of their feeding activities and the diseases transmitted by them, was US$13-18 per head of cattle at 1996 prices (de Castro, 1997). A review by Norval (1990) on the effects of *Amblyomma hebraeum* Koch (Acari: Ixodidae) and *R. appendiculatus* on meat and milk production in Zimbabwe using computer simulation models indicated that reductions in live weight gain caused by each adult female tick completing its feeding was approximately 10 g for *A. hebraeum* and 4 g for *R. appendiculatus* and losses in milk production amounted to 7 g for every female of both species that became engorged. In Australia, losses due to infestation by *B. microplus* were estimated at US$62 million in 1974 (Springell, 1983). Brazil loses around US$2 billion per year (Grisi *et al.*, 2002). In a recent study carried out in Tanzania, the total annual national loss due to tick-borne diseases was estimated at US$364 million with an estimated mortality of 1.3 million cattle (Kivaria, 2006).

1.2.6 Tick Control

1.2.6.1 Chemical control

Currently method of tick control is largely achieved through the use of chemical acaricides (organophosphate, amitraz, fipronil) and repellents (DEET and permethrin). Even though chemical acaricides are effective (Basu *et al.*, 1986; Rinkanya *et al.*, 1992; Davey *et al.*, 1998; Davey *et al.*, 2001; Schuele *et al.*, 2009), repeated utilization of these chemicals has led to the development of tick resistance (Ducornez *et al*, 2005; Baxter & Barker 2002). For example, the Mexican strain of *B. microplus* is resistant to many classes of synthetic acaricides including chlorinated hydrocarbons (eg DDT), pyrethroids, organophosphates and formamidines (Foil *et al.*, 2004).

Furthermore, there are risks of environmental pollution by these chemical acaricides (Li *et al.*, 2003). For instance, pyrethroids and organotins have shown different degrees of toxicity to some cyanobacteria and aquatic algal species (Ma, 2005). Abou-Donia (1996) reported that tick repellents nn diethyl-m-toluamide and permethrin were toxic to hens and they suggested that humans with low plasma esterase activity could be more susceptible to acaricide toxicity.

1.2.6.2 Vaccination

Ticks produce antigens which facilitate the acquisition of blood from their hosts and these antigens can activate the production of antibodies in the hosts against internal organs of ticks (da Silva Vaz *et al.*, 1996). Some of these antigens might be useful in the development of anti-tick vaccines (Imamura *et al.*, 2008). For example, a glicoprotein of 89 kDa (Bm86), linked to the gut of *R. microplus*, induced the production of antibodies directed against a critical protein in the tick gut. This protein has been synthesized using recombinant technology and was registered for commercial use in Australia (Willadsen *et al.*, 1995). In a related development, Boué *et al.* (1998) developed a vaccine (Gavac™) in Cuba, which is effective against *R. microplus* but its efficacy against other species of ticks could not be guaranteed. According to Willadsen (2005), current vaccines could be improved by inclusion of additional tick antigens. For example, immunization of cattle with a cocktail of recombinant proteins (RAS-3 and -4) and (RIM36) significantly increased immune protection against *R. appendiculatus* in vaccinated group compared to control group (Imamura *et al.*, 2008). Research in tick vaccine development is fairly recent and it opens interesting opportunities for future research.

1.2.6.3 Plant extracts

Plant materials are used traditionally for the treatment of both endo and ectoparasites of livestock and humans in parts of Africa and Asia (Lans and brown, 1998; Madge, 1998). Research on ethnoveterinary plants has led to the scientific validation of effects of some species of plants with repellent and toxic properties (Lwande *et al.*, 1999; Borges *et al.*, 2003; Kaaya, 2000a). Neem (*Azadirachta indica)* extracts cause growth inhibition and mortality in ticks (Abdel-Shafy and Zayeb, 2002). Stjernberg and Beglund (2000) found a reduction in tick bites in individuals who fed on garlic (*Allium sativum*) compared to those fed on a placebo. Currently, herbal products containing essential oils such as citronella oil are available as commercial repellents (Fradin and Day, 2002). Pyrethrum obtained from *Chrysanthemum* spp. is a widely used natural pesticide (Hansen, 2005). Limited, unpublished information on botanical products indicates some protection from ticks under field conditions. Even though plant extracts may be bioactive against ticks in the laboratory, their stability and efficacy under field conditions has not been widely demonstrated.

1.2.6.4 Entomopathogenic fungi

1.2.6.4.1 General biology

The most common fungal pathogens of arthropods widely studied for biological control belong to the hyphomycetous fungi (Class Hyphomycetes; Order Moniliales) and include species of *Beauveria, Metarhizium, Isaria (= Paecilomyces), Hirsutella, Lecanicillium* (formerly *Verticillium*), *Culicinomyces, Tolypocladium* and *Nomuraea.* Recently, Inglis *et al.* (2001) reviewed the use of Hyphomycetes for managing insect pests. Among the hyphomycetous fungi, *Beauveria, Metarhizium* and *Isaria* genera are considered 'nonspecialist' pathogens for Acari (Chandler *et al.*, 2000); they are, however, the most studied for biological control of ticks.

1.2.6.4.2 Mode of infection

Metarhizium anisopliae (Metsch.) Sorok. (Ascomycota: Hypocreales) invades *R. microplus* by a process which involves the adhesion of conidia to the cuticle, conidia germination, formation of appressoria and penetration through the cuticle, with penetration occurring 72 h post-inoculation (Arruda *et al.*, 2005). Penetration of the cuticle is aided by secretion of the chitinolytic enzyme CHIT 30 by *M. anisopliae* (Da Silva *et al.*, 2005). Other enzymes implicated in the degradation of the tick cuticle by *M. anisopliae* during the infection process include chitinases (endo- and N-acetylglucosaminidases) and proteases (subtilisin and trypsin-like proteases) (de Moraes *et al.*, 2003). According to Goettel *et al.* (1989), penetration of the epicuticle primarily occurs by enzymatic degradation, while penetration of the procuticle involves both enzymatic degradation and mechanical separation of the lamella. Differences in tick cuticular lipids and hydrocarbons can influence tick susceptibility to fungal infection, which may account for differential susceptibility of *A. maculatum* Koch and *A. americanum* to *M. anisopliae* and *B. bassiana* (Kirkland *et al.*, 2004a). Nymphs of *appendiculatus* are more susceptible than adults (Kaaya, 2000b; Kirkland *et al.*, 2004b). Kershaw *et al.* (1999) proposed that at least two possible virulence strategies existed among their isolates: (i) a "toxin strategy" in which the fungus has limited growth in the host haemolymph and produces destruxins in sufficient quantity to kill the host (ii) a "growth strategy"in which an isolate exhibits profuse growth in the haemolymph which causes a disruption of homeostasis and starvation leading to host death, although these strategies may differ among fungal isolates.

1.2.6.4.3 Natural infection of ticks by fungal pathogens

Early studies on natural mycoses in ticks revealed that soil-dwelling stages of ticks are susceptible to mitosporic fungi such as *M. anisopliae* and *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) (Chandler *et al.*, 2000). *M. anisopliae* and *B. bassiana* have been observed infecting soil-dwelling stages of the cattle ticks *R. microplus* in Brazil (Verissimo, 1995). In a comprehensive study of mycoses occurring in natural populations of *Ixodes ricinus* L. (Acari: Ixodidae) in Denmark, fungi appeared to be an important regulator of tick populations with engorged females being the most susceptible (Kalsbeek *et al.*, 1995). Similarly in Brazil, strains of entomopathogenic fungi (49 *B. bassiana* and 20 *M. anisopliae*) occurring naturally on engorged females of *B. microplus* ticks were isolated (da Costa *et al.*, 2001)

1.2.6.4.4 Potential of fungal pathogens for tick control

The pathogenicity to various fungal species against ticks has already been demonstrated in the laboratory. For example, Samish *et al.* (2001) showed that *M. anisopliae*, *B. bassiana*, *M. flavoviride* and *I. fumosorosea* were pathogenic against *Rhipicephalus sanguineus* L. (Acari: Ixodidae). In an *in vitro* bioassay comparison of the efficacy of *M. anisopliae*, *M. flavoviridae*, *B. bassiana*, *I. fumosorosea* and *L. lecanii* against engorged female *B. annulatus* ticks, Gindin *et al.* (2001) found that *M. anisopliae* and *B. bassiana* were the most effective, killing 85 to 100 percent of ticks within 7 to 10 d of treatment and preventing egg production before death. An isolate of *B. bassiana* (Bb 28) caused high larval mortality and low hatching of the eggs among fungus-treated *B. microplus* eggs and larvae (Fernandes *et al.*, 2003). Investigating the effects of 12 isolates of *M. anisopliae*, infected and non-infected by dsRNA viruses, on engorged *B. microplus* females, Frazzon *et al.* (2000) observed that the most pathogenic isolates caused 100% mortality in engorged females of *R. microplus* 13 days post-treatment at a test concentration of 10^7 conidia ml^{-1}. They also found that dsRNA mycovirus-free isolates of *M. anisopliae* were more infective than the ones taken from experimentally infected ticks. Isolates of *M. anisopliae* killed laboratory populations of engorged females, eggs and larvae of *B. microplus* and affected their oviposition behaviour (Bittencourt *et al.*, 1992; Bittencourt *et al.*, 1994a,b). Fungal suspensions of either *B. bassiana* or *M. anisopliae* at 10^8 conidia ml^{-1} caused 50–70% mortality in adult *I. scapularis* and *R. sanguineus*, but less than 20% mortality in *D. variabilis* ticks at 28 days post treatment (Kirkland *et al.*, 2004b)

The potential of entomopathogenic fungi for tick control has also been demonstrated in the field. When ticks were treated with entomopathogenic fungi and maintained in potted grass, oil-based formulations of *B. bassiana* and *M. anisopliae* of 10^9 conidia ml^{-1} induced 100% mortality in *R. appendiculatus* and *A. variegatum*, larvae, between 80 and100% mortality in nymphs and 80 and 90% mortality in adults (Kaaya and Hassan, 2000). In a more recent study, post engorgement and egg mass weights were 33 and 55% lower, respectively, in females of *I. scapularis* treated with *M. anisopliae* in the field before they were allowed to feed on laboratory rabbits (Hornbostel *et al.*, 2004). Kaaya (2000b) and Benjamin *et al.* (2002) reduced tick populations of *R. appendiculatus* larvae and *I. scapularis* unfed adults respectively in vegetation by spraying with aqueous suspension of *M. anisopliae*. Benjamin *et al.* (2002) determined the efficacy of the fungal suspension by counting the number of *I. scapularis* collected in both the fungus-treated and control treated plots after 4 weeks by the drag sampling method. Spray treatments of *M. anisopliae* and *B. bassiana* to *R.(B.) decoloratus* Koch on cattle induced tick mortality and resulted in a significant reduction in egg viability (egg hatchability). In the control group, egg viability was 98% compared with 30% and 50% in *B. bassiana* and *M. anisopliae*-treated groups, respectively (Kaaya and Hassan, 2000). In another study conducted by Alonso-Díaz *et al.* (2007) *R. microplus* population on naturally-infested cattle was significantly reduced following multiple applications of an aqueous suspension of *M. anisopliae* titred at 10^8 conidia ml^{-1} compared to the control on days 0, 1, 3, 5, 7 and 14 post-treatment.

1.2.6.4.5 Factors that influence efficacy of fungal pathogens

1.2.6.4.5.1 Environmental factors

Several climatic factors may influence the infectivity of entomopathogenic fungi for ticks. These factors include solar radiation, especially UVA and UVB, temperature and humidity (Ignoffo, 1992). For example, Fargues *et al.* (1996: 1997) reported that UV-B was the main factor causing mortality in *I. fumosorosea* and *M. anisopliae*. A similar observation was reported by Moore *et al.* (1993) with *M. anisopliae* var. *acridum* (=*Metarhizium flavoviride*) conidia in water on glass under laboratory conditions. Biochemically, UV-B causes a direct effect on DNA and proteins through sorption by aromatic amino acids (Pourzand and Tyrrell, 1999). Reactive oxygen species (ROS) generation is associated with modifications of aromatic amino acids. According to Miller *et al.* (2004), another major effect of UV-A and UV-B is a reduction in catalase–peroxidases isozyme level. Impaired growth of *M. anisopliae* after near-UV exposure may be related to reduce abilities to handle oxidative stress (Miller *et*

al., 2004). Research on the UV protective effects of oil revealed that fungal conidia suspended in oil are significantly protected from UVB in controlled-environment studies (Moore *et al.*, 1993; Inglis *et al.*, 1995). However, oil alone does not appear to enhance the survival of conidia on foliage in the field and this has been attributed in part to the absorption of oil into leaf tissues (Inglis *et al.*, 1995). A variety of strategies have been applied to increase the persistence of entomopathogenic Hyphomycetes. Incorporation of conidia into soils is thought to increase their survival by protecting propagules from solar radiation and buffering them from extremes of temperature and moisture (Gaugler *et al.*, 1989). However, incorporation of of propagules into soil to enhance persistence will depend not only on climatic factors such as ambient temperature but also on other factors such as shading or the depth to which propagules are incorporated (Inglis *et al.*, 2001). High humidity close to saturation favours germination and pathogenicity of entomopathogenic fungi, whereas low humidity produces the opposite effect (Lazzarini *et al.*, 2006). Fungal germination and growth are affected by temperature with optimum growth ranging between 25°C and 30°C and at higher temperatures (beyond 35 °C) growth is slowed (Ekesi *et al.*, 1999; Leemon *et al.*, 2008). However, fungal growth varies among fungal isolates and Polar *et al.* (2005b) demonstrated the importance of selecting suitable isolates for tick control on cattle. According to Inglis *et al.* (2001), the thermal characteristics of isolates should be matched with the microhabitats in which they will be deployed for optimum performance.

1.2.6.4.5.2 Effects of culture conditions

The viability, persistence and virulence of entomopathogenic fungi can be affected by culture conditions (Butt and Geottel, 2000; Leland, 2001). For example, when growing in a liquid medium for *M. anisopliae* containing limited nitrogen and excess carbon, *M. anisopliae* produces sporogenous cells and spores ("submerged conidia") that are morphologically indistinguishable from environmentally stable aerial conidia (Jenkins and Prior, 1993) and are relatively more stable than the blastospores (Jenkins and Goettel, 1997). In bioassays with *Galleria mellonella* larvae at different relative humidities, conidia with large amount of glycerol and erythritol were more virulent than conidia grown on rich nutrient substrates (Hallworth and Megan, 1994). Kirkland *et al.* (2005) demonstrated that presence of oxalic acid, which is also secreted by *B. bassiana* in a medium act as a key factor during pathogenesis and can affect fungal virulence and lethality against tick.

1.2.6.4.5.3 Host specificity

There is a strong relationship between the host and the pathogen, which is modulated by factors inherent to both the pathogen and the host, as well as to environment (Inglis *et al.*, 2001). This may explain the varied response of different tick species to different fugal isolates. Kirkland *et al.* (2004a) observed differential susceptibilities of *A. maculatum* and *A. americanum* to the entomopathogenic fungi *B. bassiana* and *M. anisopliae.* Polar *et al.* (2005c) noted that *M. anisopliae* was more pathogenic against *R. microplus* than *Simplicillium lamellicola* and *I. farinosa*, producing shorter average survival times for engorged adults and larvae, and longer average hatching times. The fungus was also found to be pathogenic towards engorged adult *R. sanguineus* but not against the larvae. In another study, Polar *et al.* (2005b) compared the pathogenic activity of two isolates of *M. anisopliae* against *R. microplus.* Although both isolates reduced the *R. microplus* population on cattle more than the control, one isolate produced a greater reduction in tick numbers than the other. The susceptibility of a host to fungal infection also varies according to the developmental stage exposed. For instance, the first nymphal instar of the fowl tick, *Argas persicargas persicus* Oken (Acari: Argasidae), is more susceptible to *B. bassiana* and *M. anisopliae* than the second nymphal-instar (Sewify and Habib, 2001). Nymphs of *R. sanguineus* and *Dermacentor variabilis* (Acari: Ixodidae) are more susceptible than the corresponding adult stages to conidia and blastospores of *B. bassiana* and *M. anisopliae*; while *I. scapularis* nymphs are slightly less susceptible than adults to the same fungal species (Kirkland *et al.*, 2004b). Pathogens and hosts are intrinsically linked ecologically but their evolutionary interests diverge: selection on the pathogen is for greater exploitation of the host and selection on the host is for exclusion of the pathogen (Bush *et al.*, 2001). In general, hosts can resist pathogen infection through immune defense mechanisms, for example the honey bee immune system, like other species of holometabolous insects, depends on two main categories of defense reactions against entomopathogenic fungi: the cell-mediated responses such as phagocytosis and encapsulation of foreign objects (Gliñski and Buczek, 2003)

There are several reports on the innocuous nature of microbial control agents toward beneficial insects and other non-target organisms (Gröner, 1990; Lacey and Siegel, 2000; Shapiro-Ilan and Cottrell, 2005). Premature death of the host due to infection is one of the main antagonistic interactions between entomopathogens and parasitoids (Brooks, 1993). Introduction of a fungal pathogen to an ecosystem may cause significant destabilization and have long-term consequences that affect the parasitod complex interaction with its hosts

(Furlong and Pell, 2005). However, there is evidence for behavioural and biochemical mechanisms that minimize the negative interactions between entomopathogens and insect parasitoids (Brooks, 1993; Begon *et al.*, 1999). There is a diverse assemblage of genotypes within *Metarhizium* and *Beauveria* and individual isolates can have very restricted host ranges (Goettel *et al.*, 2000). Physiological susceptibility in the laboratory does not necessarily translate into field ecological susceptibility, where the impact of a given fungus on a susceptible predator or parasitoid may be minimal (Jaronski *et al.*, 1998). Field studies by Baltensweiler and Cerutti (1986) and Parker *et al.* (1997), revealed that application of *B. brongniartii* (Ascomycotina: Hypocreales) and *B. bassiana*, respectively to forest environments produced low incidence fungal disease in non-target arthropods. Studies by Lacey *et al.* (2003) showed both antagonism and complementary activity between the entomopathogenic nematode *Steinernema carpocapsae* (Rhabditida: Steinernematidae) and two ichneumonid idiobiont parasitoids (Hymenoptera: Ichneumonidae) of codling moth (Lepidoptera: Tortricidae). This research demonstrated the ability of parasitoid females to detect and avoid laying eggs on nematode-infected cocooned codling moth larvae.

1.2.6.4.5.4 Screening bioassay

Bioassays are central to the successful development of fungi as microbial control agents. According to Butt and Goettel (2000), the ultimate challenge is to develop bioassay that can be used to predict field efficacy. Several factors can influence the pathogenicity of entomogenous fungi besides culturing conditions in bioassays; rearing conditions; handling of insect; insect life cycle; environmental factors such as temperature and humidity; inoculation methods; the location where the inoculum lands on the cuticle; dose-mortality relationships; control mortality and attenuation of virulence due to successive subculturing on artificial (Butt and Goettel, 2000). The most widely used method for inoculation of ticks in the laboratory is by immersion (Frazzon *et al.*, 2000; Onofre *et al.*, 2001; Samish *et al.*, 2001; Kirkland *et al.*, 2004a). However, contamination of ticks by dipping in a conidial suspension can provide misleading data since ticks walk on their tarsi or pulvilli. In addition, dipping in conidial suspensions can cause blockage of the spiracles of the host, resulting in high mortalities (Soarés, 1982). Poor results observed in field experiments can partly be explained by the technique used to infect the target host during screening trials in the laboratory, which may either underestimate or overestimate the virulence. Adaptation of assays to the specific needs of a host, pathogen, and application strategy might provide meaningful results during the screening process. It is therefore important to optimize strain selection through the use of

appropriate and more predictive methods of inoculation when screening fungal isolates against different life stages of ticks.

1.2.6.4.5.5 Formulations

Formulation of entomopathogens is a critical step towards their implementation as biocontrol agents. Formulation must be devised combination of ingredients to ensure that the active component effectively reaches the target pest (Soper and Ward, 1981). The proper formulation of a fungal biocontrol agent, therefore, requires an understanding of the life cycle of the pathogen, and effects of environmental factors on its biology, combined with knowledge of the target host's biology (Feng *et al.*, 1994). Water is a useful formulating agent because it is non-toxic, readily available, cheap, and can be dispersed using simple hydraulic sprayers (Polar *et al.*, 2005a). On the otherhand oil-based carrier can minimize the negative effect of environmental stress on fungal conidia and enhance infection (Prior *et al.*, 1988; Kaaya *et al.*, 1996; Kaaya and Hassan, 2000; Batta, 2003). It has also been suggested that formulation of conidia in oil ensures that conidia are viable for longer periods and have higher infectivity (Milner *et al.*, 1997). Pure oil formulations are compatible with ultra low volume (ULV) spray technology (Prior *et al.*, 1988). They are non-volatile and can be used to obtain very small droplets (50–100 μm mean diameter) required for ULV spraying, which is suitable for large-scale field application. This is not possible with water formulations due to the high evaporation rate of small water droplets (Bateman and Alves, 2000). Oil-water emulsion may provide free water and nutrients to stimulate the germination of the *Metarhizium* spores on the surface of ticks (Leemon and Jonsson, 2008). The use of emulsifiable oil adjuvants allows for the conidia and oil droplets to be suspended in a continuous, aqueous phase. This imparts some of the beneficial properties of an oil formulation in a less costly water based environment (Polar *et al.*, 2005a). Oil-water emulsions and aqueous formulations are compatible with hydraulic spray technology, which is relatively more costly compared to ULV for large-scale applications (Bateman and Alves, 2000). The development of formulations that is inexpensive, that minimize the susceptibility of fungi to environmental stress, and are compatible with suitable application technologies may enhance the efficacy of entomopathogenic fungi as biocontrol agents.

1.2.6.4.5.6 Application strategies

Inundative and augmentative releases are the main methods employed for the introduction of entomopathogens, including fungi, into the ecosystem (Lacey and Goettel, 1995). For

example, Kaaya (2000b) and Benjamin *et al.* (2002) reported reduction of populations of *R. appendiculatus* and *I. scapularis*, respectively, following spray application of aqueous formulation of *M. anisopliae* on the vegetation. Ticks that quest from vegetation could be targeted by spraying the vegetation with fungal suspension. Alonso-Diaz *et al.* (2007) obtained a reduction in the number of feeding *R. microplus* ticks following spray application of aqueous formulation of *M. anisopliae* on naturally infested cattle. Applying fungal suspensions on the host may not be practical in situations where livestock and wildlife share the same habitats. The use of blanket spray may lead to contamination of non-target species (Hajek and Geottel, 2000; Brownbridge and Glare, 2007). In addition, environmental factors may reduce fungus efficacy (Luz and Fargues, 1998; Polar *et al.*, 2005b). Therefore there is need for alternative techniques such as autoinoculation. Such a technology would permit the dissemination of tick pathogens among target tick population by using devices that attract host ticks to come into contact with the pathogen before retreating to the population (Maranga *et al.*, 2006). In some instances, this might be the most suitable method for example controlling "hunter" ticks such as *A. variegatum* off-host. Using a fungus-treated pheromone-baited trap, Maranga *et al.* (2006) were able to attract and infect *A. variegatum* under field conditions. Other methods have been tried, for example Hornbostel *et al.* (2005) treated nesting material of the white-footed mouse, *Peromyscus leucopus,* with *M anisopliae* to control larvae of *Ixodes scapularis* with some positive results. An understanding of the behaviour of the target tick species is advantageous in determining the best application strategy for a mycoacaricide.

1.2.6.5 Tick pheromones and host kairomones

Tick behaviour is mostly regulated by pheromones and kairomones, chemical compounds that enhance intraspecies communication. There are three known types of pheromones in ticks: sex pheromones (improves chances of mating), assembly pheromones (help safeguard the survival of the individuals by bringing conspecifics together) and aggregation-attachment pheromones (help ensure that ticks attach preferentially to a host on which they are more likely to feed) (Hamilton, 1992). The sensory organ of ticks responsible for picking up chemical stimuli is the Haller's organ (Sonenshine, 1991). In members of the genus *Dermacentor*, genital sex pheromones produced by females are perceived by the sensilla on the chelicerae of male ticks. Members of the genus *Amblyomma* actively respond to the attraction-aggregation-attachment-pheromones secreted by successfully feeding male ticks and to kairomones (for example CO_2) exhaled by their hosts (Norval *et al.*, 1987). The large

amount of pheromones produced by feeding males of the tropical bont tick (*A. variegatum*) (Diehl *et al.*, 1991, Pavis and Barré, 1993 and Price *et al.*, 1994) coupled with carbon dioxide from their large ungulate host, attracted unfed males and females from longer distances upto to 10 m (Norval *et al.*, 1991). The chemical 1-octen-3-ol, which occurs naturally in adult *A. variegatum* and is a minor constituent of cattle breath, attracts *A. variegatum* adults in the laboratory (McMahon *et al.*, 2001). According to Schoni *et al.* (1984), the best-known example of such a phenomenon is the AAAP (attraction-aggregation-attachment pheromone) of the tropical bont tick, which is comprised of a mixture of two substituted phenols, methyl salicylate and *o*-nitrophenol, and a fatty acid, nonanoic acid. In a recent study conducted by Donzé *et al.* (2004), *A. variegatum* and *I. ricinus* adults were attracted to the odour of rumen fluid collected from freshly-slaughtered cattle and the olfactory receptor cells of both ticks were stimulated by the same fraction of rumen metabolites (butanoic and isobutanoic acid).

1.2.6.5.1 Optimization of pheromone attraction of *A. variegatum*

It has been shown that CO_2 increased attraction of *Amblyomma* ticks to an AAAP source in the field (Maranga *et al.*, 2003). A blend of host odours such as CO_2 with 1–octen-3-ol and AAAP significantly increased attraction of *A. variegatum* compared to AAAP only (Toure, 2005). Osterkamp (1999) found that 1-octen-3-ol produces an additive attractive effect of hard ticks to semiochemicals. Optimization of AAAP traps with host kairomones (rumen fluid odour, carbon dioxide, 1-octen-3-ol, ammonia and rumen metabolites, which elicit attraction of *A. variegatum*) in appropriate concentrations may improve the attraction and orientation of *A. variegatum* towards the trap.

1.2.6.5.2 Potential of semiochemicals in integrated tick control

Integrated tick control is a multifaceted approach to tick control. The goal is to control ticks in a sustainable manner by making use of different methods of control while minimizing the negative impact of acaricides on the environment. Different tick control methods such as traditional hand picking of ticks, use of indigenous tick resistant cattle and ethnoveterinary plants) can be combined with chemical, biological, immunological and ecological controls (e.g. rotational grazing, proper fencing of game reserves) and semiochemical methods in order to achieve increased tick control efficacy. According to de Castro (1997), reliance on any one method of controlling ticks often fails to provide efficient, sustainable and long-term control. Carroll *et al.* (1995:1996) and Allan *et al.* (1998) have previously demonstrated the potential of kairomones released by the host and tick pheromones in integrated tick control.

Tick behaviour can be exploited for on-host or off-host pheromone-assisted tick control technology with satisfactory results (Sonenshine, 2006). A sex pheromone (2,6-dichlorophenol)-acaricide impregnated decoy attracted and killed mate-seeking males of *Dermacentor variabilis* on rabbits (Hamilton and Sonenshine, 1989). Allan *et al.* (2001) developed a technology that kills *I. scapularis* in vegetation, by incorporating components of arrestment pheromones (elicit adult assembly) and permethrin into oil droplets. In another study, plastic tail tags impregnated with AAA pheromone and flumethrin or cyfluthrin coupled with the CO_2 produced by the cattle was effective for the control of the tropical adult bont tick *A. hebraeum* (Norval *et al.*, 1996). Similarly, Maranga *et al.* (2006) used this concept to attract adults of *A. variegatum* from the vegetation to a CO_2 and pheromones-baited traps to infect them with an entomopathogen. The combination of mycoacaricides with pheromones may also enhance efficacy by attracting ticks to the pathogen, in a species-targeted control method, concurrently reducing potential environmental contamination.

Although some work has been done in this regard, no commercial products combining tick semiochemicals with an acaricide or biological control agent are available in the market. This may be partially attributed to the lack of adequate information on tick responses at a species level to subtle changes in the concentrations of single pheromones or constituents of pheromone blends, and the absence of suitable application techniques for a mycoacaricide. The aim of this thesis was to address the different aspects that could play a role in developing effective products by integrating the different aspects discussed above.

1.3 Hypothesis

- Entomopathogenic fungi cause mortality in *R. pulchellus* and *A. variegatum*
- Ticks are effectively attracted to pheromones and kairomones, which could result in the development of a contamination trap in the field.
- A combination of tick pheromones with host kairomones with conidia of entomopathogenic fungi in a trap will attract more ticks and infect them before they return to the environment.

1.4 Overall aim of study

To develop formulations and delivery systems for microbial control of ticks.

1.4.1 Specific objectives

(i) To identify fungal isolates which are pathogenic against *R. pulchellus in vitro*.

(ii) To identify the most appropriate techniques for laboratory inoculation of nymphal and adult stages of *Rhipicephalus* ticks with fungal conidia.

(iii) To optimize attraction of *A. variegatum* adults by combining AAAP (pheromone) with 1-octen 3-ol and butyric acid (kairomones)

(iv) To evaluate the effects of formulations on the efficacy of a fungus-treated semiochemical-baited trap for control of *A. variegatum* population under field conditions.

(iv) To evaluate the performance of a *M. anisopliae*-treated semiochemical-baited trap to control *Amblyomma variegatum* in the field.

CHAPTER TWO

Laboratory screening of entomopathogenic fungi against *Rhipicephalus pulchellus* Gerstäcker (Acari: Ixodidae)

2.1 Introduction

The Zebra tick *R. pulchellus* is one of the most widely distributed tick species in Kenya, Northern Somalia and Eastern Ethiopia (Pegram *et al.*, 1971; Pegram, 1976; Walker *et al*, 2003). It is closely associated with wildlife and recent studies carried out in Haller Park along the coastline of Kenya indicated that *R. pulchellus* was the most abundant tick species (Wanzala and Okanga, 2006). It transmits Crimean Congo haemorrhagic fever virus and *Rickettsia conorii* Brumpt (Rickettsiales: Rickettsiaceae), which causes Mediterranean spotted fever and Kenyan tick typhus in humans and Nairobi sheep fever virus in livestock (Walker *et al.*, 2003). The immature stages of the tick readily bite humans (Walker *et al.*, 2003).

Ticks are mostly controlled by chemical acaricides (Mekonnen *et al.*, 2002). However, extensive use of these chemicals has favoured development of acaricide resistance in ticks (Baker and Shaw, 1965; Solomon *et al.*, 1979; Alonso-Díaz *et al.*, 2006) and has led to heightened concerns over human health and environmental impacts (Dipeolu and Ndungu, 1991). This has encouraged the search for alternative tick control strategies that are environmentally benign. Several biocontrol agents such as nematodes, predators, parasitic hymenoptera, are currently being considered for use against ticks (Chandler *et al.*, 2000; Samish *et al.*, 2004). Entomopathogenic fungi have shown potential for the management of Ixodid ticks (Samish *et al.*, 2004). Despite many studies on the susceptibility of members of the genus *Rhipicephalus* to *M. anisopliae* and *B. bassiana*, no previous report were found in the literature on the pathogenicity of these fungi against *R. pulchellus*. The objective of the current chapter is to identify isolates of *M. anisopliae* and *B. bassiana* possessing good virulence against unfed *R. pulchellus* adults in the laboratory.

2.2 Materials and methods

2.2.1 Ticks

Unfed adult ticks were collected from vegetation in the Mwea Game Reserve and were used to establishment of a tick colony. Ticks were reared in the International Centre of Insect Physiology and Ecology (*icipe*) insect rearing unit, and F_1 individuals were used in our study. Larvae, nymphs, and females were fed on New Zealand white rabbits and incubated in Perspex chambers at 26°C ± 1 and 85% RH at 12:12 L:D photoperiod. Three to four-week old unfed adult ticks were used.

2.2.2 Fungi

Fifteen isolates of arthropod-pathogenic fungi (12 isolates of *M. anisopliae* and 3 isolates of *B. bassiana*) were screened. Their origin is presented in Table 2.1. All the isolates were obtained from the *icipe's* Arthropod Germplasm Centre. The fungal isolates were stored under mineral oil. They were cultured on SDA plates at 26± 2 ° C. Conidia were harvested by scraping 3-4 week-old cultures and suspended in 20-ml universal bottles containing 0.05% Triton X 100 or corn oil (Chef cooking oil). Approximately 10 sterile glass beads (3 mm in diameter) were introduced into the conidial suspension and the suspension was mixed vigorously on a vortex shaker for 5 minutes to ensure separation of spores. Viability of conidia was determined using the technique described by Goettel and Inglis (1997). Viability of the conidia was determined before each bioassay by spread-plating 0.1 ml of conidial suspension titrated at 1 x 10^6 conidia ml^{-1} on SDA plates and examined under a light microscope 18 hours later. Conidial germination was determined from 100-spore counts with four replicates. Over 90 % of conidia germination was regularly obtained. Conidial germination > 90% was obtained and was considered suitable for the experiment.

2.2.3 Bioassays

A batch of 20 unfed adult ticks were individually inoculated topically with 1 µl of aqueous or oil suspension containing 1 x 10^9 conidia ml^{-1} using a microapplicator (Arnold Hand Microapplicator Burkard Manufacturing Co Ltd. Rickmansworth England). Inoculation was done around the anterior region on the joint between the idiosoma and basis capitulum of the tick (Figure 2.1). In the control treatment, ticks were treated with 1 µl of the carriers (0.05% Triton X 100 or corn oil) only. Ticks were handled using sterilized pairs of soft forceps. Twenty ticks were then placed in a 9-cm diameter Petri dish and incubated at 85 ± 5% RH,

26 ± 1 °C and 12:12 L:D photoperiod. Each treatment was replicated 6 times for each fungal isolate and for the control and there were 20 ticks per replication. Tick mortality was recorded after 4 weeks. Ticks that were immobile were assessed to confirm if they were dead or not. The ticks were breathed upon to stimulate them with CO_2, and any that remained immobile were then physically pressed on their underside with a pair of forceps. If the legs extended and then retracted tightly or moved back and forth independently, the tick was considered alive. If the legs did not retract fully, the tick was considered dead. Most of the dead ticks appeared wasted and dehydrated. Dead ticks were surface sterilized by dipping successively in 2.5% sodium hypochlorite, 70% ethanol for one minute and then rinsed twice in sterile distilled water. The ticks were then placed in Petri dishes lined with moistened filter paper to promote fungal outgrowth and conidiation. Mortality due to mycoses was confirmed by microscopic examination of hyphae and conidia developing on the surface of the cadaver.

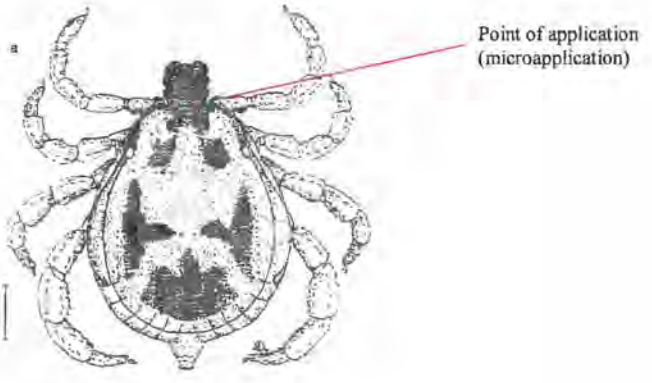

Figure 2.1 The point of inoculation of *R. pulchellus* with fungal conidia using a microapplicator (Walker, 1960)

Table 2.1 Origins of *M. anisopliae* and *B. bassiana* isolates that were assayed against *R. pulchellus*

Isolates	Locality	Host species/origin	Year of isolation
B. bassiana			
ICIPE 50	Rusinga island, Homa Bay Kenya	*Rhipicephalus appendiculatus*	1996
Bb Mbita	ICIPE field station, Mbita, Kenya	Soil	2004
Bb Kericho (ICIPE 279)	Kericho, Kenya	Soil	2005
M. anisopliae			
ICIPE 30	Kendu Bay, Kenya	*Busseola fusca*	1989
ICIPE 49	Mt. Kenya, Nyeri, Kenya	Soil	2005
ICIPE 20	Migori, Isbania, Kenya	Soil	1989
ICIPE 41	Migori, Isbania, Kenya	Soil	2005
ICIPE 55	Embu, Kenya	Soil	1990
ICIPE 60	Kakelo, Kisumu, Kenya	Soil	1996
RIRA/MA (ICIPE 7)	Rusinga island Homabay	*Amblyomma variegatum*	1996
ICIPE 62	Kinshasa, Democratic Republic of Congo (DRC)	Soil	1990
ICIPE 69	Matete, Kinshasa, DRC	Soil	1990
ICIPE 78	Ungoy, Mbita Kenya	*T. nigroplagiatae*	1990
ICIPE 31	Madagascar	*Locusta migrotoria capita*	2003
ICIPE 51	Embu, Kenya	Soil	2005

2.2.4 Data analyses

Tick mortality was adjusted using Abbott (1925) formula. ANOVA was performed on arcsin-squareroot-transformed percentage mortality data and means separated by Tukey's (HSD) test at $P = 0.05$ significance level. All analyses were performed with the SAS (2001) package.

2.3 Results

Figure 2.2 Mycosed *Rhipicephalus pulchellus* cadaver following infection by *Metarhizium anisopliae* in the laboratory

No mortality was recorded among ticks in the controls at four weeks post-treatment. Ticks were susceptible to fungi formulated in oil, however, four *M. anisopliae* isolates (ICIPE 78, ICIPE 69, ICIPE 62 and ICIPE 60) out of the 15 assayed were virulent against *R. pulchellus*. These were; ICIPE 78 (76.1 ± 5.9%), ICIPE 69 (62.6 ± 5%), ICIPE 62 (49.8 ± 5.8%) and ICIPE 60 (49.6 ± 7.7%) (Table 2.2). Only ICIPE 78, ICIPE 69 induced significantly higher (F value = 11.86; DF = 14, 75; $P < 0.0001$) tick mortalities compared to the other 11 less virulent fungal isolates that were assayed. Fungal conidiation was observed on tick cadavers held under conditions of high humidity (Figure 2.2). Similarly, no mortality was recorded for ticks treated with aqueous suspensions of *M. anisopliae* and *B. bassiana*.

Table 2.2 Percentage mortality (mean ± SE) of *R. pulchellus* adults induced by conidia of fungal isolates formulated in oil at 1 x 10^9 conidia ml^{-1} 4 weeks post-treatment.

Fungi isolates	Percentage mortality (mean ± SE)
ICIPE 50	21.0 ± 12.0 bc
Bb Mbita	15.9 ± 7.3.0 bc
ICIPE 279	15.9 ± 2.6 bc
ICIPE 30	17.5 ± 7.3 c
ICIPE 49	16.0 ± 5.5 c
ICIPE 20	15.9 ± 5.2 bc
ICIPE 41	2.6 ± 2.6 bc
ICIPE 55	1.6 ± 1.6 c
ICIPE 60	49.6 ± 7.0 ab
ICIPE 7	5.8 ± 3.2 c
ICIPE 62	49.8 ± 5.8 ab
ICIPE 69	62.6 ± 5.0 a
ICIPE 78	76.1 ± 5.9 a
ICIPE 31	18.9 ± 3.9 bc
ICIPE 51	16.1 ± 4.6 bc

Means with same lowercase letter within column are not significantly different at 0.05 level following Tukey's test.

2.4 Discussion

The result obtained in this study is in agreement with previous results that demonstrated the susceptibility of *Rhipicephalus* ticks to *M. anisopliae* isolates under laboratory conditions (Mwangi *et al.* 1995; Kaaya *et al.*, 1996; Barbosa *et al.*, 1997; Monteiro *et al.*, 1998a: 1998b). The results showed that *Metarhizium* isolates were relatively more virulent than *Beauveria* isolates; the most effective isolate was *M. anisopliae* ICIPE 78. Gindin *et al.* (2002), showed that *M. anisopliae* isolates were more virulent than fungi species from other genera; i.e., *Beauveria*, *Paecilomyces* and *Verticillium*. In the current study, *M. anisopliae*-ICIPE 7 induced low mortality (5%) in *R. pulchellus* adults whereas high mortality levels (65%) were obtained with the same isolate against *R. appendiculatus* adults in vegetation (Kaaya and Hassan, 2000). The origin, stability and culture history of individual isolates of entomopathogenic fungi may lead to significant variability in their virulence and host specificity (Cherrya *et al.*, 2005).

In the present study, ticks were killed when conidia were formulated in oil but no infection occurred after treatment with aqueous suspensions, indicating that oil formulation enhances virulence (Prior *et al.*, 1988; Kaaya and Hassan, 2000). Oils are reasonably effective in sticking spores to the hydrophobic cuticle of arthropod (Inglis *et al.,* 2002). The presence of a waterproof chitinous cuticle (Walker *et al.*, 2003), which is also hydrophobic may prevent adhesion of conidia or penetration of an aqueous treatment to susceptible regions of the cuticle.

This study demonstrated the susceptibility of *R. pulchellus* to some isolates of *M. anisopliae* Optimization of laboratory assay techniques is essential to provide predictive data on the entomopathogenic fungi. In the next chapter, development of procedures to optimize tick infection with the selected fungal strains will be presented.

CHAPTER THREE

Optimizing modes of inoculation of *Rhipicephalus* ticks (Acari: Ixodidae) with a mitosporic entomopathogenic fungus in the laboratory

3.1 Introduction

Strain selection is the primary step in the development of fungal pathogens for biological control (Soper and Ward, 1981). Several modes of inoculation have been used to evaluate the virulence of mitosporic Hyphomycetes. These include spraying conidia directly onto the target; exposing arthropods to treated leaves; dipping arthropods into titrated conidial suspensions; and treating the substrate (Hall and Papierok, 1982). Another technique was developed for screening fungal pathogens against locust consists of applying inoculum on the pronotum of the insect (Prior *et al.*, 1995). The most common method used to inoculation ticks has been by immersing them into titrated conidial suspensions (Frazzon *et al.*, 2000; Onofre *et al.*, 2001; Samish *et al.*, 2001; Kirkland *et al.*, 2004a). Dipping assays have shown that *M. anisopliae* is a virulent pathogen of members of the genus *Rhipicephalus* (Acari: Ixodidae) (Samish *et al.*, 2001; Gindin *et al.*, 2002). However, inoculation of ticks by dipping optimizes exposure to a pathogen and may not allow the most virulent strains to be readily differentiated from the less virulent ones. Dipping in conidial suspension can cause blockage of the spiracles of the host, resulting in high mortalities (Soarés, 1982). Furthermore, dipping of the ticks in conidial suspension does not closely "mimic" ULV spraying technique, which might be suitable for application of fungal suspensions over large area of grass vegetation infested with host seeking ticks ("ambusher"). In reality, these ticks will acquire the conidia directly or indirectly from the foliage when crawling over the contaminated substrate in a ULV spray application without being immersed in the suspension. Three-host ticks spend most of their life in soil and vegetation, thus development of safe and effective methods for controlling free-living ticks is warranted (Benjamin *et al.*, 2002).

The development of oil-based formulations for entomopathogenic fungi has improved the control of many arthropod pests (Lomer *et al.* 2001; Kaaya and Hassan, 2000; Shi *et al.*, 2008). Pure oils are compatible with the established ULV spray technique that is suitable for large-scale field application (Prior *et al.*, 2005a). Considering that large areas, may require treatments, as well as the costs involved with spore production and labour, pure oil formulation may be more effective compared to 10% water-oil emulsion; since it is

compatible with ULV application technique. Most workers investigating the biological control of ticks using *Metarhizium* have only reported using aqueous formulations (Mwangi *et al.*, 1995; Monteiro *et al.*, 1998a,b; Correia *et al.*, 1998). Others have evaluated pathogenicity of entomopathogenic fungal isolates suspended in oil-water emulsions (Kaaya and Hassan, 2000; Leemon and Johnson, 2008). To my knowledge only Polar *et al.* (2005a) have investigated pathogenicity of *M. anisopliae* suspended in pure oils against ticks. Optimizing inoculation procedure for infecting ticks with conidia formulated in pure oil may facilitate strain selection in the context of ULV application of mycoacaricide for tick control. Here, different methods of applying *M. anisopliae* onto nymphs and adults of the hard tick *R. pulchellus* were evaluated in laboratory in order to define superior methods to screen for the most effective fungal isolates.

3.2 Materials and methods

3.2.1 Tick

Ticks were obtained from the Animal and Quarantine Rearing Unit at *icipe*. The initial colony was established from adult ticks collected from the vegetation in Mwea Game Park reserve, Kenya, in 2006. Larvae, nymphs, and females were fed on New Zealand white rabbits and incubated in clear Perspex chambers at 26°C ± 1 and 85% ± 5 RH for 12:12 L:D photoperiod. Three to four week-old unfed adult ticks and 2-3 week old unfed nymphs were used for this study.

3.2.2 Fungus

Metarhizium anisopliae (ICIPE 60) used in this study was obtained from the *icipe's* Arthropod Germplasm Centre. The strain was originally isolated from soil in Kakelo Kisumu, Kenya in 1996 and was previously reported to be infective against *R. pulchellus* (see Chapter 2). The fungus was cultured on SDA plates at 26 ± 2°C. The virulence of the fungal strain was maintained by regular passage through the *R. pulchellus* (Schaerffenberg, 1964). Two to three week-old aerial conidia of *M. anisopliae* were harvested by scraping and suspended into corn oil (CHEF cooking oil, Premier Oil Mills Ltd) in a universal bottle containing glass beads. The suspension was then mixed in a vortex shaker for more than 5 minutes to homogenize the suspension. Conidial concentration was determined using an improved Neubauer haemocytometer and different test concentrations (10^6, 10^7, 10^8, 10^9 and 10^{10} conidia ml^{-1}) were obtained by serial dilutions in corn oil. Viability of the conidia was determined before each bioassay by spread-plating 0.1 ml of conidial suspension titrated at 1 x 10^6 conidia ml^{-1} onto SDA plates which were examined under a light microscope 18 hours later. Conidial germination was determined from 100-spore counts with four replicates. Germination rates of >90 % were regularly obtained. Attempts to include petroleum oils (kerosene and diesel) as carriers or in a mixture with corn oil in the current study was abandoned because thick swirling cloud of droplets was produced following spray by Burgerjon's spray tower, which may contaminate the laboratory.

3.2.3 Inoculation procedures

Two inoculation methods were initially tested: (i) via a Burgerjon spray tower (Burgerjon, 1956); (ii) using a microapplicator. In preliminary experiment, oil-based formulation of *M. anisopliae* (ICIPE 60) titred to 10^9 conidia ml^{-1} was applied to *R. pulchellus* adults using both

application methods. Since comparatively lower infection rates were obtained following treatment by microapplicator, all further treatments were delivered via Burgerjon's sprayer. In order to estimate the number of propagules on each tick 1 unfed male tick (size of tick was approximately 4 mm long including mouth parts) and 1 nymph were placed into separate 10-ml vial containing 0.05% Triton X-100 respectively. Vials were subjected to vigorous shaking by a vortex shaker for 5 minutes to dislodge conidia from the tick surface. The number of conidia ml^{-1} was determined using an improved Neubauer haemocytometer. The treatment consisted of 25 replicates per inoculation procedure. This method was not sensitive enough to detect the number of propagules on ticks at lower concentrations of conidial suspensions, especially for the nymphal stage.

3.2.3.1 Microapplication

Adult individual ticks were inoculated with 1 µl of conidial suspension titred at 10^9 conidia ml^{-1} formulated in oil applied around the anterior region on the joint between the idiosoma and basis capitulum using a 1 ml-syringe fixed to a microapplicator (Arnold Hand Microapplicator Burkard Manufacturing Co Ltd. Rickmansworth England) (Figure 3.1). In the control, ticks were treated with oil without conidia. Test-ticks were transferred to 9cm-diameter Petri dish after treatment and maintained at 25 ± 1 °C and 85 ± 5% RH. Mortality was recorded weekly for four weeks. Mortality caused by fungus was confirmed by microscopic examination of hyphae and spores on the body of dead ticks following incubation under high humid condition for 4-5 days. There were 20 ticks per replicates and 6 replicates in total per treatment group.

Figure 3.1 Inoculating tick with fungus using a microapplicator in the laboratory

3.2.3.2 Burgerjon's spray tower

The tower was fitted with an air-atomising nozzle connected to a regulator valve providing a constant airflow under 4 bar pressure, resulting to a deposit of approximately 3.8×10^6 conidia cm^2 (Figure 3.2). In the preliminary bioassays, 10 ml of conidial suspension titred at 1×10^9 conidia ml^{-1} was sprayed directly on ticks placed on a Petri dish (9 cm in diameter) lined with filter paper and ticks immediately transferred to clean Petri dishes (9 cm in diameter). The ticks were placed with the dorsal side uppermost while spraying. In the subsequent bioassays, three methods were used to infect ticks with the inoculum. The inoculation methods were designed to "mimic" the possible routes of inoculation (i.e. directly, indirectly or a combination of both direct and indirect routes) of ticks with fungus in an oil suspension following ULV spray-application under field conditions. The criteria for selecting the most appropriate method for assaying fungal isolates suspended in pure oil was based on mortality in both the control and fungus treated ticks.

(i) Direct spray of ticks and the substrate (filter paper lined at the bottom of a Petri dish) on which ticks were placed. Ticks were maintained in the treated Petri dish for 12 hours before being transfer to clean Petri dishes (SS).

(ii) Direct spray of ticks and substrate as above but ticks were transferred to clean dishes immediately (between 0-5 minutes) contamination was completed (SP).

(iii) Contamination of the substrate (filter paper). Petri dish lined at the bottom with a filter paper was sprayed and were then placed in an upside down position to allow ticks to climb up to the contaminated substrate naturally. Ticks were then

introduced into contaminated dishes where they were held for 12 h before removal to clean dishes (SW).

In all cases ticks were maintained at 25 ± 1 °C and 85 ± 5% RH for 2 weeks (nymphs) and 3 weeks (adults). In the control treatments, ticks were treated with oil only. Treatments were block randomized and consisted of 20 ticks per replicate and the experiment was replicated 6 times.

Figure 3.2 Burgerjon's spray tower used for infecting *Rhipicephalus pulchellus* in the laboratory

3.2.4 Data analyses

Tick mortality was adjusted using Abbott (1925) formula. ANOVA was performed on arcsin-squareroot-transformed Abbott percentage mortality data, log base 10 (x) transformations of number of spores on tick and means separated by Tukey's (HSD) test. Comparison for significance was at P = 0.05 significance level. LC_{50} was determined using probit analysis and their 95% Confidence intervals (IC) were used to evaluate significant difference of LC_{50} between different modes of inoculation. The analyses were performed using the SAS (2001) package.

3.3 Results

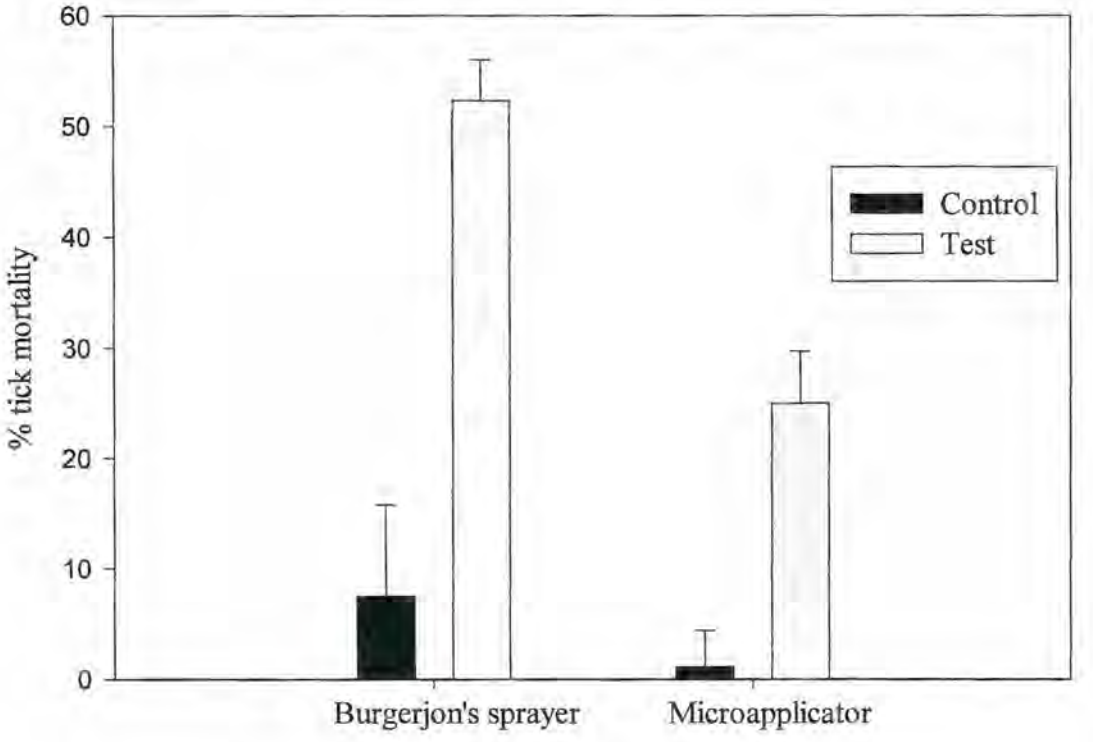

Figure 3.3 Mortality (%) of adult *R. pulchellus* following inoculation with oil suspension of *M. anisopliae* titred at 1 x 10^9 conidia ml^{-1} applied by Burgerjon's spray tower, or using a microapplicator at 4 weeks post-treatment.

Table 3.1 Percentage mortality (mean ± SE) of *R. pulchellus* nymphs to varying concentrations of oil-based formulation of *M. anisopliae* following different methods of exposure to conidia, 14 days post-treatment. (SS = direct spray of the inoculum on the tick and substrate, SP = direct pray on substrate and tick then transferred to a clean Petri dish and SW = exposure of ticks to contaminated substrate).

Concentration of conidia ml^{-1} before spraying on ticks	Mean ± SE number of spores on ticks Mode of exposure		
	SS	SW	SP
Control	24.2 ± 1.5	0.0 ± 0.0	2.5 ± 1.1
10^6	29.3 ± 10.4 bA	0.0 ± 0 cB	25.7 ± 5.8 Ba
10^7	28.3 ± 8.9 bA	2.5 ± 2.5 cA	26.8 ± 15 Ba
10^8	100.0 ± 0 aA	14.2 ± 2.4 bC	57.6 ± 16.1 abB
10^9	100.0 ± 0 aA	96.7 ± 2.1 aA	66.1 ± 14.7 abB
10^{10}	100.0 ± 0 aA	100.0 ± 0 aA	100.0 ± 0 Aa
LC_{50} (CI)	1.4 x 10^7 conidia ml^{-1} (1.2 x 10^7 – 1.6 x 10^7 conidia ml^{-1})	5 x 10^8 conidia ml^{-1} (4.7 x 10^8 - 5.3 x 10^8 conidia ml^{-1})	5.7 x 10^8 conidia ml^{-1} (5.2 x 10^8 - 6.3 x 10^8 conidia ml^{-1})

Means with same lowercase letter in each column and uppercase letter in each row are not significantly different at $P = 0.05$ following analysis with Tukey's test. CI signifies confidence interval.

Table 3.2 Number of *M. anisopliae* spores (mean ± SE) on nymphs and adults of *R. pulchellus* after application of oil suspension of 10^{10} conidia ml^{-1} by different modes of exposure to conidia. (SS = direct spray of the inoculum on the tick and substrate, SP = direct pray on substrate and tick, tick then transferred to a clean Petri dish; and SW = exposure of ticks to contaminated substrate)

Life stage of tick	Number of spores on ticks (mean ± SE) Mode of exposure		
	SS	SW	SP
Nymph	$1.5 \times 10^4 \pm 0.9 \times 10^3$ A	$4.4 \times 10^3 \pm 3.9 \times 10^2$ C	$9.9 \times 10^3 \pm 6 \times 10^2$ B
Adult	$1.3 \times 10^5 \pm 1.1 \times 10^4$ A	$2.3 \times 10^4 \pm 1.3 \times 10^3$ C	$9.2 \times 10^4 \pm 4.5 \times 10^3$ B

Mean with same lowercase letter in each row is not significantly different at 0.05 level of significance following analysis with Tukey's test.

Table 3.3 Percentage mortality (mean ± SE) of *R. pulchellus* adults to varying concentrations of oil-based formulation of *M. anisopliae* following different methods of exposure to conidia, 21 days post-treatment. (SS = direct spray of the inoculum on the tick and substrate, SP = direct pray on substrate and tick, tick then transferred to a clean Petri dish; and SW = exposure of ticks to contaminated substrate).

Concentration of conidia ml^{-1} before spraying on ticks	Mean ± SE number of spores on ticks Mode of exposure		
	SS	SW	SP
Control	23.3 ± 2.7	0.0 ± 0.0	12.5 ± 2.5
10^7	25.4 ± 4.9cA	0.0 ± 0bB	17.0 ± 5.9cA
10^8	72.0 ± 7.4bA	0.0 ± 0bC	31.8 ± 7.9bcB
10^9	100.0 ± 0aA	0.0 ± 0bC	53.0 ± 11.7abB
10^{10}	100.0 ± 0aA	15.0 ± 3.2aC	70.9 ± 5.8aB
LC_{50} (CI)	6.7×10^7 conidia ml^{-1} (6.3×10^7 - 7.2×10^7 conidia ml^{-1})	$>1 \times 10^{10}$ conidia ml^{-1}	5.3×10^9 conidia ml^{-1} (4.9×10^9 - 5.8×10^9 conidia ml^{-1})

Means with same lowercase letter in each column and uppercase letter in each row are not significantly different at 0.05 level of significance following analysis with Tukey's test. CI signifies confidence interval.

3.3.1 Preliminary experiment comparing different application methods

Control mortality in preliminary experiment was 4.2 and 7.5% when the control treatment was applied by microapplicator and Burgerjon's spray tower, respectively. Mortality from the fungal treatments was 25.0 and 52.3 % following application by microapplicator and spray tower, respectively at 4 weeks post treatment (Figure 3.3). The estimated mean number of fungal propagules on *R. pulchellus* adults was $1.5 \times 10^4 \pm 1.1 \times 10^3$ conidia ml^{-1} after spraying by Burgerjon's spray tower compared to 1×10^6 conidia ml^{-1} obtained with the microapplicator.

3.3.2 Nymph assays

Mortality of nymphs that were directly exposed to a fungal spray (SS) was 24.2% in the control but ranged from 29.3% (lowest concentration) to 100% (highest concentration), 2 weeks after fungus treatment (Table 3.1). Mortality of nymphs that were sprayed and transferred into clean Petri dishes (SP) was 2.5% in the control and ranged from 25.7% (lowest concentration) to 100% (highest concentration) in the fungus-treatment, 2 weeks post-treatment (Table 3.1). Mortality of nymphs that were exposed indirectly (SW) was 0.0% in the control and ranged from 0.0% (lowest concentration) and 100% (highest concentration) in the fungus treatment, 2 weeks post-treatment (Table 3.1). Mortality of *R. pulchellus* nymphs increased significantly (df = 4, 25; $P < 0.001$) with increasing concentrations of conidia irrespective of the method of exposure (SS, SW and SP). The fungus was most effective when application was by direct exposure (SS) (LC_{50} = 1.4 x 10^7 conidia ml^{-1} and CI = 1.2 x 10^7 – 1.6 x 10^7 conidia ml^{-1}) followed by SP (LC_{50} = 5.7 x 10^8 conidia ml^{-1} and CI = 5.2 x 10^8 – 6.3 x 10^8 conidia ml^{-1}) and SW (LC_{50} = 5 x 10^8 conidia ml^{-1} and CI = 4.7 x 10^8 - 5.3 x 10^8 conidia ml^{-1}), 14 days post-treatment. However, there was a significantly higher (df = 2, 74; $P < 0.0001$) mean number of fungal propagules on ticks after spraying 10^{10} conidia ml^{-1} by SS (1.5 x 10^4 ± 0.9 x 10^3 conidia ml^{-1}) compared to SP (9.9 x 10^3 ± 6 x 10^2 conidia ml^{-1}) and SW (4.4 x 10^3 ± 3.9 x 10^2 conidia ml^{-1}) (Table 3.2).

3.3.3 Adult assays

Mortality of adults that were directly exposed to fungal sprays (SS) was 23.3% in the control and ranged from 25.4% (lowest concentration) to 100% (highest concentration) in the fungus treatments, 3 weeks after spraying (Table 3.3). Mortality of adults that were directly exposed to fungal spray and then transferred into clean plates (SP) was 5.8% in the control and ranged from 17.0% (lowest concentration) to 70.9% (highest concentration) in the fungus-treated lots, 3 weeks post-treatment (Table 3.3). Mortality of adults that were exposed to treated substrate (SW), and ranged from 0.0% (lowest concentration) to 15.0% (highest concentration) in the fungus-treated lots, 3 weeks after treatment (Table 3.3). Mortality increased significantly (df = 3, 20; $P < 0.001$) with increasing concentrations in all the three techniques (SS, SW and SP) three weeks post-treatment (Tables 3.3). The fungus was most effective when application was by direct exposure (SS) (LC_{50} = 6.7 x 10^7 conidia ml^{-1} and CI = 6.3 x 10^7 – 7.2 x 10^7 conidia ml^{-1}) followed by SP (LC_{50} = 5.3 x 10^9 conidia ml^{-1} and CI = 4.9 x 10^9 – 5.8 x 10^9 conidia ml^{-1}) and ($LC_{50} > 10^{10}$ conidia ml^{-1}), 21 days post-treatment. The

mean number of fungal propagules on ticks following direct spray (SS) ($1.3 \times 10^5 \pm 1.1 \times 10^4$ conidia ml^{-1}) of 10 ml of fungal suspensions titred at 10^{10} conidia ml^{-1} was significantly higher (df = 2, 72; $P < 0.0001$) compared to SP ($9.2 \times 10^4 \pm 4.5 \times 10^3$ conidia ml^{-1}) and SW ($2.3 \times 10^4 \pm 1.3 \times 10^3$ conidia ml^{-1}) (Table 3.2).

3.4 Discussion

Scant information on the optimization of assay techniques for *Rhipicephalus* ticks ("ambusher") and the use of pure oils as formulation agents for assaying entomogenous fungi against ticks was found in literature, prompting the current series of experiments. In the preliminary experiment, application of fungi via a Burgerjon's spray tower resulted in higher mortality than inoculation with a microapplicator. The former technique was therefore selected for further studies. High mortality levels were recorded in the control treatments when nymph and adult ticks were exposed to sprays and maintained in the same Petri dish (SS). This may be explained by the toxic effect of oil to arthropods (Goettel and Inglis, 1997; Moslim *et al.*, 2004) and the prolonged exposure time compared to the other treatments. Previously, Polar *et al.* (2005a) reported that pure coconut oil caused high mortality in control-treated *R. microplus*. They suggested that high mortality caused by the pure coconut oil might have contributed to tick mortality induced by *M. anisopliae* formulated in coconut oil. Although mortality in the control-treated ticks can be accounted statistically, bioassay results may become meaningless if control mortalities are too high (Butt and Goettel, 2000).

Significant differences in tick mortality following treatment with fungi were observed among the different methods of inoculation used, implying that the way the pathogen is applied can influence host mortality. For example, tick mortality recorded following exposure to spray via Burgerjon's spray tower was higher than in microapplicator, although the actual number of fungal propagules on tick was higher in the later. Samish *et al.* (2001) suggested that differences in techniques of contamination (immersing adults in a conidial suspension or placing the preimaginal stages on paper soaked with conidial) may induce different levels of infection in the various stages. According to Fernandez *et al.* (2001), distribution of conidia on the host is likely to differ according to the method of exposure, and successful infection is more likely via some parts of the cuticle than others. For example, penetrations of the cuticle at the many leg joints, mouthparts, spiracles or setae is easier than other parts of the tick cuticle and were more likely to be contaminated by droplets applied by the spray tower than the microapplicator. These parts could present easy points for germinating tubes and their exposure to spray droplets by Burgerjon's spray tower might have resulted in the higher tick mortality than the microapplicator where the conidial suspension was applied on one joint, i.e. between the idiosoma and basis capitulum. In ticks, it has been observed that the setae and cuticular ridge are easily penetrable by the fungus (Leemon and Jonsson, 2008).

Scanning electron micrograph analysis of infected ticks showed that *M. anisopliae* conidia preferred attachment sites at certain junctions, like those present in the tick legs (Arruda *et al.*, 2005). According to Sonenshine (1991), the cuticle is thinner in these areas. Cuticular folds in arthropods exoskeletons may also be sites of high moisture (Inglis *et al.*, 2001). Other workers such as Kirkland *et al.* (2004a) observed conidial germination varied by body region.

The calculated LC_{50} values showed that higher infection levels were obtained when nymphs and adults of *R. pulchellus* were sprayed directly and maintained on treated substrate for 12 hours (SS) compared to when sprayed directly and transferred to clean dishes (SP) or indirectly treated by exposure to contaminated substrate (SW). The higher dose of the acquired inocula in SS could be responsible for the higher mortality recorded since mortality was dose-dependent. Tick mortality increases with conidial concentrations (Zhioua *et al.*, 1997; Frazzon *et al.*, 2000). Increased in conidia concentrations could have led to corresponding increases in the actual number of conidia deposited on the tick, thus inducing higher infection levels. Eventhough nymphs acquired lower dose of fungal propagules than the adults, they were relatively more susceptible compared to the adults is the three inoculation methods. The relatively smaller size of nymph and the lack of thick cuticle compared to adult tick could have favoured the high levels of infection. It appears that the number of spores on the cuticle must reach a certain threshold to induced high levels of tick mortality. Previously, Zhioua *et al.* (1997) recorded sharp increase in mortality of *R. microplus* engorged larvae and females at concentrations above 10^6 conidia ml^{-1} and minimal mortality below the concentration. It is thus important researchers obtain estimates of the number of propagules on tick during bioassay to minimize variation in results obtained in different laboratories with the same fungal isolates.

Based on the behaviour of *Rhipicephalus* ticks ("ambushers"), all the inoculation methods were appropriate for screening of entomopathogenic fungal isolates in the pure oil carrier against adults and nymphs. However, while conidia can be applied by direct spraying and ticks retained within the assay arena (SS), the unacceptable high levels of control mortality obtained ruled this method out for routine work. Direct spray treatment followed by removal of ticks to a clean container (SP) is thus a more suitable method for screening entomopathogenic fungi in an oil carrier against adult and nymph and indirect exposure to treated substrate (SW) is suitable for immature stages. The main setback with the indirect inoculation method (SS) is the difficulty to ensure presentation of a precise dose, which will

reduce variability in the number of propagules picked by the individual tick (Butt and Goettel, 2000). However, free living immature stages of *R. pulchellus* prefer questing from on grass in nature and instinctively climbed up to the fungus-treated surfaces few minutes after treatment in this study. Furthermore, the lowest tick mortalities in control treatments were recorded following indirect spray (SW).

Having established the most suitable procedures for infecting ticks ("ambushers"), different fungal formulations, and delivery systems could then be evaluated with a goal of identifying ways of minimizing environmental contamination and increasing efficacy against "hunter" ticks. The next chapter addresses the potential of combining fungus and semiochemicals in baited trap for reduction of *A. variegatum* tick population under semifield conditions.

CHAPTER FOUR

The use of a semiochemical bait to enhance exposure of *Amblyomma variegatum* (Acari: Ixodidae) to *Metarhizium anisopliae* (Ascomycota: Hypocreales)

4.1 Introduction

Amblyomma variegatum Fabricius 1794 (Acari: Ixodidae) or the tropical bont tick is the most widely distributed tick species across the African continent (Walker *et al.*, 2003) and was recently introduced into the Caribbean (Barré *et al.*, 1995). It transmits *Ehrlichia ruminantium* (Rickettsiales: Anaplasmataceae) a rickettsia that causes heartwater in ruminants, and the bacterium *Dermatophilus congolensis* van Saceghem 1915 (Actinobacteria: Dermatophilaceae), an acute bovine dermatophilosis. *Amblyomma variegatum* is also a vector of *Rickettsia africae* Kelly *et al.* (1996) sp. nov. (Rickettsiales: Rickettsiaceae), the causative agent of African tick-bite fever (Morita *et al.*, 2004). Although endemic to sub-Saharan Africa (Kelly *et al.*, 1996), the disease is spreading rapidly and is regarded as the most widely distributed of all the rickettsial spotted fevers known to be pathogenic to humans (Raoult and Roux, 1997). Infestation by ticks can also cause considerable losses to the livestock industry in Africa and the Caribbean (Uilenberg *et al.*, 1984; Kvaria, 2006).

Current methods of tick control rely heavily on conventional chemical acaricides and repellents. There are many problems associated with their widespread use including development of resistance to these synthetic chemicals, and negative impacts on human health and the environmental (Mukhebi and Perry, 1992; George, 2000; Jonsson *et al.*, 2000; Tingle *et al.*, 2000). This has prompted the search for alternative methods of tick control that can be used within integrated tick management programmes.

Controlling *Ambyomma* ticks off-host might be difficult because they are "hunters"and do not climb up the vegetation in search of their host. Hence, an alternative strategy for fungus application off-host would be to lure the ticks from the vegetation into contact with fungus in traps baited with semiochemicals. *Amblyomma* ticks respond to a number of semiochemicals including the attraction-aggregation-attachment-pheromone (AAAP) emitted by feeding males and carbon dioxide (kairomone) exhaled from their hosts (Sonenshine, 2006). An

infection device designated to deliver entomopathogenic fungi to *Amblyomma* ticks in the field was first described by Maranga *et al.* (2006). However, the device is costly to fabricate and difficult to use, which reduces its potential for widespread implementation. There is need to develop a simple low-cost contamination device or modify the existing one. Bryson *et al.* (2000) developed a simple device for tick monitoring. The device, which uses solvent extracts of fed male ticks as baits could be adopted. The attraction of ticks to the trap could be increased by optimization of the pheromone AAAP (attraction-aggregation-attachment pheromone) and carbon dioxide. The chemical 1-octen-3-ol, which occurs naturally in adult *A. variegatum*, is a minor constituent of cattle breath, attracts *A. variegatum* adults in the laboratory (McMahon *et al.*, 2001). Butyric acid, a constituent of rumen metabolites of vertebrates also elicits response in ticks under laboratory conditions (Donzé *et al.*, 2004). The objectives of the present chapter were (i) to see if 1-octen 3-ol and butyric acid would augment the AAAP pheromone in the attraction of *A. variegatum* adults; (ii) to test the performance of the trap described by Bryson *et al.* (2000) baited with a synthetic combination of effective semiochemicals; and (iii) to compare the performance of fungal formulations that could be used with a semiochemical-baited trap for effective control of *A. variegatum* in the field.

4.2 Materials and Methods

4.2.1 Ticks

Engorged *A. variegatum* females collected from cattle originating from the Marsabit area of Kenya in 2006 were used to start a fresh tick colony. Ticks were reared at the Animal Rearing and Quarantine Unit, International Centre of Insect Physiology and Ecology (*icipe).* All life stages of the tick were fed on New Zealand white rabbits. The different instars were maintained in perspex chambers at 26°C ± 1 and 85 ± 5 % RH under a 12:12 L: D photoperiod. Three to four-week old unfed adults were used, in all the experiments.

4.2.2 Fungus

The *M. anisopliae* isolate (R1/RA) used in this study was obtained from the *icipe* Arthropod Germplasm Centre. The strain was originally isolated from an engorged *A. variegatum* female collected from Rusinga Island, Kenya in 1996 and has previously been reported to be virulent against *A. variegatum* (Kaaya *et al.*, 1996). It was maintained under mineral oil. The virulence of the isolated fungus was restored by passage through adult *A. variegatum.* Conidia were produced on long rice as substrate (Milner R.J., unpublished data). Viability of conidia was determined using the technique described by Goettel and Inglis (1997) before they were used in the field trials. Conidial germination > 90% was obtained and was considered acceptable for field application.

4.2.3 Source of semiochemical candidates

The synthetic components of the attraction-aggregation-attachment pheromone (ortho-nitrophenol, methyl salicylate and nonanioc acid), dicloromethane (DCM), butyric acid and 1-octen-3-ol were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Dry ice used as a source of CO_2 was obtained from Carbacid, Kenya.

4.2.4 Optimization of semiochemical blend

A two-choice T-tube olfactometer bioassay method was used to assess relative tick attraction to two odour sources. The olfactometer consisted of two square tubular glass arms (1 x 1 x 10 cm) and a stem (1 x 1 x 5 cm) connected tightly to the glass arms (Figure 4.1). The extreme end of each of the arms and stem was connected to a square glass chamber (3 x 3 x 3 cm). The chamber of the stem served as the release point for the tick. Air entered each arm of the

olfactometer and after flowing over the respective odour source [filter paper (2 x 2 cm) + pheromone and/or kairomones or control] held in the chamber, at a flow rate of 5 ml/s. The doses of semiochemicals in DCM solvent on the test filter paper that were evaluated for attraction are presented in Table 4.1. Control filter paper had DCM only. One tick was in the release chamber at a time and had to move upwind as in nature. At the T-junction ticks choose between the control and treatment arms. Each tick was allowed a minimum of 0.5 seconds and a maximum of 5 minutes to move. Ticks failing to respond after 5 minutes were removed. One randomly selected tick representing one replicate was bioassayed separately. Twenty ticks (10 males and 10 females) were successfully assayed for each test dose. The olfactometer was rinsed with DCM and air dried at 40 °C for 10 minutes after each determination. The position of the treatment and control chambers at the end of the arms were changed for each assay, by alternating placement of the control and test filter papers. The olfactometer was placed horizontally during the experiment to "mimic" the natural questing behaviour of *A. variegatum*. Each tick was used only once. The experiment was conducted at 26 ± 1 °C and 70 ± 5% RH.

Table 4.1 Doses of semiochemicals that were evaluated in T-tube olfactometer

Semiochemicals	Dose			
AAAP[1]	1100 µg	44 µg	22 µg	14.7 µg
Butyric acid	0.010 µg	0.001 µg	0.0005 µg	-
1-Octen-3-ol	0.032 µg	0.016 µg	0.008 µg	-

Table 4.1 Doses of semiochemicals that were evaluated in T-tube olfactometer

[1]AAAP = O-nitrophenol, methyl salicylate and nonanoic acid in the ratio 2:1:8

Figure 4.1 Two-choice olfactometer. (Air is passed over the left and right chambers containing the source of volatiles by sucking air out of the front central chamber containing the ticks)

4.2.5 Attraction of ticks to semiochemical-baited traps in experimental field plots

A modification of the trap described by Bryson *et al.* (2000) was used in this study. It consisted of a 900 cm^2 area made of four 10 cm-wooden pegs hammered to the ground. A 2-cm^2 rubber sponge impregnated with 0.016 µg of 1-octen-3-ol and 22 µg of AAAP was fixed onto the top of each of the four wooden pegs per trap (Figure 4.2). These doses were selected based on the results obtained in the T-tube olfactometer bioassays. Blocks of dry ice (approx. 70 g) in a plastic beaker were placed in the centre of the trap served as a source of CO_2. Traps were placed upwind of the release ticks on the chosen sites to allow ticks to move upwind. Traps were located in open areas (225 m^2) with approximately 5 cm high grass. Test and control traps were placed 2 m from each other. Ticks were placed 1, 2, 3, 4, 5, 6, 7 and 8 meters from the traps at an angle of 90° and midway between the test and control traps. Unfortunately, it was not possible to measure the wind speed at the time of testing, but it was a light breeze. Ten ticks (5 males and 5 females) were used for each distance. Each tick was marked with a distinct colour spot (Rowney Georgian oil colour, made in England, London HA 35 RH) applied topically and ventrally depending on their distance from the trap. The experiment was carried out from 13 to 30 October 2006 within *icipe*'s Nairobi Headquarters premises and was allowed to run for 90 minutes in mornings between 0800 and 0930 h. Ticks were active in the morning. The experiment was replicated five times. The temperature above ground during the experimental period ranged from 25 to 29 °C (under the sun) and the relative humidity was between 50 and 70%.

4.2.6 Relative efficacy of different fungal formulations on tick reduction under field conditions

Three formulations of fungal spores were evaluated in the semiochemical-baited trap described above:

(i) Dry spore powder (approx. 50 g) spread on the grass within the trap by hand.

(ii) High volume emulsifiable formulation (HV) titrated at 10^9 conidia ml^{-1} (consisting of 49.5% sterile distilled water, 49.5% corn oil [CHEF cooking oil, Premier Oil Mills LTD] and 1% Tween 80). Two hundred and fifty millilitres was sprayed within the trap using a pressurized hand-held sprayer (C 1.5 model) at an estimated output of 150 l per ha.

(iii) An ultra-low volume (ULV) oil formulation at 1 x 10^9 conidia ml^{-1}. One hundred millilitres was sprayed using a Micron-ULV sprayer (Micron Sprayers, Bromyard, UK) at the output of 1 l per hectare (Figure 4.3).

(iv) Control treatments consisted of the same formulations without the fungus, except in the case of treatment (i), where grass served as the control.

After the traps were prepared, atleast 24 ticks were placed 4 m away from the traps. Ticks were attracted to the pheromone-baited trap where they were contaminated with the fungus. Ticks were then transferred from the trap to plastic basins (41 cm top diameter x 29 cm bottom diameter x 24 cm height) previously planted with *Pennisetum clandestinum* (kikuyu grass). The height of the grass was approximately 12 cm. The top of the basin was covered with mosquito net (supplied by Amiran Kenya) held tightly in place with bands which prevented the ticks from escaping (Figure 4.4). The experiment was allowed to run for 5 weeks after which surviving were recovered and counted. These ticks were then transferred to 9-cm diameter Petri dishes and brought to the laboratory where they were maintained at 26 ± 1 °C, 85 ± 5 % RH and a 12:12 h L:D photoperiod for 2 weeks, after which mortality was recorded. Dead ticks were surface-sterilized with 2.5% sodium hypochlorite and 70% alcohol, rinsed twice in sterile distilled water and were then placed into 9-cm diameter Petri dishes lined with moistened filter paper to promote growth and confirm death due to mycosis on the surface of the cadaver. Petri dishes were sealed with paraffin and maintained at room temperature (25 ± 2 °C). Five plastic basins were used for each treatment, which represented 5 replicates. The entire experiment was repeated twice: from September to October 2007 and from December 2007 to January 2008, which corresponded with the rainy and dry seasons, respectively. The average daytime temperatures and relative humidity within buckets during

the 1st trial ranged from 24.8 ± 0.7 °C to 29.3 ± 0.5 °C and 86.3 ± 2.4% to 89 ± 1.8% respectively. The average daytime temperature and relative humidity ranged from 25.0 ± 0.3 °C to 27.4 ± 0.2 °C and 53.5 ± 3.4% to 66.4 ± 3.4% respectively for the 2nd trial. The grass was watered from below with 1l of water after 2 weeks into the experiment.

Figure 4.2 A fungus-treated semiochemical-baited trap. Note the high volume pressurized (HV) sprayer located to the left of the trap.

Figure 4.3 ULV micron applicator

Figure 4.4 Plastic basins containing planted *Pennisetum clandestinum* grass into which fungus or control-treated ticks were transferred. The nets prevent ticks from escaping.

4.2.7 Effect of fungal formulation on tick infection

The objective was to estimate the number of conidia that a single tick picked up while visiting the semiochemical-baited trap immediately after treatment and 48 h later following transfer to the plastic basins. Four ticks per replicate and per treatment were selected at random and each was transferred into individual 10-ml vials and brought to the laboratory. Five millilitres of 0.05% Triton X-100 was added to each vial which was then shaken vigorous on a vortex shaker for 5 minutes to dislodge conidia from the tick cuticle. The number of conidia ml^{-1} was determined using an improved Neubauer haemocytometer. Counts were taken from 5 replicates of 4 ticks from each treatment. The percentage of conidia that germinated, 0 and 48 hours post-treatment was determined in the 2^{nd} trial only. The treatment consisted of 5 replicates of 4 ticks each and the experiment was repeated twice.

4.2.8 Data analysis

The percentage attraction in the T-tube assays was determined using the following formula: (number of ticks attracted to kairomone/pheromone - number of ticks in control)/(number of ticks in control + attracted to kairomone/pheromone) x 100. The significance of the difference in the proportion of ticks (males and females pooled) attracted to the test and control treatments amongst different concentrations of each pheromone/kairomone was determined with Fisher's exact method at $P = 0.05$ (Robertson, 1960). A one-way ANOVA was performed on arcsin-square root transformed tick control efficacy (%) data, arcsin square

root transformation of percentage attraction of *A. variegatum* in the field, arcsin square root transformation of tick mortality (%) in the laboratory and log base 10 (x) transformations of number of spores on ticks and number of germinating conidia. Means were separated by Tukey (HSD) test using the SAS (2001) package at $P = 0.05$. The relative tick reduction (%) in traps baited with semiochemicals and the three fungal formulations was calculated using the formula [(number of alive ticks in control- number of alive ticks in test)/number of alive ticks in control] X 100 (European Medicines Agency, 2004).

4.3 Results

Table 4.2 Percentage relative attraction of *A. variegatum* adults (pooled data for males and females) to semiochemicals in a two-choice olfactometer

Semiochemical	% Relative attraction Pooled Males and Females
AAAP	
1100 µg	-50*
44 µg	20
22 µg	50
14.7 µg	30
Butyric acid	
0.010 µg	-20
0.001 µg	-20
0.0005 µg	-10
1-octen-3-ol	
0.032 µg	-30
0.016 µg	50
0.008 µg	20

* Denotes attractive response was significantly different compared to the other concentrations for each semiochemical within the column at $P < 0.05$. No significant difference in attraction was observed between males and female, hence data was pooled.

Table 4.3 Relative attraction of adult *Amblyomma variegatum* to a trap baited with a blend of AAA pheromone at dose of 22 µg, 1-octen-3-ol at dose of 0.016 µg and CO_2 under field conditions when placed at different distances from the trap.

Distance (m)	Percentage (Mean ± SE) tick attraction
1	100 ± 0 a
2	100 ± 0 a
3	96 ± 2.5 a
4	100 ± 0 a
5	96 ± 2.5 a
6	94 ± 6 a
7	66 ± 5.1 b
8	24 ± 5.1 c

Mean tick attraction between distances ($F = 36.4$; $df = 7,32$; $P < 0.0001$). Means within a column bearing the same letter are not significantly different at the 0.05 level as determined with Tukey's test.

Table 4.4 Percent relative tick reduction (mean ± SE) following exposure to grass treated with different formulations of *Metarhizium anisopliae* applied in pheromone-baited areas against *Amblyomma variegatum* during the first and second trials over the controls

Formulation	(%) Relative tick reduction (mean ± SE)	
	1st trial	2nd trial
Powder	38.0± 6.2 ab	24.4 ± 3.9 b
High volume (HV emulsifiable)	54.7 ± 4.2 a	46.5 ± 5.5 a
ULV oil	32.0 ± 5.1 b	23.8 ± 3.3 b

Mean relative tick reduction during the 1st trial ($F = 4.94$; $df = 2,12$; $P = 0.02$) and 2nd trials ($F = 8.47$; $df = 2,12$; $P = 0.005$). Means bearing the same letter within column a are not significantly different at the 0.05 level as determined with Tukey's test.

Table 4.5 Mean ± SE number of spores carried on ticks immediately post-contamination (day 0) in the pheromone-baited trap and two days post-contamination (day 2). Data of two experiments were pooled.

Formulation	mean ± SE (day 0)	mean ± SE (day 2)	Spore loss (%)
Powder	$6.7 \times 10^6 \pm 8.4 \times 10^5$ aA	$7.3 \times 10^5 \pm 6.5 \times 10^4$ aB	89.1
High volume (HV emulsifiable)	$4.6 \times 10^5 \pm 5.7 \times 10^4$ bA	$3.7 \times 10^5 \pm 4.2 \times 10^4$ bA	17.1
ULV oil	$2.3 \times 10^5 \pm 2.3 \times 10^4$ cA	$1.5 \times 10^5 \pm 3.0 \times 10^4$ cB	33.3

Mean number of spores picked up by individual ticks at day 0 and day 2 in areas treated with spore powder ($F = 106.6$; df =1, 78; $P < 0.0001$), oil ($F = 14.49$; df =1, 78; $P < 0.0003$) and emulsifiable ($F = 0.35$; df =1, 78; $P = 0.55$) formulations. Means bearing the same lowercase letter within column and uppercase letter within rows are not significantly different at the 0.05 level as determined using Turkey's test.

Table 4.6 Percentage of spore germination (mean ± SE) recorded on ticks, 2 days post-contamination (day 2) in pheromone trap in the 2nd trial.

Formulation	Percentage of spore germination (day 2) Mean ± SE
Powder	0.11 ± 0.059 b
High volume (HV emulsifiable)	27.78 ± 4.5 a
ULV oil	0.98 ± 0.56 b

Means bearing the same lowercase letter within column is not significantly different at the 0.05 level as determined with Turkey's test.

4.3.1 Optimization of semiochemical blend

In olfactometer bioassays, AAAP at the doses of 14.7 µg, 22 µg and 44 µg, and 1-octen–3-ol at a dose of 0.016 µg attracted both sexes of *A. variegatum*. However, ticks significantly ($P < 0.05$) avoided AAAP at a concentration of 1.1 mg in the olfactometer assay (Table 4.2). Butyric acid repelled unfed *A. variegatum* ticks (Table 4.2).

Optimum attraction was observed at doses 22 µg and 0.016 µg of AAAP and 1-octen-3-ol, respectively' and both doses were selected for field trials.

4.3.2 Attraction of adult *A. variegatum* to semiochemical-baited traps in field plots

No single tick moved to the control trap (No AAAP, carbon dioxide or 1-octen-3-ol) in the field. Ticks were attracted from up to a distance of 8 m (Table 4.3). No significant difference was observed in the attraction of groups of ticks released between 1 and 6 m away from the trap. However, fewer ticks were attracted to the semiochemical-baited trap beyond 6 m ($F = 36.4$; $df = 7, 32$; $P < 0.0001$). There was no significant difference ($P > 0.05$) in the attraction between males and females to the blend. Some ticks were observed spending several seconds within the trap before leaving, whilst others displayed arrestant behaviour. Most of the ticks were attracted to the trap in less than 90 minutes and this could enhance efficacy of an entomopathogenic fungus applied to a limited field area.

4.3.3 Relative efficacy of different fungal formulation on tick reduction under field conditions

Emulsifiable conidial formulation caused significant ($F= 4.94$; $df = 2,12$; $P = 0.02$) tick reduction (54.7%) compared to the powder formulation (38.0%) and the ULV oil formulation (32.0%) during the first trial carried out in the rainy season. However no significant difference in tick reduction was obtained between powder and emulsifiable formulation in the first trial. In the second trial in the dry season, emulsifiable formulation (46.5%) also caused significant ($F= 8.47$, $df = 2,12$ and $P = 0.005$) tick reduction compared to powder (24.4%) and ULV oil (23.8%) formulations (Table 4.4). Ticks that survived infection in the field succumbed to fungal infection two weeks after they were moved to the laboratory: 46.8 and 53.0% mortality in emulsifiable formulation treatments, 1st and 2nd trial, respectively; 39.0% and 34.4% in oil formulation, 1st and 2nd trial, respectively; 4.2 and 6.8% from powder

formulation, 1st and 2nd trial, respectively. Mortality was significantly higher in emulsifiable and oil formulations in both trials (F= 24.93; df = 2,12; $P < 0.0001$) for the trial in the rainy season) and (F = 29.88; df = 2,12; $P < 0.0001$) for the trial in the dry season) than the powder formulation. Fungus-treated semiochemical-biated trap significantly reduced tick population in buckets compared to the control treated traps.

4.3.4 Effect of fungal formulation in contaminating ticks

No significant differences ($P > 0.05$) were observed between the treatments in the first and second trials, thus the data were pooled. The number of conidia picked up by individual ticks immediately after the exposure was significantly higher (F = 170.66; df = 2,117; $P < 0.0001$) in the powder formulation ($6.7 \times 10^6 \pm 8.4 \times 10^5$ conidia), followed by the emulsifiable formulation ($4.6 \times 10^5 \pm 5.7 \times 10^4$ conidia) and oil formulation ($2.3 \times 10^5 \pm 2.3 \times 10^4$ conidia) (Table 4.5). However, 48 h postinfection, ticks exposed to the powder formulation had a significant (F = 106.6; df =1, 78; $P < 0.0001$) loss of 89.1% compared to 17.1% and 33.3% with the emulsifiable (F = 0.35; df =1, 78; $P > 0.55$) and oil (F = 14.49; df =1, 78; $P < 0.0003$) formulations, respectively (Table 4.5). A significantly (F = 78.4; df = 2, 57; $P< 0.0001$) higher percentage of spores that were dislodged from ticks treated with fungus in the emulsifiable formulation (27.78 ± 4.5%) had germinated 48 h post-treatment compared to the ULV oil (0.98 ± 0.56%) and spore powder formulation (0.11 ± 0.059%) in the 2nd trial (Table 4.6). No spore had germinated at 0 day post-treatment in the 2nd trial.

4.4 Discussion

AAAP attracted unfed adults of *A. variegatum* in laboratory assays as previously reported (Schoni *et al.*, 1984; Touré, 2005). Schoni *et al.* (1984), found the activity threshold of the synthetic AAAP pheromone in laboratory assays to correspond to an equivalent of 1% of the pheromones produced by a fed male. One fed adult *A. variegatum* male produces 2 µg o-nitrophenol, 1 µg methyl salicylate and 8 µg nonanoic acid. In this study, the olfactometer was placed horizontally, which is consistent with the natural hunter behavior of host seeking *A. variegatum* and optimum attraction was recorded at 22 µg of AAAP (Table 4.2). Higher doses of AAAP appeared to repel the ticks. Previously, Toure (2005) reported attraction at a higher dose (1100 µg) in a T-tube olfactometer placed vertically. Response to an attractant varies with its ambient concentration gradients; for example, *A. variegatum* ticks were reported to be insensitive to high levels of 1-octen-3-ol but were attracted to lower levels (McMahon and Guerin, 2000).

Of the two additional candidate semiochemicals evaluated in the laboratory in this study, butyric acid appeared to be repellent to unfed *A. variegatum* ticks at the three concentrations tested and was therefore not included in the field experiments. The attraction responses of *A. variegatum* adults to 1-octen-3-ol observed in this study is in agreement with the findings of McMahon *et al.* (2001) and Touré (2005). In field plots, Touré (2005) also showed enhanced attraction of *A. variegatum* to a combination of 1-octen-3-ol and AAAP. In the present study, the combination attracted *A. variegatum* adults from up to 8 m away (24.0% of ticks attracted), which is further than those reported in previous studies, where ticks were attracted to a maximum distance of 5 or 6 m and with a lower efficiency (Barré *et al.*, 1997; Maranga *et al.*, 2003). Maranga *et al.* (2003) used 6600 µg/trap of AAAP to elicit the optimum attraction of *A. variegatum* without 1-octen-3-ol in the field. This dose of AAAP far exceeded the dose of 88 µg/trap (equivalent to 8 fed-male ticks per trap) that was used in the current study in field plots. The fact that adults of *A. variegatum* were lured from 8 m and congregated within the baited-trap in our study, demonstrates the superior efficacy of a combination of AAAP and 1-octen-3-ol (22 µg x 4 = 88 µg and 0.016 µg x 4 = 0.064 µg, respectively). 1-Octen-3-ol is a significant constituent of cattle odour (Osterkamp *et al.*, 1999). Thus, the enhanced attraction of *A. variegatum* adults to a combined treatment comprised of AAAP and 1-octen-3-ol is not surprising.

Although ticks that were attracted to traps treated with *M. anisopliae* powder initially picked up the highest number of spores ($6.7 \times 10^6 \pm 8.4 \times 10^5$ conidia) compared to oil and emulsifiable formulations of the fungus ($2.3 \times 10^5 \pm 2.3 \times 10$ and $4.6 \times 10^5 \pm 5.7 \times 10^4$ conidia respectively), the ticks also lost the highest numbers of spores (89.1%) 48h post-treatment. Fungal formulations that are electrostatically charged or have lipophylic characteristics may enhance adherence of conidia to the cuticle of the tick, and thereby resulting in better control. Emulsifiable formulation showed the least spore loss (17.1%) in the current study. This could be explained by the higher percentage spore germination recorded in emulsifiable formulation compared to spore powder and ULV oil formulations (Table 4.6). The germinated spores might have adhered more to the cuticle of the ticks. Germination and formation of appressorium occur within 11 h of inoculation (Leemon and Jonsson, 2008). The availability of free water in the emulsifiable formulation could have favoured the relatively higher percentage spore germination than the 100% oil formulation in the dry season. However, Samson *et al.* (1988) noted that although free moisture is required to initiate germination, continuation of the process is dependent upon the availability of suitable exogenous nutrients. This requires further research. In the present study, the emulsifiable formulation caused greater reduction in tick numbers (54.7 and 46.5% in the rainy and dry season, respectively) than the others. Maranga *et al.* (2006) recovered 33.77% of *A. variegatum* ticks released in plots that had semiochemical-baited trap with a mixture of *B. bassiana* and *M. anisopliae* spores in an oil-based formulation compared to 76.33-84.08% in control plots. The high tick mortality obtained under laboratory conditions in the present study demonstrated the virulence of the *M. anisopliae* strain used.

Oil is known to enhance adhesion between conidia and the hydrophobic cuticle of insect and it seems logical to assume that oil formulation promote adhesion to tick cuticle (Prior *et al.*, 1988). The difference in the relative tick reduction between emulsifiable and oil formulations in this study could partially be explained by the fact that spray droplets released under pressure from a HV application had better penetration into the grass than a ULV oil formulation. Ekesi *et al.* (1998) reported similar results with *M. anisopliae* against the flower, *Megalurothrips sjostedti* (Trybom) and attributed it to better penetration by the infective propagules as a result of the large sray volumes applied to the plant. Thus ticks were more likely to encounter infective conidia on the foliage as they move from one plant to the other. Also, infective inocula may easily come into contact with the easily penetrable parts of the

cuticle, such as leg joints, setae and spiracles as the ticks forced their way through the vegetation.

In summation, the present study demonstrates the potential of semiochemical-baited trap for delivery of entomopathogenic fungi for control of *Amblyomma* spp. This strategy could be valuable for reducing *Amblyomma* population around water points, camping sites and paddock facilities. Further studies are required to validate these results in large-scale field experiments and are discussed in the next chapter.

CHAPTER FIVE

Performance of *Metarhizium anisopliae*-treated semiochemical-baited trap in reducing *Amblyomma variegatum* populations in the field

5.1 Introduction

Inundative and augmentative releases are widely used methods for the introduction of entomopathogens into an ecosystem for control of arthropod pests including ticks (Lacey and Geottel, 1995). For example, Kaaya (2000b) and Benjamin *et al.* (2002) were able to reduce populations of *R. appendiculatus* and *I. scapularis*, respectively, following spray application of aqueous formulations of *M. anisopliae* onto the vegetation. Alonso-Díaz *et al.* (2007) reduced in the number of feeding *R. microplus* ticks after spraying naturally-infested cattle with an aqueous formulation of *M. anisopliae*. However, there are challenges to the direct application of biopesticides to control ticks on cattle; temperature and host secretions (eg sweat), may affect the virulence of entomopathogenic fungi (Polar *et al.*, 2005b). Blanket sprays of the vegetation may also affect non-target organisms (Hajek and Geottel, 2000; Brownbridge and Glare, 2007) and are expensive requiring significant quantities of materials to treat a large area. Furthermore species of ticks such as members of the genus *Amblyomma* that actively seek their hosts by crawling on the soil below the vegetation may not come into contact with an entomopathogen in blanket spray. Alternative, target methods of applying fungal pathogens to the environment to control ticks are needed. There are opportunities to use autodissemination device to deliver pathogens to ticks (Maranga *et al.*, 2006). Such devices uses visual cues, pheromones and kairomones to attract host pests to a pathogen source (Vega *et al.*, 2007). Using a fungus-treated pheromone-baited trap, Maranga *et al.* (2006) was able to attract and infect *A. variegatum* under field conditions.

In the previous chapter the performance of the trap described by Bryson *et al.* (2000) baited with the attraction-aggregation-attachment pheromone (AAAP) and treated with the fungus *M. anisopliae* in semi-field experiment was evaluated. Ticks attracted to the trap were infected and killed by the fungus, with a subsequent reduction in the tick population. Here, trials to evaluate the performance of the fungus-treated trap baited with both the pheromone and kairomone blends against adult of *A. variegatum* populations in the field are described.

5.2 Materials and Methods

5.2.1 Field site

The experiment was carried out at the National Veterinary Research Centre, Muguga-Kenya Agriculture Research Institute: Latitude 1° 13' S, Longitude 36° 18' E, Altitude 2070 m (Obiri *et al.*, 1994). The climate is a modified equatorial type with a mean monthly temperature of 18 °C. Mean annual rainfall is 1005 mm distributed bi-modally with peaks in April and October (Obiri *et al.*, 1994).

The study area was a 2-acre paddock and the vegetation was predominantly red oat grass, *Themeda triandra* (Liliopsida: Poaceae). The height of the grass was maintained between 5 and10 cm. The experiment was done during the rainy season, from April to May of 2008. The climatic conditions were as follows: mean maximum temperature, 22.75°C; mean minimum temperature, 13.84 °C; 86.5% RH at 6 AM; 57.3% RH at noon; and 177.2 mm rainfall (Department of Meteorology, Kenya). Prior to the start of the experiment, semiochemical-baited traps were placed in the paddock to trap ticks and ensure that the area was free of *A. variegatum*. No *A. variegatum* tick was found in the plot; however a few *R. appendiculatus* were attracted to the traps. The field was divided into 6 plots of 100 m^2 (10 m x 10 m) each, of which three served as controls and three were assigned to fungal treatments. Plots were allocated to both treatments randomly. Plots were separated from each other by a 40 m buffer zone.

5.2.2 Ticks

Engorged *A. variegatum* females collected from infested cattle from the Marsabit area of Kenya in 2006 were used to established tick colony. Ticks were reared at the Animal Rearing and Quarantine Unit, *icipe*. All life stages of the tick were fed on New Zealand white rabbits. The different instars were maintained in Perspex chambers at 26 °C ± 1 and 85 ± 5% RH under 12:12 L: D photoperiod. Three to four-week old unfed adults were used, in the trials.

5.2.3 Fungus

The *Metarhizium anisopliae* isolate (R1/RA) used in this study was obtained from the *icipe*'s Arthropod Germplasm Centre. The strain was isolated from an engorged female *A. variegatum* collected from Rusinga Island, Kenya in 1996 and was previously reported to be

virulent against *A. variegatum* (Kaaya *et al.*, 1996). Conidia were mass produced using long rice as a substrate (Milner R.J., unpubl.). Blastospores were cultured in liquid medium containing glucose (30 g/l), peptone (10 g/l) and yeast extract (30 g/l) in a 250 ml Erlenmeyer flask maintained in a shaker at 100 rpm and 26 ± 2° C for 3 days. Glucose, peptone and yeast extract were obtained from Sigma. The contents of the flask were autoclaved for 30 minutes at 121°C and allowed to cool before inoculation with the conidia. Two kilograms of rice per plastic bag was autoclaved for 1 h at 121°C, transferred to polyethylene autoclavable bags and inoculated with the 3-day old culture of blastospores (50 ml). The blastospores suspension was thoroughly mixed with the rice to distribute the inoculum throughout the substrate and incubated between 26 and 30°C and 60–75% R.H. for 21 days. The polyethylene bag was then opened and the culture was allowed to dry for 5 days at room temperature to approximately 10 - 15% moisture content. Conidia were harvested by sifting the substrate through a sieve (295 µm mesh size) and approximately 200 g of spores was produced per bag. Dry conidia were stored in a refrigerator (4–6°C) prior to use. The viability of conidia was determined using the technique described by Goettel and Inglis (1997) before before being used in the field trials. Germination rates > 90% after 24 h on Sabouraud dextrose agar was considered adequate for use in the field trials. One litre of emulsifiable conidial suspension containing 1 x 10^9 conidia ml^{-1} was prepared for the trials (consisting of 49.5% sterile distilled water, fungal conidia, 49.5% corn oil [CHEF cooking oil, Premier Oil Mills LTD] and 1% Tween 80). One litre of a control solution was also prepared in a similar manner without the fungus for use in the control plots.

5.2.4 Semiochemicals and traps

The synthetic components of the attraction-aggregation-attachment pheromone (ortho-nitrophenol, methyl salicylate and nonanioc acid), dicloromethane (DCM) and 1-octen-3-ol were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Dry ice, which was used as a source of CO_2 was obtained from Carbacid Kenya. The semiochemical-baited trap used in this study was similar to the one described in Chapter 4. Briefly, the trap consisted of a 900 cm^2 area demarked by four 10 cm-wooden pegs hammered into the ground. A 2 x 2 x 2 cm^3 rubber sponge impregnated with 16 ng of 1-octen-3-ol and 0.022 mg of AAAP was attached on the top of each of the four wooden pegs per trap with dry ice dispenser (plastic cup) of CO_2 placed in the centre.

5.2.5 Treatments

Three days before application of the treatments, 118 laboratory-reared adult *A. variegatum* (59 males and 59 females) were seeded in the vegetation in each plot and allowed to acclimatize. Five semiochemical-baited traps were placed at different positions within each plot (1 central trap and 4 diagonal opposed traps). The positions of the diagonally opposed traps were moved to new positions by rotating clockwise (45°) while the centrally placed trap was shifted 1 m to the north after 14 days. The positions of the 4 diagonally opposed traps were moved again after 4 weeks, moving them 1 m (towards the centre) and clockwise direction by 45°; the central trap was moved 2 m to the south. The area within the confines of each trap in the fungal test plots were treated with 250 ml of emulsifiable conidial suspension using a high volume HV applicator (1.5 l Model) at a rate of approximately 150 l/hectare prior to the attachment of the sponges and impreganation of the sponges with the synthetic semiochemicals. In the control plots, traps were treated with the emulsifiable carrier only. All treatments were applied again to previously untreated foliage after 14 and 28 days immediately after rotating the trap as described above. The experiment lasted for 6 weeks.

5.2.6 Evaluation of the efficacy of treatments

At 6 weeks post-treatment, 5 semiochemical-baited traps were deployed in each plot in the morning hours (9.00-12.00 am) in order to attract surviving ticks from the vegetation within the plots. The positions of the traps were also changed daily and collections were made over three consecutive mornings. Ticks collected in each plot were transferred to labelled 9 cm diameter plastic Petri dishes (atmost 10 ticks per dish) and brought to the laboratory where they were maintained at 26 ± 1 °C, 85 ± 5 % RH and 12:12 h L:D photoperiod for two weeks. Mortality was recorded after two weeks. Dead ticks were surface-sterilized with 2.5% sodium hypochlorite and 70% alcohol, rinsed twice in sterile distilled water, and then placed into 9-cm diameter Petri dishes lined with moistened filter paper to promote outgrowth of fungi from cadaver to confirm death due to mycosis.

5.2.7 Fungal Persistence

The persistence of inoculum on the treated foliage was also investigated during the trial. One uppermost flag leaf of grass that was directly exposed to sunlight was cut within each fungus-treated trap immediately and two weeks after treatment using a pair of sterile scissors. Grass samples were kept separately in 9 cm diameter Petri dishes before being transferred to

universal bottles containing 10 ml of 0.05% Triton X-100. The samples were shakened vigorously on a vortex shaker for 5 minutes to dislodged conidia from the treated foliage. The concentration of the fungal suspensions was determined using an improved Neubauer haemocytometer and was diluted to a concentration of 1.0×10^6 conidia ml^{-1}; 100 µl of the suspension was spread over a SDA plate and a sterile microscope cover slip placed on each plate. Plates were incubated at 25 ± 2 °C, and germination was determined after 24 h, by counting 100 conidia/plate (Goettel and Inglis, 1997). No *M. anisopliae* conidia were recovered from flag leaf collected from control traps, hence the uninoculated control treatment were excluded from the analyses of conidia persistence. Five replicates representing 5 traps in each plot were used.

5.2.8 Data analysis

A student's t-test was used to compare the following arcsin square root-transformed data at P = 0.05 significance level: (i) percentage of ticks recovered from control and fungus-treated plots; (ii) percentage tick mortality in the laboratory of ticks recovered from control and fungus-treated plots; and (iii) percentage germination of conidia recovered from treated foliage, 0 and 14 days post-spray. All analyses were performed using the SAS (2001) package. The relative (%) reduction of tick populations in fungus-treated plots was calculated using the formula [(number of surviving ticks recovered from control plots - number of surviving ticks recovered from fungus-treated plots)/number of surviving ticks recovered from control plots] X 100 (European Medicines Agency, 2004).

5.3 Results

Table 5.1 The number of *Amblyomma variegatum* ticks recovered from plots containing control and fungus-treated traps, 6 weeks post-treatment and mortality of recovered ticks from control and fungus-treated plots that were maintained under laboratory conditions for 2 weeks.

Treatments	Replicates	Number of ticks recovered from plots after 6 weeks post-treatment	Number of recovered ticks that died 2 weeks later under laboratory conditions
Control	Plot 1	98	5
	Plot 2	108	3
	Plot 3	97	2
Test	Plot 1	39	35
	Plot 2	46	44
	Plot 3	25	24

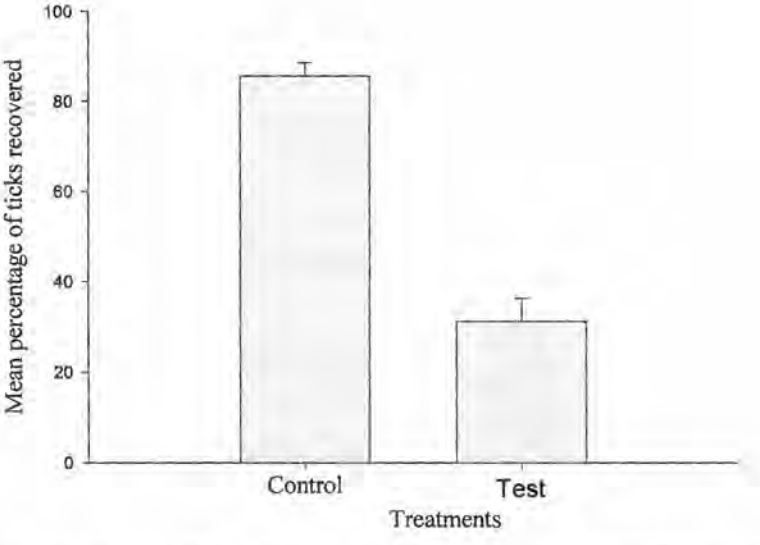

Figure 5.1 Mean percentage of ticks recovered from fungus-treated traps and control plots, 6 weeks after spraying

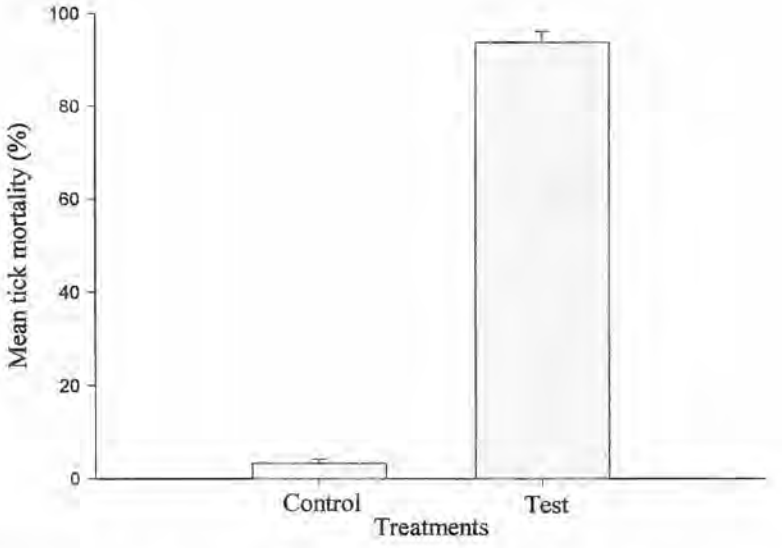

Figure 5.2 Percent mean tick mortality of ticks recovered ticks from control and fungus-treated plots. Collected ticks were maintained in the laboratory, for two weeks to ascertain mortality levels.

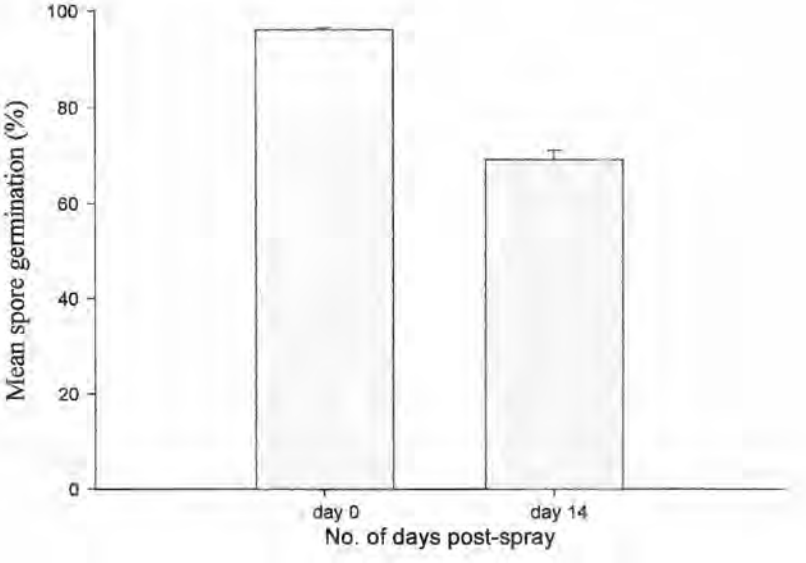

Figure 5.3 Percentage germination of *M. anisopliae* conidia applied to foliage in emulsifiable formulation immediately after application (day 0) and 14 days later (day 14).

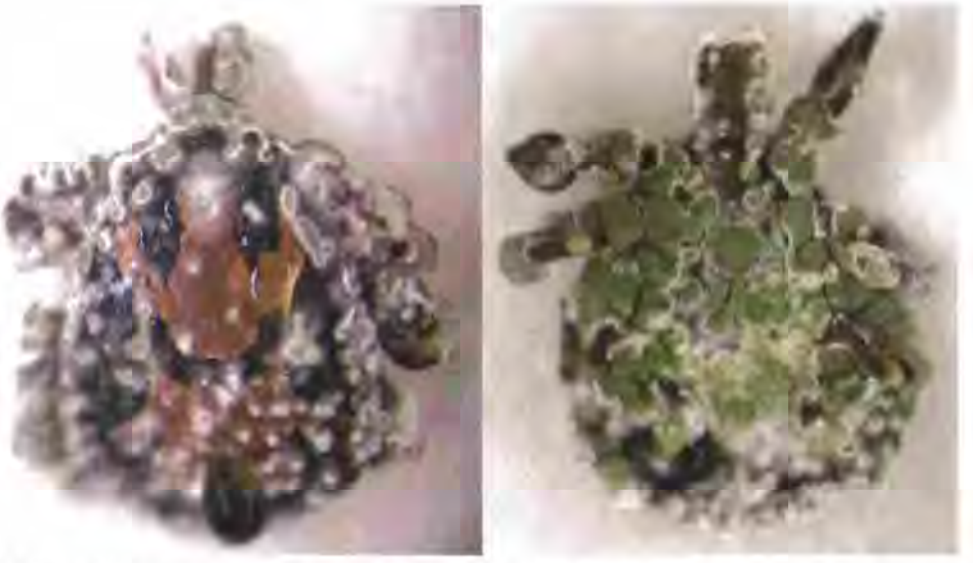

Figure 5.4 *Metarhizium anisopliae* conidia developing on *Amblyomma variegatum* cadaver; viewed from the dorsal (left) and ventral (right) sides. Symptoms developed when ticks were held under high conditions of humidity after collection from the field.

More ticks were recovered in the control plots compared to the fungus-treated plots at 6 post-treatment (Table 5.1). The mean percentage of ticks recovered after 6-weeks in the fungus-treated plots (31.1 ± 5.2%) was significantly lower ($F = 66.48$; $df = 1,4$; $P = 0.01231$) than the proportion recovered as a result of fungal activity from the control plots (85.6 ± 3%) (Figure 5.1), representing a relative reduction of 63.7%. Mortality of ticks that were collected from the field plots after 6 weeks was significantly higher ($F= 586.32$; $df = 1,4$; $P < 0.0001$) among those collected from fungus-treated plots than the controls (Table 5.1). Ninety-four (94%) per cent of the ticks recovered from the fungus-treated plots succumbed to infection compared to 3% in the controls (Figure 5.2). The germination of conidia washed from treated foliage was 96.1 ± 0.41% immediately after spraying but had decreased to 69.2 ± 1.9% after 14 days, representing a reduction of 28% (Figure 5.3). All dead ticks among those recovered from fungus-treated plots were covered with conidia but no fungal growth was observed on dead ticks collected from control plots (Figure 5.4).

5.4 Discussion

The high number of ticks recovered from the control plots (85.6%) compared to the fungus treated plots (31.1%) demonstrates the efficacy of the semiochemical-baited trap. Maranga *et al.* (2006) reported similar results using a pheromone-baited trap and kairomone; 76-84% of *A. variegatum* ticks were recovered in the control plots compared to 33.8% in *B. bassiana* and *M. anisopliae*-treated plots. While the semiochemical blend used in the present study were attractive to *A. variegatum*, it may not represent the full complement of tick and host odour constituents that *A. variegatum* requires to locate its preferred hosts. Further studies might reveal additional components, the use of which might enhance the performance of fungus-baited traps. A major advantage of using semiochemicals to attract ticks is that they might facilitate rapid contamination of *Amblyomma* ticks with the fungus used. Most of the ticks that survived infection in the field and were brought to the laboratory succumbed to fungal infection, indicating that further mortality may occur beyond the 6 weeks experimental period

The viability of conidia decreased by 28% at two weeks post-treatment in the present study. A variety of environmental factors may influence the viability of entomopathogenic fungi (Ignoffo, 1992). However, it has been demonstrated that an oil-based carrier can minimize the negative effects of environmental stress on fungal conidia compared to aqueous carriers (Inglis *et al.*, 1995). Addition of potential sunscreens and antioxidants to formulations could further increase persistence (Moore *et al.*, 1993), although sunscreens do not appear to promote persistence by a significant amount and the extra cost does not seem to justify their inclusion (Hunt *et al.*, 1994).

In conclusion, this study demonstrates that fungus-treated semiochemical-baited traps can control *A. variegatum* under field conditions. Such traps could also be used to control other *Amblyomma* species and other tick species. However, further studies are required for operational strategies to be developed, and to improve trap efficacy. For example, refinements to the formulation may promote conidial persistence, and optimization of attractant components may enhance field performance.

CHAPTER SIX

General Discussion, Conclusion and Recommendations

6.1 General Discussion

For entomopathogenic fungi to become viable biological control agents for ticks, considerable research is required to promote product development. This includes appropriate assay to ensure selection of the most effective fungal strain, and development of suitable formulations and delivery systems. The current study was initiated to address these aspects and to generate information relevant to the practical use of fungi in tick management strategies. The primary objectives of the study were;

(i) To identify fungal isolates which are pathogenic against *R. pulchellus in vitro*.

(ii) To identify the most appropriate techniques for laboratory inoculation of nymphal and adult stages of *Rhipicephalus* ticks with fungal conidia.

(iii) To optimize attraction-aggregation-attachment pheromone (AAAP) by combining with 1-octen 3-ol and butyric acid for attraction of *A. variegatum* adults.

(iv) To evaluate the effects of formulations on the efficacy of fungus-treated semiochemical-baited traps for control of *A. variegatum* under field conditions.

(v) To evaluate the performance of *M. anisopliae*-treated semiochemical-baited traps to control *Amblyomma variegatum* in the field.

In the present study, out of twelve *M. anisopliae* and three *B. bassiana* isolates assayed, only four *M. anisopliae* isolates (ICIPE 60, ICIPE 69, ICIPE 78 and ICIPE 62) were pathogenic to unfed *R. pulchellus* adults. Considerable variation in pathogenicity among isolates for a specific tick species has been demonstrated (Kirkland *et al.*, 2004a: 2004b and Samish *et al.*, 2001). The current study also demonstrated that inoculation method directly affects tick mortality. Inoculation by spray using a Burgerjon's spray tower caused higher infection levels than inoculation by microapplicator although more conidia were deposited on ticks by microapplication than spraying by Burgerjon's spray tower. The different inoculation methods could have influenced the spatial distributions of propagules on the tick cuticle. Localization of propagules on the intergument can affect the success of an entomopathogen, for example, the median lethal dose of *B. bassiana* conidia applied in oil to grasshopper abdomens is much lower than when conidia are applied beneath the pronotal shield (Inglis *et al.*, 2002). Scanning electron micrograph analysis of infected ticks showed that *M.*

anisopliae conidia preferred attachment sites at certain junctions, like those present in the tick legs (Arruda *et al.*, 2005).

It was also demonstrated in the current study that the method of application of conidia influence tick mortality by variation in the actual number of fungal propagules deposited onto the tick. Calculated LC_{50} values showed that higher infection levels of nymphs and adults were obtained when fungus was sprayed directly on ticks and substrate and incubated for 12 h (SS) compared to when fungus was directly sprayed on ticks and substrate and ticks were immediately transferred to clean Petri dishes (SP) and indirect exposure (SW). However, a significantly higher number of fungal conidia were found on ticks when fungus was sprayed directly on the ticks and substrate and incubated for 12 h (SS) compared to when fungus was directly sprayed on ticks and substrate and ticks were immediately transferred to clean Petri dish (SP) and indirect contamination (SW) although the spore concentrations were the same before spraying. Future research should consider determination of the actual number of fungal conidia on the tick after inoculation in bioassays.

Delivery of oil formulated conidia via a spray tower (SP) was the best method for treating adults and nymphs of *Rhipicephalus* spp., but exposure of immature stages to contaminated surface (SW) also provided an appropriate assay technique with minimal control mortality. To my knowledge, only one study (Polar *et al.*, 2005a) had evaluated pathogenicity of fungal conidia suspended in pure oils against tick. The current study also highlights the need to develop assays that "mimic" spray application of fungus under field conditions and looks at the possibility of developing bioassays that simulate application of fungal conidia in an oil carrier using a ULV spray technique.

According to Osterfield *et al.* (2006) the efficacy of *M. anisopliae* and *B. bassiana* as tick control agents has been sufficiently well established to warrant aggressive pursuit of efficient and safe delivery systems. Technologies such as ULV and hydraulic sprayers have been used for field application of fungus against insects (Wraight and Carruthers, 1999). These technologies might be applied for inundative applications against tick species that spend most of their life (three-host ticks) questing from vegetation. Research on developing efficient technologies for application of fungus against hunter ticks is still in its infancy. Thus, one of the objectives of the current study was to evaluate the possibility of a baited-trap with an optimized combination of AAAP (attraction-aggregation-attachment pheromone), 1-octen-3-

ol and carbon dioxide, treated with *M. anisopliae* isolate (ICIPE 7) for suppression of *A. variegatum* under field conditions. The proportion of the test population recovered in the *M. anisopliae*-treated plots (31.1%) was significantly lower than that recovered in the control plots (85.6%) after 6 weeks. This represented a relative tick reduction of 63.7% by the fungus-treated traps compared to the control traps. Using a fungus-treated pheromone-baited trap, Maranga *et al.* (2006) were able to attract and infect *A. variegatum* under field conditions. The fungus-treated semiochemical-baited trap used in the current study is less costly and the trap was more efficient in attracting adults of *A. variegatum*. Ticks were attracted from 8 m compared to 5 m achieved by Maranga *et al.* (2003) under field conditions. This trap can be used against *A. variegatum*, around water points and near salt licks. The traps may also be useful against other tick species with some modifications.

Results obtained in the present study also demonstrated that fungal conidia suspended in an emulsifiable formulation induced higher mortality compared with powder and oil formulations under field conditions. According to Leemon and Jonsson (2008), 10% emulsion can yield similar results to those obtained using a 100% oil formulation of *Metarhizium* conidia. Previous research has demonstrated that emulsifiable formulations of conidia can enhance fungi activity against arthropods (Kaaya and Hassan, 2000).

Other field studies addressing the use of fungi in tick control have focused on the inundative application by spraying vegetation (Kaaya, 2000b; Benjamin *et al.*, 2002; Hornbostel *et al.*, 2004) and on ticks attached to the host (Alonso-Díaz *et al.*, 2007). However the most recognised concern is the risk of infection posed to non–target species. Experiments have shown *M. anisopliae* and *B. bassiana* isolates to be non-pathogenic to natural enemies and beneficial soil insect (Jaronski *et al.*, 1998; Thungrabeab and Tongma, 2007). There is no documented case where a fungal pathogen introduced for classical biological control of an insect pest caused substantial mortality to non-target species or had caused negative effect on human and animal health, or any significant impact on the environment (Hajek *et al.*, 2003). However, native pathogen present in natural pest population can be augmented by inoculation or inundation to establish epizootics (Shah and Pell, 2003). Information on physiological host range, and, hopefully, ecological host range might be required before introduction of native or exotic fungal strain in an area for tick control.

An important constraint to blanket-spraying vegetation is cost. It costs US$17 (1997 price) to produce of 100 g of spores of Green Muscle (a commercial biopesticide based on *M. anisopliae* var. *acridum*.) sufficient to treat one hectare for locust control (de Groote, 1997). In comparison, an estimated 20 g of *M. anisopliae* spores per hectare would be needed if traps such as those used in this study were used in tick control programme. Even, considering the additional cost of the pheromone and other attractants, a fungus-treated semiochemical-baited trap is a cheaper method of treatment and, cost-wise competes favourably with an inundative spray. Use of semiochemical-baited traps has an added advantage in that it may increase the rate of contamination of *A. variegatum*, thus the efficacy of the control strategy is increased. Under favourable field conditions (during morning periods when there is reduced sunlight and a gentle breeze), ticks closer to the traps start moving upwind towards the trap in less than 2 minutes and the semiochemicals remained active for 90 minutes under field conditions.

Despite the promising results obtained, there are additional hurdles that need to be overcome. For example, many traps were needed to cover a relatively small area; there are difficulties in obtaining storing and transportation of CO_2 in the form of dry ice, which would limit their widespread use. The limited persistence of conidia under field conditions also means that areas may have to be treated over the course of a season. I also recognise that the fungus-treated semiochemical-baited trap may be species-specific and not all tick species are "hunters". This technology may not be suitable for control of some tick species ("ambushers") in vegetation. Inundative spray such as ULV might be more suitable for entomopathogen application over large areas. According to Shah and Pell (2003), the use of inundative mycopesticides may not be as sustainable as non-inundative strategies but they are more likely to be commercially successful, depending on the value of the target market. It seems many application strategies are required for simultaneous control of different tick species occurring in an area. I have addressed some challenges in the recommendation that follows.

6.2 Conclusion

This study demonstrated the susceptibility of the zebra tick, *R. pulchellus*, to the entomopathogenic fungus *M. anisopliae* for the first time and also identified appropriate bioassays for screening entomopathogenic fungal isolates against adults and nymphs of this species. Furthermore, this study has demonstrated the efficacy of a simple semiochemical-baited trap that is inexpensive and efficiently delivers entomopathogenic fungi for the control of *Amblyomma* spp. With further development, this provides a valuable control tool for ticks in Africa, which hosts several *Ambyomma* spp.

6.3 Recommendations

- The results obtained in this study clearly demonstrate the potential value of fungal pathogens for tick control and the need for more research to select virulent isolates. Screening of naturally occurring fungal isolates from a particular locality or ecosystem where tick control is envisaged may minimized potential risks posed by exotic strains.
- Because appropriate *in vitro* bioassays as well as field trials are imperative for selection of virulent and effective fungal isolates, there is need to optimise the infection techniques for different life stages of ticks to facilitate comparative analyses of efficacy and susceptibility.
- Since many species of hard ticks can be found on vegetation at any given time, it is important to determine tick populations, behaviours of the different tick species, biology and ecology of different tick species occurring in a targeted locality and adapt the control strategy to target these species.
- As immature life stages of ticks are generally more susceptible to fungi than adult stage, targeting immature stages could improve on efficacy of a control strategy incorporating entomopathogens and reduce the need for multiple species and/or isolates (entomopathogenic fungi), since host specificity may be less of a limiting factor when targeting these stages.
- The use of semiochemicals (tick pheromones and host kairomones) and entomopathogenic fungi in a trap has demonstrated potential for tick control off-host. This has the additional advantage in that it reduces the risks of transmission of tick-borne diseases by killing ticks before they become attached to a host. Furthermore,

this technology would minimize the area treated with a mycoacaricide. However, the limiting factor with this technology is that it could be species-specific or less efficient for some species, and different semiochemicals may be needed to control different tick species in vegetation. Research to identify the full range of kairomones produced by the hosts that are attractive against different target tick species is important.

- Use of an emulsifiable formulation enhances the efficacy of a fungal treatment under conditions of low humidity and its compatible with lipophylic conidia. There is need to develop technologies for practical application of small droplets of conidia in oil-water emulsion. This may reduce the cost involved with hydraulic technologies that are compatible with oil-water emulsion.
- The viability of applied conidia declined over two weeks under natural field conditions (Chapter Five). Inclusion of additives in the formulation, e.g., sunscreens and antioxidants may prolong conidial persistence.
- For some tick genera, including *Rhipicephalus* and *Ixodes*, larval stages of these ticks feed on small mammals such as mice. The use of wood nest boxes attached to tree trunks at a height of 1.5 m as a platform has been used to deliver fungi (Hornbostel *et al.*, 2005). This may be a component of an integrated tick management strategy, particularly to control of some *Rhipicephalus* ticks, which wait on grass ("ambushers") for suitable hosts. ULV application technology could be used for delivery of fungal conidia suspended in pure oil for control of some *Rhipicephalus* ticks ("ambushers").

In order to achieve sustainable control of ticks in the future, it is important to apply suitable and practical control technologies and to assess the economics of all possible tactics. Technologies that are inexpensive, user-friendly, and environmentally benign and have proven tick control efficacy would provide a solid base for a sustainable management strategy for livestock farmers.

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