PATHOGENICITY OF THE ENTOMOPATHOGENIC FUNGUS *METARHIZIUM ANISOPLIAE* TO TICKS AND THE PROTECTION OF CONIDIA FROM ULTRA-VIOLET RADIATION DAMAGE

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Abstract

The potential of the entomopathogenic fungus, *Metarhizium anisopliae* to serve as a bio-control agent of ticks has been studied and promising results reported. In this study, efficacy of *M. anisopliae* conidia formulated in either oil or water, in inducing mortalities to various developmental stages of ticks was evaluated. A fungal concentration of 10^8 conidia/ml induced in *Rhipicephalus evertsi evertsi* mortalities of: 87% in eggs, 100% in unfed larvae, 72% in engorged nymphs, 91% in unfed adults, and 97% in fed adults when formulated in oil, and 77% in eggs, 95% in unfed larvae, 64% in engorged nymphs, 83% in unfed adults, and 90% in fed adults when formulated in water. Various stages of *Amblyomma hebraeum*, *Amblyomma variegatum*, *Rhipicephalus appendiculatus* and *R. e. evertsi* were also found to be highly susceptible to *M. anisopliae* in water and oil formulation. Furthermore in this study, conidia were protected with 3% chemical sunscreens, Everysun and E45, respectively, and exposed to UV radiation for 1, 2, 3, 4, and 5 hours. Survival of conidia after 5 hrs of exposure to UV in oil formulation was: 4% in control, 29% in conidia protected with Everysun, and 40% in conidia protected with E45. In comparison, survival of conidia formulated in water was: 0% in control, 13% in conidia protected with Everysun, and 24% in conidia protected with E45. Furthermore, effects of the 2 sunscreens on viability and on pathogenicity of conidia to in various stages of *R. e. evertsi* were evaluated. The 2 sunscreens were found to confer protection to conidia against damage by UV radiation without interfering with their pathogenicity to ticks. This study has therefore indicated that *M. anisopliae* has a potential as a myco pesticide to control different developmental stages and species of tick populations. Results of this study have also shown that oil and chemical sunscreens can protect conidia from UV radiation damage, and they do not reduce the ability of *M. anisopliae* conidia to germinate, or infect ticks and induce mortalities in different stages of *R. e. evertsi*.

Keywords: *Metarhizium anisopliae*, Ultra-violet radiation, Sunscreens, *Amblyomma hebraeum, Amblyomma variegatum, Rhipicephalus appendiculatus, Rhipicephalus evertsi evertsi*, Ticks, Germination, Infection, Mortality, Formulation.

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List of Abbreviations

LC_{50}- Lethal concentration required to induce 50% mortality

PI-Post infection

RH-Relative humidity

SDA- Saboraud's dextrose agar

SD-Standard deviation

SE-Standard error

SPF- Sun protection factor

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Declarations

I, Marius Hedimbi, hereby declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institute of higher education.

…… Date……..

Marius Hedimbi

CHAPTER 1: INTRODUCTION

1.1. General Introduction

Ticks are blood feeding external parasites of mammals, birds, and reptiles throughout the world. They belong to phylum Arthropoda, class Arachnida, order Acari, and suborder Ixodidae (Rajput *et al.,* 2006). Approximately 850 species have been described worldwide (Barker and Murrell, 2004). Ticks are important vectors of disease causing agents to humans and animals throughout the world. Ticks cause great economic losses to livestock in the world and have adverse effect on the livestock host in several ways (Graf *et al.,* 2004) and parasitize a wide range of vertebrate hosts, and transmit a wider variety of pathogenic agents than any other group of arthropods (Rajput *et al.,* 2006).

Ticks are the most important ecto-parasites of livestock in tropical and sub-tropical areas, and are responsible for severe economic losses in livestock (Bittencourt *et al.,* 1994). The major losses, however, caused by ticks are due to their ability to transmit protozoan and rickettsial diseases to livestock, which are of great economic importance world-wide (Mathysse and Colbo, 1987). Tick-borne protozoan diseases (e.g. Theileriosis and Babesiosis) and rickettsial diseases (e.g. Anaplasmosis) and cowdriosis and tick-associated dermatophilosis are major health and management problems of livestock in many developing countries (Mathysse and Colbo, 1987). The economically most important ixodid ticks of livestock in tropical regions belong

to the genera of *Hyalomma*, *Boophilus*, *Rhipicephalus* and *Amblyomma* (Frans, 2000).

1.2. Tick feeding and life cycle

All feedings of ticks at each stage of their life cycle are parasitic. Ticks feed only on the blood of their hosts. They crawl onto their host and attach to the skin using a combination of cutting mouthparts (chelicerae) which penetrate the skin and often an adhesive structure is secreted in the saliva to aid attachment to the skin. The chelicerae break the capillary blood vessels very close to the surface of the skin and the tick feeds on the released blood. On the ventral surface of the mouthparts is the hypostome which is barbed with teeth to grip the host. A feeding tube is formed between the hypostome and the sheath surrounding the chelicerae (Mathysse and Colbo, 1987).

Hard ticks have four distinct life stages. Larvae which emerge from the egg have six legs. After obtaining a blood meal from a vertebrate host, they molt into a nymph and acquire eight legs. Nymphs resemble the adult tick in that they have eight legs. They do not, however, have a genital opening. Nymphs feed and molt to the next and final stage, the adult which also has eight legs (Walker *et al.,* 2003). Adult ticks may require several days of feeding before they are able to reproduce. Male hard ticks usually die soon after mating, and females die soon after laying eggs. Mating usually occurs while adult ticks are on the body of the host animal (Walker *et al.,* 2003).

Only one blood meal is taken during each of the three life stages. The time to completion of the entire life cycle may vary from less than a year in tropical regions to over three years in cold climates, where certain stages may enter diapause until hosts are available. Many hard ticks can go for several months without feeding until they find suitable hosts (Norval *et al*., 1992). The life cycle in hard ticks can be divided into three distinct types depending on how many hosts the tick attaches to during its life cycle: one-host tick life cycle e.g. *Boophilus* spp (Figure 1); two-host tick life cycle e.g. *Rhipicephalus evertsi eversti* (Figure 2); and three-host tick life cycle e.g. *Dermacentor* spp (Figure 3); (Coetzer and Justin, 2004).

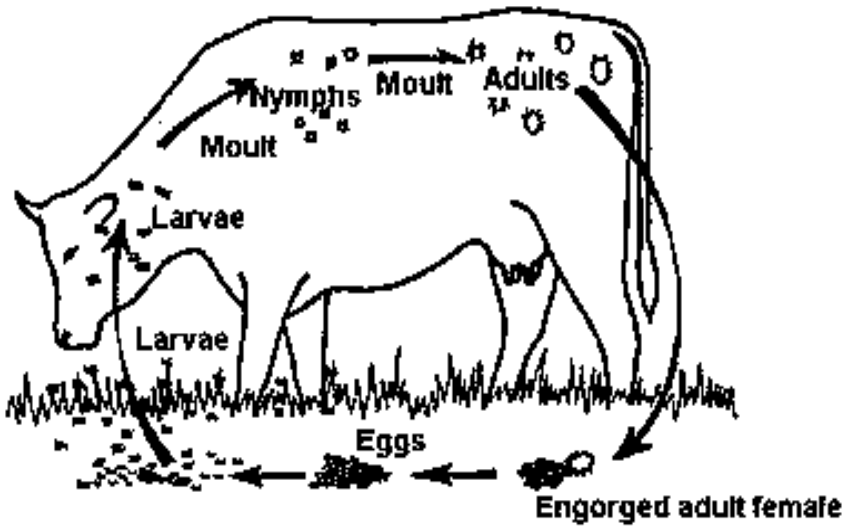


Figure 1: Schematic illustration of a one-host tick life cycle (e.g. *Boophilus microplus)*

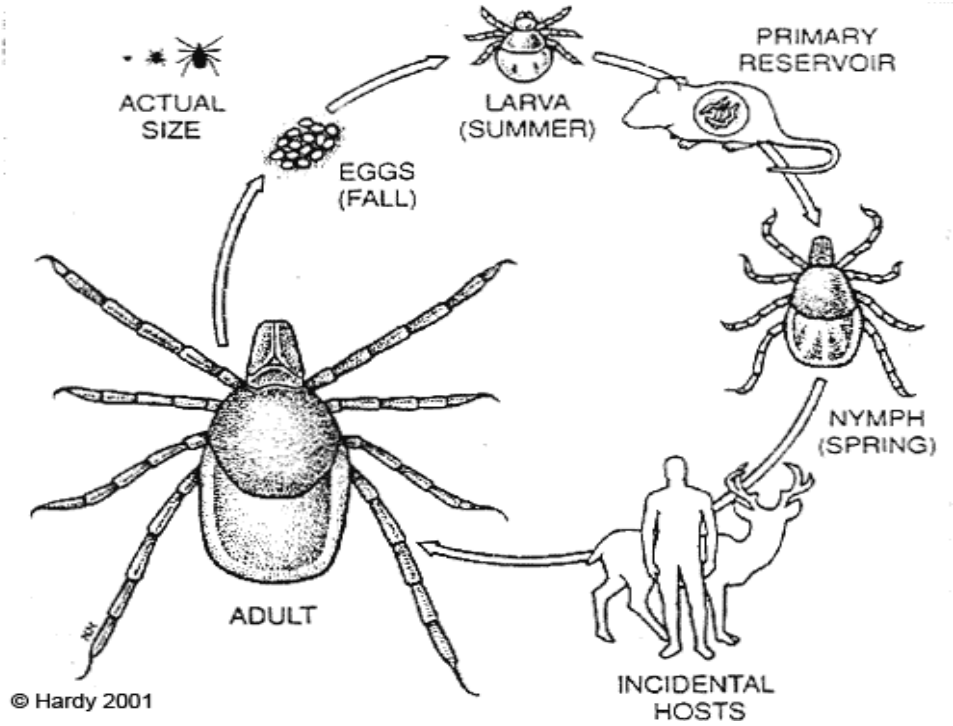


Figure 2: Schematic illustration of a two-host tick life cycle (e.g. *Rhipicephalus evertsi evertsi*)

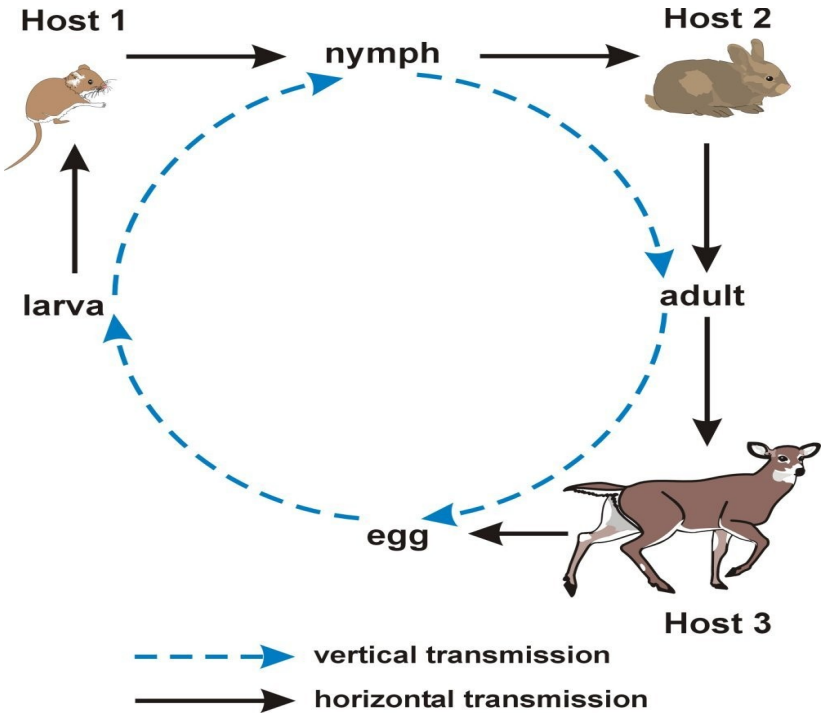


Figure 3: Schematic illustration of a three-host tick life cycle (e.g. *Dermacentor andersoni*).

1.3. Ticks as disease carriers

Ticks harm their hosts by sucking blood, injuring the skin, causing irritation and pain, and by transmitting diseases (Bittencourt *et al*., 1994). Ticks are important vectors of disease-transmiting agents to humans and animals, and must be controlled if livestock production is to meet world needs for animal protein (Rajput *et al.,* 2006). Certain ticks carry the causal organisms of animal diseases such as: Lyme disease, Babesiosis, Rocky Mountain spotted fever (RMSF), Tularemia, Relapsing fever (Cattle tick fever), Texas fever, East Coast fever, Heartwater, Redwater, Typhus, Rickettsial pox, Colorado tick fever, Corridor disease, Piroplasmosis, Anaplasmosis, and Texas cattle fever (Mathysse and Colbo, 1987).

Another health threat posed by certain ticks attacking humans and animals is tick paralysis and toxicosis. Tick paralysis is quite distinct from tick-borne diseases in that the causal factor is a toxic substance, not a disease organism (Coetzer and Justin, 2004). Several different mammals may be paralyzed by single tick species and several tick species can cause paralysis in a particular host. Paralysis, which is not a common condition in cattle, is caused by introduction of toxins into the body of the host with the salivary secretions of the tick during feeding. The severity of the paralysis depends on the susceptibility of the host and the quantity of toxins introduced. In cattle, paralysis is often proportional to the size of tick infestation, whereas in human one tick may cause severe toxicosis (Coetzer and Justin, 2004).

1.4. Chemical control of ticks

Chemical control with acaricides was considered as one of the best methods for controling ticks, but it has been shown that ticks have developed resistance against a range of acaricides (Martins *et al*., 1995). Furthermore, these chemicals are toxic and costly. Problems of acaricide resistance, chemical residues in food and the environment and the unsuitability of tick resistant cattle for all production systems make the current situation unsatisfactory, which is why there is a need for the development of an alternative control method (Martins *et al*., 1995), such as the use of entomopathogenic fungi (Kaaya *et al.,* 1996). Tick resistance to acaricides is an increasing problem and economic threat to the livestock and allied industries. The successive evolution of resistance of ticks to acaricides in each chemical group with the concomitant reduction in the usefulness of a group of acaricides is a major reason for the diversity of acaricides (George *et al*., 2004).

According to Rajput *et al.* (2006), acaricides can be toxic to livestock and humans, can create residues in tissues of animals, and may be destructive to the environment if they are not used and handled in a safe and correct manner (Garcia-Garcia *et al.,* 2000). Licking behavior and environmental contamination arising from pour-on ivermectin for cattle was studied by Laffont *et al*. (2001) and was found to be associated with unexpected residues in meat and dairy products and as an environmental contaminant via cattle dung. They recommended that the route of potential contamination of parasiticides be taken into account during product registration.

1.5. Alternative methods of tick control

At present, ticks are mainly controlled by chemical acaricides. However, this method is fraught with various problems such as environmental safety and human health, the increasing cost of chemical control and the increasing resistance of ticks to pesticides (Garcia-Garcia *et al.,* 2000). Consequently, alternative methods e.g. biological control that will alleviate these problems are needed (Norval *et al*., 1992). Kaaya and Hassan (2000) reported that the use of entomopathogenic fungi to control ticks may reduce the frequency of chemical acaricide use and the need for treatment for tick-borne diseases. They also concluded that mycopesticides are safer for the environment than conventional acaricides. Entomopathogenic fungi display either a very broad host spectrum like *M. anisopliae* or have a very narrow host range like *Aschersonisspa*, which can only infect scale insects and white flies (Samson *et al*., 1988).

CHAPTER 2: LITERATURE REVIEW

2.1. Background

Ticks have numerous natural enemies, but only a few species have been evaluated as tick bio-control agents. Since the beginning of the 20th century, investigators have documented numerous potential tick bio-control agents, such as pathogens, parasitoids and predators of ticks (Mwangi *et al*., 1991; Samish and Rehacek, 1999; Kaaya, 2003). Several authors have reviewed specific groups of natural enemies of ticks, including pathogens (Chandler *et al.,* 2000; Kaaya, 2000), nematodes (Samish *et al*., 2000; Samish and Glazer, 2001; Kaaya, 2003), parastoids (Mwangi and Kaaya, 1997; Knipling and Steelman, 2000), and predators (Mwangi *et al*., 1991; Kok and Petney, 1993; Samish and Alexseev, 2001). The first attempt to control ticks with natural enemies started in the late 1920s, when the parasitic wasp *Ixodiphagus hookeri* was transferred to both Russia and the USA (Copping, 2001).

2.2. Use of entomopathogenic fungi to control ticks

Fungi have been reported to be major pathogens of ticks because of their wide-dispersal, wide spectrum of hosts, ability to enter the tick via the cuticle, and relatively specific virulence of a single strain to one or small groups of pests (Samish and Rehacek, 1999). Comparing egg production of *Ixodes ricinus* females and the viability of their eggs in different habitats indicated that in a forest biotope, tick populations are low because of the presence of fungi (Samish and Rehacek, 1999). Studies in Europe indicate that fungal infection may cause the death of up to 50% of

Dermacentor, Ixodes, and other ticks (Kalsbeek *et al*., 1995). However, representatives from only 6 genera out of 57 major entomopathogenic fungi are known to attack ticks (Sewify and Habib, 2001). More recently, Kaaya *et al*. (2007) observed significant mortalities in *Amblyomma variegatum* ticks feeding on rabbits treated with neem oil. Furthermore, they also observed that *A. variegatum* fed on goats which were fed on neem seed powder diet exhibited inability to attach, and feeding, molting and overall development were significantly prolonged. Kaaya *et al*. (2007) also obsereved that eggs of *Rhipicephalus appendiculatus* and *Boophilus decoloratus* exhibited reduced hatchability when exposed to neem oil.

Several fungal species were found to be lethal to ticks, among them *Beaveria bassiana, Metarhizium anisopliae, Paecilomyces farinosus* (Kalsbeek *et al*., 1995), *Paecilomyces fumosoroseus*, and *Verticillium lecanii* (Sewify and Habib, 2001). *Aspergillus ochraceus, Curvulvaria lunata*, and *Rhizopus arrhizus* were isolated from naturally- infected *R. sanguineus* ticks and were found to kill them (Estrada-Pena *et al*., 1990; Casasolas-Oliver, 1991). Mwangi *et al.* (1991) isolated species of the fungi belonging to the genera *Aspergillus*, *Fusarium*, and *Mucor* from engorged female *R. appendiculatus* after they were kept on grass for 8 days.

Entomopathogenic fungi especially strains of *M. anisopliae* and *Beauveria bassiana* are major pathogens of ticks. *M. anisopliae* and *B. bassiana* have high virulence, wide dispersal and ability to penetrate the host via the cuticle (Mwangi *et al*., 1991; Samish and Rehacek, 1999), but their potential in reducing tick populations is not clear (Sewify and Habib, 2001). They are used against terrestrial insects because of

their wide geographic spread and host range as well as their exceptional ability to germinate even at a relatively low humidity (Samish and Rehacek, 1999). Various species of ticks were treated with *M. anisopliae* or *B. bassiana* with satisfactory results (Kaaya, *et al*., 1996; Barci, 1997).

2.3. Prevalence of tick-fungus interaction

In nature, 20 species of fungi have been reported to be associated with ticks. Some 13 species of ticks from 7 genera were found to be infected by fungi (Mwangi *et al*., 1995; Zhioua *et al*., 1999; Guerra *et al*., 2001). Ticks collected in north east USA were infected primarily with *Verticillium spp*. and *B. bassiana*; 10 species of fungi were isolated in Europe and 3 in Africa (Mwangi *et al*., 1995; Kalsbeek *et al*., 1995; Zhioua *et al*., 1999). Of the engorged female *B. microplus* ticks collected from soil in Brazil, 24.5% were infected with *B. bassiana*, 10% with *M. anisopliae* (Da Costa *et al*., 2001), and 22% of the *R. sanguineus* nymphs were contaminated with fungi from 5 genera (Guerra *et al*., 2001).

The percentage of ticks infected by fungi in collections from natural areas varies considerably, largely according to the stage and species of the tick and to the ecological conditions at the sampling sites. For instance, 7.5% of the adult *Ixodes ricinus* collected in central Europe during winter time were contaminated by fungi, compared to over 50% of ticks collected during the summer (Sewify and Habib, 2001). In northern Europe, between 6 and 32% of engorged *I. ricinus* females were infected by fungi in nature, depending on season (Kalsbeek *et al*., 1995). Only 1.7% of the engorged *R. appendiculatus* females collected in Kenya died from fungal

infection (Mwangi *et al*., 1995), and in the northern USA, 4.3% of the collected unfed female *Ixodes scapularis* were infected (Zhioua *et al*., 1999). In nature, a higher percentage of adult ticks seem to be infected by fungi than their pre-imaginal stages and engorged females seems to be most readily infected (Kalsbeek *et al*., 1995; Zhioua *et al*., 1999).

Besides the infection via the insect cuticle, there are other infection routes via the insect mouth, wounds or the respiration openings. According to Alves *et al.* (1998), penetration through the insect cuticle is the predominant infection route. During the infection process through the cuticle, the whole integument has to be penetrated. The insect cuticle consists of thin chitin-free epicuticle, the chitin containing exo and endo cuticle and the cellular epidermis. The intersegmental skins contain much thinner chitin layers than other parts of the insect body so that these parts are more easily penetrated by the fungal hyphae. *M. anisopliae* prefers to infect at these locations (Alves *et al*., 1998).

The process of germination and the hyphae growth on the insect cuticle is dependent on environmental conditions like temperature and relative humidity. The temperature conditions needed for infection are different and vary between fungal species depending on their natural habitats (Samson *et al*., 1988). Some species need high humidity, whereas others need only a thin water film for germination. The nutrients necessary for germination and growth of the germ tube can be provided by enzymatic degradation of the insect integument (Carnley and St. Leger, 1991).

2.4. Laboratory bio-assays of fungal efficacy

When various fungal genera, species and strains were tested under optimal laboratory conditions, *M. anisopliae* and *B. bassiana* exhibited the strongest tick pathogenicity (Gindin *et al*., 2001; Samish *et al*., 2001). In most cases, *M. anisopliae* strains were more virulent than those of *B. bassiana* (Mwangi, *et al*., 1995; Kaaya and Hassan, 2000; Sewify and Habib, 2001). Under laboratory conditions, at least 15 ixodid tick species and two argasid ticks were found to be susceptible to fungi (Gindin *et al.,* 2003). Studies comparing the susceptibility of unfed larvae of *B. annulatus*, *Hyalomma anatolicum excavatum*, and *R. sanguineus* to 12 fungal strains, from species of fungi, gave the general impression that *Boophilus* and *Hyalomma* larvae showed similar susceptibility while *Rhipicephalus* larvae were more resistant. In contrast, engorged *H. a. excavatum* females were far more resistant to entomopathogenic fungi than females of the other two tick species (Gindin *et al*., 2003).

2.5. Formulation of fungal propagules

An important problem associated with microbial applications of entomopathogenic fungi relates to the hydrophobicity of the aerial conidia. This characteristic renders technical powders extremely dusty and difficult to suspend in water (Kaaya *et al.,* 2000). Many important constraints to the commercial development of entomopathogenic fungi are being addressed through formulation of fungal spores (Mwangi *et al.,* 1995).

Oils (both vegetable and petroleum-derived) are inherently compatible with lipophilic conidia and make superior spray carriers. They are essential ingredients for ultra-low-volume applications, capable of being atomized into small droplets (50-100 μm) that do not evaporate before hitting the target (Wraight *et al.,* 2001). Liquid oil formulations are easily measured and dispensed under operational field conditions. Those containing emulsifiers suspend quickly in water with minimal agitation (Ibrahim *et al*., 1999).

With respect to safety, oil formulants eliminate the dust hazards associated with dry spores (Goettel and Jaronski, 1997). Elimination of dust greatly reduces the risk of inhalation exposure and contact with eyes. This is especially significant with respect to reducing the allergenic capacity of fungal propagules. For these reasons, use of oil is attractive not only for spray applications, but also in the harvesting of aerial conidia from mass culture (Goettel and Jaronski, 1997). Formulations to improve the efficacy of fungal spores can be viewed as having two broad objectives: (i) improving the persistence of infectious propagules or making them attractive (e.g. using baits) to increase the chances of host contact; and (ii) improving the infectivity of propagules after host contact (Butt *et al*., 1998).

2.6. Efficacy testing of formulations

In most laboratory tests, fungal spores were suspended only in water with a small amount of dispersing agent. Kaaya and Hassan (2000) compared the anti-tick effect of a suspension of *M. anisopliae* spores in water with 1% Tween-80 with or without 15% peanut oil. Unfed nymphal and adult ticks were immersed in the suspension and

then kept outdoors on vegetation (Kaaya and Hassan, 2000). Treating *Anocentor nitens* infested on ears of horses with *B. bassiana* suspension in water resulted in less than 20% tick mortality, while their application in a polymerized pulp gel resulted in more than 50% mortality (Bittencourt *et al*., 1999).

The mortality of *A. variegatum* treated with the oil suspension with *M. anisopliae* was 30% higher for nymphs and 2.7 times higher for adults, and *R. appendiculatus* 15% higher for nymphs and 4.3 times higher for adults than in applications with the water carrier without oil. The influence of oil itself on the ticks has to be clarified (Kaaya and Hassan, 2000). In another trial, a powder formulation was prepared by mixing 1 volume of *M. anisopliae* spores with 9 volumes of various powders and was tested against *R. appendiculatus* adults feeding on cattle ears. The spore mixture with millet powder caused 100% mortality, maize 79%, sorghum 64%, and starch 53% (Kaaya and Hassan, 2000).

Submerged conidia in oil emulsion or suspended in water were equally effective. Also, in field tests of aerial conidia of *B. bassiana* against whiteflies, wettable powder formulations suspended with organosilicone surfactants were as effective as emulsifiable oil formulation (Inglis *et al*., 1995). The reason for the different laboratory and field results is not known. However, in field tests of *B. bassiana* formulated with Tinopal and applied to crested wheat-grass, linear regression showed that numbers of colony-forming units (CFU) declined by an average of 73% within 24 h and 88% within 48 h compared with 87 and 96%, respectively for water-formulated (Inglis *et al*., 1995).

Ticks ingest body fluids through their mouthparts, but they also ingest water vapor, through both mouthparts and cuticle for hydration (Samish *et al*., 2004). One mechanism involves a highly hygroscopic fluid secreted by the tick that is hydrated by water vapor from the surrounding atmosphere, and is then re-ingested into the tick. Therefore, it is plausible that spraying or dipping ticks in highly concentrated fungal solutions could result in tick mortality if sufficient fluid was ingested to elicit pathogenic effects in midgut (Samish *et al*., 2004). However, other mechanisms of pathogenicity are also plausible, including: the actions of other toxins such as *B. thuringiensis* exotoxins; negative effects associated with fungi invasion of the haemocoel, and the physical blocking of spiracles or other openings by fungal spores (Dubois and Dean, 1995).

2.7. Effects of UV radiation on spore germination

Ultraviolet light from sunlight (particularly UVB, 280-320 nm) causes direct structural effects on DNA or indirect damage caused by the formation of reactive oxygen molecules, with the indirect action by the reactive oxygen molecules likely being the most significant damaging factor (Ignoffo, 1992; Ignoffo and Garcia, 1994). The half life of most entomopathogenic fungal conidia ranges from 1 to 4 hr in simulated sunlight and 4 to 400 hrs in natural sunlight on foliage; the difference likely being the effects of shielding provided by the plant or sampling time in field studies (Ignoffo, 1992).

The reduction in the ozone layer and the consequent increase in UV-B, particularly at wavelengths between 290 and 315nm, may aggravate the problem (Caldwell *et al.,*

1998; McKenzie *et al.,* 1999). It has been demonstrated that an increase in UV-B irradiance drastically reduces the culturability of conidia of various species of the genus *Metarhizium* (Braga *et al.,* 2001) and that the effect of increased irradiance is greater during the growth phase in which the fungus is more susceptible to radiation, such as the end of germination (Braga *et al.,* 2001). It is well established that UV-B is the fraction of the spectrum that presents the highest biological effectiveness and the highest potential to damage entomopathogens within the wavelength that reach the earth's surface (Moore *et al.,* 1993; Fargues *et al.,* 1997; Braga *et al.,* 2001). In a recent series of experiments, it has been demonstrated that solar UV-A also reduces the cultuarability and delays the germination of *M. anosopliae* conidia (Braga *et al.,* 2001).

Both the conidial inactivation and delay in the germination of the survivors are mainly caused by DNA damage induced by UV radiation. While UV-A usually causes only indirect damage to DNA through catalyzing the formation of chemical intermediates such as sensitizer radicals or reactive oxygen species, UV-B acts directly in DNA inducing the formation of several photoproducts (Friedberg *et al.,*1995; Griffiths *et al.,*1998). The most common photoproduct induced by UV-B is the cyclobutane pyrimidine dimer (Sancar, 1994). This kind of derived product is removed by systems such as nucleotide excision repair (NER) and by photolyases, which are light-dependent enzymes that directly revert pyrimidine dimers into the original pyrimidines (Eker *et al.,* 1994; Sancar, 1994; Deisenhofer, 2000). However, the relative importance of these repair systems varies considerably among different biological systems (Sancar, 1994; Kim and Sundin, 2001).

2.8. Improving persistence of fungal spores

Fungal spores are extremely susceptible to solar radiation and therefore efforts have for many years been focused on the development of economical ultraviolet (UV) protectants that do not interfere with the host infection process. Many materials have been identified in laboratory studies that significantly increase survival times of irradiated spores; however, fungal propagules exhibit such extreme sensitivity to solar radiation (most are killed within 2 h by direct exposure) that even many-fold increases in survival times may not translate into improved efficacy under field conditions (Shah *et al*., 1998). Among the most promising UV protectants identified so far are stilbene brighteners, especially Tinopal LPW (Calcofluor white). These materials can afford high levels of protection to conidia exposed to artificial UV sources in the laboratory and statistically significant protection in the field (Inglis *et al*., 1995).

A few studies have been conducted on the use of spreaders and stickers to improve persistence of fungal propagules. Inglis *et al*. (1995) reported a 28-61% loss of unformulated conidia of *B. bassiana* from treated wheat and Lucerne foliage exposed to simulated rain. Most entomopathogenic fungi do not infect their hosts through the alimentary canal, and an important concern with respect to use of stickers is that the spores should not adhere to foliage so strongly as to prevent them being dislodged and inoculated on to the host cuticle. Oil carriers are excellent spreaders/stickers that apparently do not interfere with (and may actually enhance) host inoculation (Ibrahim *et al*., 1999; Inyang *et al*., 2000).

2.9. Protecting fungal spores from UV damage

The deleterious effects of UV radiation have been demonstrated in several genera of entomopathogenic fungi such as *Metarhizium, Beauveria,* and (Morley-Davis *et al.,* 1995; Fargues *et al.,* 1996; Braga *et al.,* 2001). Although the Hyphomycete species *V. lecanii* and *Aphanocladium album* are used for the control of various agricultural pests in different UV environments (Lacey *et al.,* 1996; Steenberg and Humber 1999), little is known about the effects of UV on these genera. In general, a few hours of direct exposure to radiation of an intensity frequently encountered in the environment are sufficient to fully inactivate the conidia of all the species studied (Morley-Davis *et al.,* 1995).

In general, attempts have been made to reduce the negative effects of UV radiation on entomopathogens by adding photo-protective agents to formulations (Moore *et al.,* 1993; Alves *et al.,* 1998) and by selecting strains more tolerant to radiation. Poor protection by chemical sunscreens in the field may result from evaporation or absorption of the carrier, leaving an ineffective, thin deposit of the protectant (Butt *et al*., 1998). Strain selection has been facilitated by the wide intra-specific variability in tolerance observed in species such as *M. anosopliae* (Fargues *et al.,* 1996; Braga *et al.,* 2001), *M. flavoviride* (Morley-Davies *et al.,* 1995), *B. bassiana* (Fargues *et al.,* 1996), and *Paecilomyces fumosoroseus* (Fargues *et al.,* 1996). Part of the variability in UV tolerance may be explained by the adaptation of the strains to specific habitats (Bidochka *et al.,* 2001) and to different environmental UV levels associated with the latitude of the sites of origin (Braga *et al.,* 2001).

Fargues *et al.* (1996) compared the susceptibility of aerial conidia from 65 isolates of *B. bassiana*, 23 isolates of *M. anisopliae*, 14 isolates of *M. flavovide*, and 33 isolates of *Paecilomyces fumosoroseus*. Overall, isolates of *M. flavovide* were the most tolerant to simulated sunlight followed by *B. bassiana* and *M. anisopliae*, with *P. fumosoroseus* being the least tolerant. Following the longest exposure time (8 hr) survival of *M. flavovide* and *M. anisopliae* ranged from 0.1 to 11%, and from 0 to 1%, respectively. Survival of *M. anisopliae* var. *acridum* (IMI 330189) aerial conidia was 46, 23, 8, and 5% following 1, 2, 4, and 8 hrs of exposure to simulated sunlight, respectively (Fargues *et al.,* 1996).

2.10. Statement of the problem

Ticks cause big economic losses in livestock production world wide. They contribute to unthriftiness and anaemia; they damage hides and subject livestock to secondary infection; they cause toxicoses and paralysis by the injection of their salivary secretions; and most importantly, they transmit pathogenic agents that cause diseases, many of which result in debility and death. *Amblyomma* and *Rhipicephalus* are economically important pests of livestock in most parts of Africa. *Rhipicephalus* spp. carries causal organisms for Babesiosis, Piroplasmosis, Redwater, Cattle Tick Fever, Texas Fever (*Babesia equi, B. bigemina, B. argentina, B. bovis,* and *B. divergens*), Theileriosis, East-Coast Fever, Coridor disease (*Theileria parva* and *Theileria lawrencei*), and Anaplasmosis (*Anaplasma marginale)*, it causes tick toxicosis to cattle and its saliva contains toxins that cause paralysis in lambs, adult sheep and calves. *Amblyomma spp*. carries causal organisms for Rickettisial diseases (*Ricketisia ruminantium*), and Heartwater (*Cowdrium ruminantium*).

In the area of chemical tick control, much has been achieved, but much more remains to be done in the biological control area. At present, ticks are mainly controlled by chemical acaricides. However, an interest in alternative methods for the control of ticks is growing for several reasons: increasing concerns about environmental safety and human health e.g. chemical residues in food, the increasing cost of chemical control, and the increasing resistance of ticks to acaricides.

Currently, no biological control method exists for ticks. The ability of entomopathogenic fungi to induce mortality in ticks in the laboratory and field makes them promising candidates for tick control. The formulation in which the spores are applied is crucial to the level of control obtained with fungus-based anti-tick compounds, but very little has been published on the subject. Much information in formulating fungi for use against free-living ticks can be obtained from experiments on fungal conidia for the control of insect pests, but considerable research is still required to develop satisfactory formulations for the control of ticks while they are feeding on vertebrate hosts. The susceptibility of *A. hebraeum* and *R. e. evertsi* to *M. anisopliae* has not yet been reported. There is also no report on concentration-mortality relationships. Furthermore, there is no report comparing mortality induced by *M. anisopliae* in tick instars of different species and formulations.

A major obstacle in using entomopathogenic fungi under field conditions is the rapid inactivation of conidia caused by ultra-violet (UV) radiation. Fungal conidia are very susceptible to solar radiation and investigators have for many years tried to find ways of protecting conidia against damage caused by UV radiation. However, such efforts

have not been very successful. In addition to inactivating conidia, UV radiation has been shown to cause delay in the germination process of the surviving conidia. All these reduce the efficiency of fungi as biocontrol agent under field conditions where there is a strong solar irradiation. It is therefore important to search for chemicals, e.g. sunscreens that will protect the spores in order to enhance their performance. There is very little report on fungal spore protection against UV radiation to enhance spore survival using chemical sunscreens. The efficacy of formulation in enhancing spore survival is also not well documented. The effects of chemicals sunscreens on viability and pathogenicity of *M. anisopliae* conidia is also not reported.

2.11. Aims of the study

This study, therefore, aimed to investigate the susceptibility of various developmental stages (eggs and instars) of various tick species (*A. hebraeum, A. variegatum, R. appendiculatus* and *R. e. evertsi*) to infection by the entomopathogenic fungus *M. anisopliae* in water and oil formulations. The susceptibility of eggs and instars of *R. e. evertsi* to varying conidia concentrations of *M. anisopliae,* in water and oil formulations was also studied, and hence the possibility of using this fungus as a bio-control agent against ticks. Furthermore, the potential of two commercial sunscreens as improving compounds for a tick biopesticide was studied. The direct influence of the sunscreens on the ability of the conidia to germinate, and induce mortalities in eggs and instars of *R. e. evertsi* was also studied.

2.12. Specific Objectives

(i) To evaluate the susceptibility of eggs and instars of *R. e. evertsi* to different spore concentrations of *M. anisopliae,* and compare between water and oil formulations.

(ii) To determine the susceptibility of developmental stages of *A. hebraeum* (eggs)*, A. variegatum* (eggs and unfed nymphs)*, R. appendiculatus* (eggs and unfed instars) and *R. e. evertsi* (eggs and instars) to infection by the entomopathogenic fungus *M. anisopliae*, and compare susceptibility in water and oil formulations.

(iii) To investigate the efficacy of (environmentally-friendly) chemical sunscreens in protecting spores of *M. anisopliae* from ultra-violet radiation damage, and compare between water and oil formulations.

(iv) To evaluate the effects of (environmentally-friendly) chemical sunscreens on viability, and pathogenicity of *M. anisopliae* conidia to eggs and instars of *R. e. evertsi* in water and oil formulations.

2.13. Study questions

1. What is the effect of varying conidia concentrations of *M. anisopliae* formulated in water and in oil on eggs and instars of *R. e. evertsi*?
2. What is the susceptibility of developmental stages of *A. hebraeum* (eggs)*, A. variegatum* (eggs and unfed nymphs)*, R. appendiculatus* (eggs and unfed instars) and *R. e. evertsi* (eggs and instars) to infection by the entomopathogenic fungus *M. anisopliae* formulated in water and in oil?

3. What is the efficacy of various sunscreens in protecting spores of *M. anisopliae* from ultra-violet radiation damage in water and in oil formulations?
4. What effects do chemical sunscreens have on viability, and pathogenicity of *M. anisopliae* conidia to eggs and instars of *R. e. evertsi* in water and in oil formulations?

2.14. Hypotheses

1. Null hypothesis (H_o): There is no difference in susceptibility of eggs and instars of *R. e. evertsi* to different spore concentrations of *M. anisopliae* formulated in water and in oil.

   Experimental hypothesis (H_E): There is a difference in susceptibility of eggs and instars of *R. e. evertsi* to different spore concentrations of *M. anisopliae* formulated in water and in oil.
2. Null hypothesis (H_o): There is no difference in susceptibility of developmental stages of *A. hebraeum* (eggs), *A. variegatum* (eggs and unfed nymphs), *R. appendiculatus* (eggs and unfed instars) and *R. e. evertsi* (eggs and instars) to infection with the entomopathogenic fungus *M. anisopliae* formulated in water and oil.

   Experimental hypothesis (H_E): There is a difference in susceptibility of developmental stages of *A. hebraeum* (eggs), *A. variegatum* (eggs and unfed nymphs), *R. appendiculatus* (eggs and unfed instars) and *R. e. evertsi* (eggs and instars) to infection with the entomopathogenic fungus *M. anisopliae* formulated in water and oil.

3. Null hypothesis (H_o): There is no difference in efficacy of various sunscreens in protecting spores of *M. anisopliae* from ultra-violet radiation damage in water and in oil formulations.

   Experimental hypothesis (H_E): There is a difference in efficacy of various sunscreens in protecting spores of *M. anisopliae* from ultra-violet radiation damage in water and in oil formulations.

4. Null hypothesis (H_o): There is no difference in the effects of chemical sunscreens on viability and pathogenicity of *M. anisopliae* conidia in water and in oil formulations.

   Experimental hypothesis (H_E): There is a difference in the effects of chemical sunscreens on viability, and pathogenicity of *M. anisopliae* conidia to eggs and instars of *R. e. evertsi,* in water and in oil formulations.

CHAPTER 3: MATERIALS AND METHODS

3.1. Location of study

The first 3 months of the study were spent at Kimron Veterinary Institute, ARO, Volcani center in the State of Israel to learn the techniques and principles used in this study i.e. rearing and maintaining tick colonies in the laboratory, and culturing and formulating fungi. All experimental stages were conducted in laboratories in the Department of Biological Sciences and Department of Physics at the University of Namibia, Windhoek, Namibia.

3.2. Rearing of tick colonies

Eggs and instars of ticks (*Amblyomma hebraeum, Amblyomma variegatum,* and *R. appendiculatus*) used in the study were obtained from the existing tick colonies in the Department of Biological Sciences at the University of Namibia. Engorged *R. e. evertsi* were collected from zebu cattle (*Bos primigenius indicus*) in Ohangwena and Otjikoto regions in Northern Namibia. All off-host stages were maintained at 25 °C and 100% relative humidity (RH) and on-host stages were fed on rabbits free of previous contact with ticks (Kaaya *et al.*, 1996). During feeding, the ticks were restricted to the hosts by nearly transparent polyester bags fixed with non-toxic material to their shaved backs or ears, until they were fully engorged. Ticks not being used in experiments were stored at 14 °C until use (Samish *et al*., 1999).

3.3. Inoculant preparation

Metarhizium anisopliae RS2 (originally isolated from *Amblyomma variegatum*) was cultured in petri dishes for 3 weeks at 25 °C on sabourauds dextrose agar (SDA) as described by Kaaya *et al.* (1996). Conidia were harvested by rinsing agar with sterile, distilled water containing 0.05% (v/v) Triton X-100. Conidia were then washed twice in sterilized distilled water by centrifugation at 5000 revolutions/minute for 5 minutes. A hemocytometer was used to determine the concentration of conidia in the initial suspension. Serial dilutions were then made to get the desired concentration of conidia.

3.4. Preparation of formulations

Water formulations were prepared in sterile distilled water containing 0.05% Triton X-100, and Oil based (100% extra virgin olive oil) formulations were prepared in sterile distilled water containing 20% olive oil + 0.05% Triton X-100. The efficacies of oil formulations in inducing mortality to different free-living life-stages of several tick species were compared with those of aqueous formulations.

3.5. Efficacy of varying fungal concentrations to various stages of ticks

Eggs of *R. appendiculatus* and *R. e. evertsi*, and larvae of *R. e. evertsi* were infected by placing them on filter papers in disposable petri dishes (65 mm diameter) previously wetted with 1 ml of different concentrations of fungal suspensions (1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 conidia ml^{-1}) in each formulation,

respectively. Each petri dish contained between 30-40 eggs or larvae, respectively. Nymphs and adults of *R. e. evertsi* were infected by dipping them in different concentrations of fungal solution (1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7, and 1 x 10^8 conidia ml^{-1}) in each formulation, respectively, for about five seconds and placed on filter papers in disposable petri dishes (65mm diameter). Each petri dish contained between 15-20 instars of *R. e. evertsi*. Three replicates were set up in each experiment. No fungal conidia were added to either formulation in controls. All petri dishes were sealed with parafilm and incubated at 25 °C and 100% RH. All fungal infections were viewed under a dissecting microscope. Mortalities (or infection for eggs) caused by the fungi were recorded for 21 days PI and compared between water, and oil formulations.

3.6. Fungal efficacy to various stages of different tick species

Eggs or unfed larvae of either tick species were infected by placing them on filter papers in disposable petri dishes (65 mm diameter) previously wetted with 1 ml of fungal suspension (1×10^8 conidia ml^{-1}) in each formulation, respectively. The fungal concentration of 1×10^8 conidia ml^{-1} was used because it induced optimum mortalities to various stages of ticks in earlier experiments of this study. Each petri dish contained between 30-40 eggs or unfed larvae of either tick species, respectively. Nymphs and adults of either tick species were infected by dipping them in a fungal solution (1×10^8 conidia ml^{-1}) in each formulation, respectively, for five seconds and then placed on filter papers in disposable petri dishes (65 mm diameter). Each petri dish contained between 15-20 instars of either tick species, repectively. Three replicates were set up in each experiment. No fungal conidia were added to

either formulation in controls. All petri dishes were sealed with parafilm and incubated at 25 °C and 100% RH. All fungal infections were viewed under a dissecting microscope. Mortalities (or infection for eggs) induced by the fungi were recorded daily until 21 days post infection (PI) and compared between water and oil formulations.

3.7. Viewing fungal infections

Signs of eggs infection appeared when the egg darkened several days post infection (Gindin *et al.,* 2000). Infected larvae were darkened, immobile, and swollen PI (Gindin *et al.,* 2000; Samish *et al.,* 2001). Indications of infection in nymphs appeared as large dark spots below the cuticle of the tick and as external exudates PI (Samish *et al.,* 2001). Signs of infection in adults appeared as hemorrhages below the cuticle and as external exudates PI (Gindin *et al.,* 2000; Samish *et al.,* 2001).

3.8. Protection of *M. anisopliae* conidia from UV radiation damage

3.8.1. Chemical sunscreens

Two commercial sunscreens for application on people's skin were used and their chemical compositions are provided in Table 1. These types of sunscreens were chosen because they are readily available and they are relatively cheaper compared to pure chemical sunscreens like benzylcinnamate, and hence likely to be available even to peasant farmers. Sunscreens developed for humans are likely to be safer to animals and probably to the environment than pure chemicals, hence their use in this study.

Table 1. UV protectantss used in the study and their chemical compositions

UV protectant	**Sun protection factor (SPF)**	**Chemical compositions**	**Manufacturer**
Everysun® (Everysun)	30	Aqua, Ethlyhexyl Methoxycinnamate, Benzophenone-3, C12-15 Alkyl Benzoate Cinnamate, Ethylhexyl Salicylate, Cyclomethicone, 4-methyl Benzylidene Camphor, Steareth-100, Stearth-2, Glyceryl Stearate Citrate, Sucrose, Mannan, Xanthan Gum, Phenoxy Ethanol, Alkyl Stearate, Methyl Thiazolinone.	Permark International (Pty) Ltd (South Africa)
E45 Sun Block 50® (E45)	50	Aqua, Zinc Oxide, Octyl Stearate, Titanium Dioxide, Isopropyl Myristate, Isohexadecane butylenes Glycol, Polyglyceryl-3 Oleate, Cetyl Dimethionine Copolyol, Magnesium Sulphate, Phenoxyethanol, Aluminum Stearate, Alumina, Lecithin, Isopropyl Palmitate, Propylparaben 2-Bromo-2-Nitropropane-1,3-diol.	Healthcare South Africa (Pty) Ltd (South Africa)

Furthermore, the SPF values of the sunscreens used (30 and 50) are likely to confer better protection from UV radiation than sunscreens with lower SPF values, especially in harsh environmental conditions with high solar irradiation.

3.8.2. Preparation of conidia formulations

Chemical sunscreens Everysun and E45 were tested for their efficacy in protecting fungal conidia from UV radiation damage in both water (including 0.05% Triton X-100), or oil formulations (20% olive oil + 0.05% Triton X-100 in water). A concentration of 20 conidia ml^{-1} was prepared in water and oil formulations, respectively. A solution 1% or 3% (v/v) of the commercial sunscreen was prepared in water or oil formulations respectively. In the control groups, conidia were suspended in the same solutions without the sunscreens. All conidial components used in the tests started with a pre-incubation period of 30 min at standard room temperature to get all conidia in the same temperature range and humidity.

3.8.3. UV Source

An artificial UV source was used at a wavelength of 200-400 nm (AVANTES: AvaLight-DHS, Serial Nr-LS-0501001; compact and closed system). The wavelength of light emitted by the UV source was detected using AvaSpec-2048 monitor and was analyzed and adjusted using AvaSoft 6.2 software for AvaSpec Spectrometer. Samples were exposed in a closed chamber ± 1m from UV source. Temperature in the chamber was maintained at about 25 °C during exposure.

3.8.4. Effects of UV protectants on conidia viability

One ml of UV-exposed conidial suspension (200 conidia/ml) samples in water or in oil formulations with and without (1% or 3%) sunscreens were spread on SDA plates using glass beads and incubated at 25 °C and 100% RH in the dark. Germinating conidia were counted after 48 h of incubation under a dissecting microscope and recorded as percentage germination. Conidia were considered to have germinated if the germ tube was longer than half the diameter of the germinating spore. Three replicates of each sample were prepared and each experiment repeated 3 times.

3.8.5. Protection of conidia from UV damage using sunscreens

One ml samples (200 conidia/ml) of each formulation containing 1% or 3% sunscreen were placed in macro disposable cuvettes (10 × 10 × 45 mm, <1mm thick) during exposure to UV radiation. Samples were exposed to UV radiation in a compact and closed environment for 0, 1, 2, 3, 4, and 5 h, respectively. A 0.1 ml (±20 conidia/dish) of exposed samples were then cultured on SDA and spread using glass beads and incubated at 25 °C and 100% RH in the dark. Colonies developing from each sample were counted daily for 3 days under a dissecting microscope. This provided an estimate of the ability to form colonies (colony forming units, CFUs). The mean ability to form colonies (%) is reperesented as $(S_t/S_c) \times 100$, where S_t is the mean CFUs of 9 replicates at exposure time t, and S_c is the mean CFUs of the water control (no exposure) after 3 day germination (Lee *et al.,* 2006).

3.8.6. Influence of UV protectants on the pathogencity of *M. anisopliae* conidia to *R. e. evertsi* ticks

Eggs and unfed larvae of *R. e. evertsi* were infected with *M. anisopliae* conidia by placing 20-30 eggs or larvae on filter papers in disposable petri dishes (65 mm diameter) earlier wetted with 1 ml of *M. anisopliae* suspension ($1x10^8$ conidia/ml) with or without 3% of the sunscreens E45 or Everysun in oil or water formulations. A sunscreen concentration of 3% was chosen because it offered better protection to conidia against UV radiation in the range of chemicals tested. Similarly, engorged nymphs and fed adult *R. e. evertsi* ticks were infected by dipping them in conidia suspension and placing them on filter papers in petri dishes. The dishes were then incubated at 25 °C and 100% RH in the dark. Mortalities were recorded daily until 21 days post-infection.

3.9. Data manipulation and analysis

Normality of the data was tested using Kolomogorov-Smirnov test and the normally distributed data was analyzed by ANOVA and means were compared using a post-hoc Scheffé multiple comparison test, using SPSS™ for Windows®. All analysis were done at confidence interval (CI) = 95%, $\alpha = 0.05$. LC_{50} were calculated by fitting a standard curve to the normal distribution line of susceptibility of developmental stages of *R.appendiculatus,* and *R. e. evertsi* to *M. anisopliae* conidia.

CHAPTER 4: RESULTS

4.1. Efficacy of varying fungal concentrations to various stages of ticks

4.1.1. Mortality in *R. appendiculatus* unfed larvae

Variable mortalities were obsereved when unfed larvae were exposed to varying doses of *M. anisopliae* conidia in oil, and in water formulations. A 1×10^3 conidia/ml concentration induced larval mortality of 13% (±0.4SE) in water and 24% (±1.5SE) in oil formulation ($P<0.05$) (Figure 4). A concentration of 1×10^4 conidia/ml induced mortality of 30% (±0.8SE) in water and 40% (±0.8SE) in oil formulation ($P<0.05$). Mortality of 42% (±1.8SE) and 58% (±1.5SE) were recorded with the concentration of 1×10^5 conidia/ml in water and oil formulations, respectively ($P<0.05$). A concentration of 1×10^6 conidia/ml induced mortality of 54% (±1.2SE) in water and 68% (±0.7SE) in oil formulation ($P<0.05$). At concentration of 1×10^7 conidia/ml, mortality of 69% (±0.8SE) in water and 84% (±1.5SE) in oil formulation ($P<0.05$) were recorded. Mortality reached 87% (±0.6SE) in water and 96% (±2.1SE) in oil formulation ($P<0.05$) at a concentration of 1×10^8 conidia/ml. No mortality (0%) due to fungi was observed in controls of either formulation. The LC_{50} was 1×10^5 conidia/ml in oil and 1×10^6 conidia/ml in water formulation (Figure 4).

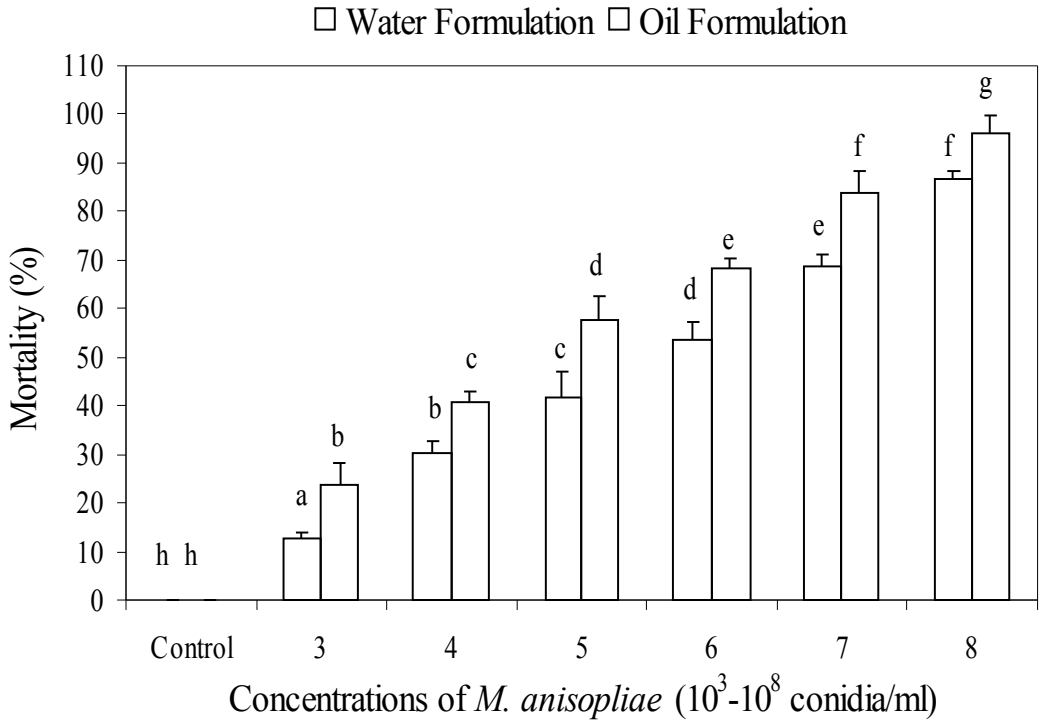


Figure 4: Mortality (%) in *R. appendiculatus* unfed larvae induced by increased concentrations of *M. anisopliae* conidia. Controls contained no fungal conidia. Means (±SD) of three replicates are presented. Means with the same letter are not significantly different from each other (Scheffé test, P>0.05).

4.1.2. Mortality induced in various developmental stages of *R. e. evertsi* by different concentrations of *M. anisopliae* conidia

4.1.2.1. *R. e. evertsi* eggs

Variable mortalities were obsereved when eggs were exposed to varying doses of *M. anisopliae* conidia in oil, and in water formulations. A 1×10^3 conidia/ml concentration induced egg infection of 8% (±0.3SE) in water and 17% (±0.3SE) in oil formulation (P<0.01) (Figure 5). A concentration of 1×10^4 conidia/ml induced infection of 25% (±0.5SE) in water and 35% (±0.4SE) in oil formulation (P<0.05).

Infection of 38% (±0.7SE) and 52% (±0.5SE) were recorded with the concentration of 1 × 10^5 conidia/ml in water and oil formulations, respectively (P<0.01). A concentration of 1 × 10^6 conidia/ml induced infection of 55% (±0.6SE) in water and 65% (±0.4SE) in oil formulation (P<0.05). At concentration of 1 × 10^7 conidia/ml, infection of 64% (±0.6SE) in water and 77% (±0.7SE) in oil formulation (P<0.05) were recorded. Infection reached 77% (±0.5SE) in water and 87% (±2.1SE) in oil formulation (P<0.05) at a concentration of 1 × 10^8 conidia/ml. No infection (0%) due to fungi was observed in controls of either formulation. Furthermore, infected egg failed to hatch into larvae. The LC_{50} was 1 × 10^5 conidia/ml in oil and 1 × 10^6 conidia/ml in water formulation (Figure 5).

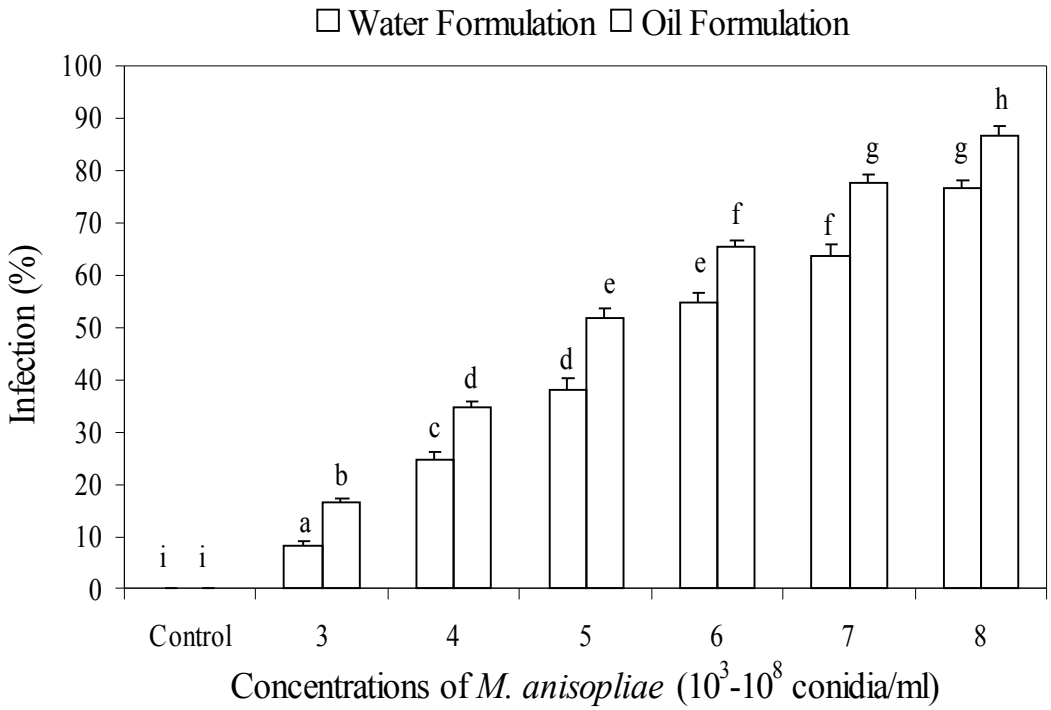


Figure 5: Infection (%) of *R. e. evertsi* eggs induced by increased fungal concentrations of *M. anisopliae* conidia. Controls contained no fungal conidia.

Means (±SD) of three replicates are presented. Means with the same letter are not significantly different from each other (Scheffé test, P>0.05).

4.1.2.2. *R. e. evertsi* unfed larvae

Variable mortalities were obsereved when unfed larvae were exposed to varying doses of *M. anisopliae* conidia in oil, and in water formulations. A 1×10^3 conidia/ml concentration induced larval mortality of 21% (±1.5SE) in water and 39% (±2.1SE) in oil formulation (P<0.05) (Figure 6). A concentration of 1×10^4 conidia/ml induced mortality of 42% (±0.7SE) in water and 52% (±0.9SE) in oil formulation (P>0.05). Mortality of 54% (±1.2SE) and 67% (±0.9SE) were recorded with the concentration of 1×10^5 conidia/ml in water and oil formulations, respectively (P<0.05). A concentration of 1×10^6 conidia/ml induced mortality of 65% (±1.5SE) in water and 76% (±1.0SE) in oil formulation (P<0.05). At concentration of 1×10^7 conidia/ml, mortality of 74% (±2.9SE) in water and 90% (±1.5SE) in oil formulation (P<0.01) were recorded. Mortality reached 95% (±5.0SE) in water and 100% (±0.0SE) in oil formulation (P>0.05) at a concentration of 1×10^8 conidia/ml. No mortality (0%) due to fungi was observed in controls of either formulation. The LC_{50} was 1×10^4 conidia/ml in oil and 1×10^5 conidia/ml in water formulation (Figure 6).

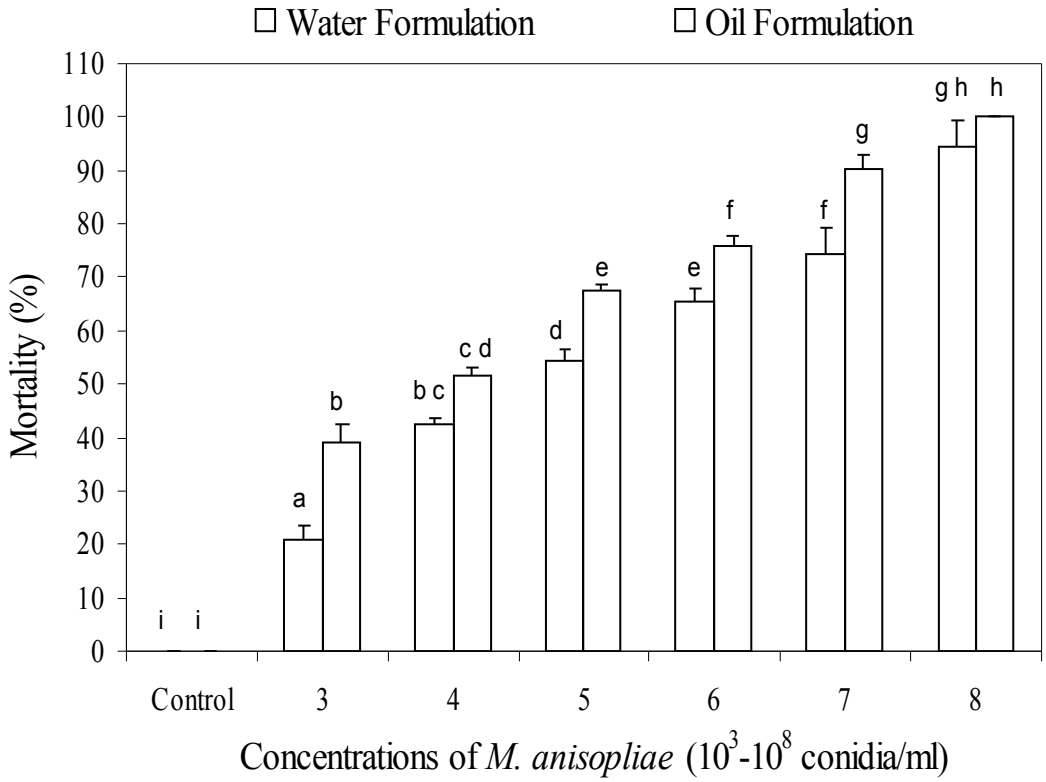


Figure 6: Mortality (%) of *R. e. evertsi* unfed larvae induced by increased fungal concentrations of *M. anisopliae* conidia. Controls contained no fungal conidia. Means (±SD) of three replicates are presented. Means with the same letter are not significantly different from each other (Scheffé test, P>0.05).

4.1.2.3. *R. e. evertsi* engorged nymphs

Variable mortalities were obsereved when engorged nymphs were exposed to varying doses of *M. anisopliae* conidia in oil, and in water formulations. A 1×10^3 conidia/ml concentration induced nymphal mortality of 5% (±1.2SE) in water and 12% (±1.8SE) in oil formulation (P<0.01) (Figure 7). A concentration of 1×10^4 conidia/ml induced mortality of 20% (±0.9SE) in water and 28% (±2.3SE) in oil formulation (P>0.05). Mortality of 25% (±0.9SE) and 41% (±1.2SE) were recorded

with the concentration of 1 × 10^5 conidia/ml in water and oil formulations, respectively (P<0.05). A concentration of 1 × 10^6 conidia/ml induced mortality of 43% (±0.9SE) in water and 55% (±2.5SE) in oil formulation (P<0.05). At concentration of 1 × 10^7 conidia/ml, mortality of 57% (±1.2SE) in water and 62% (±2.2SE) in oil formulation (P>0.05) were recorded. Mortality reached 64% (±1.8SE) in water and 72% (±1.5SE) in oil formulation (P>0.05) at a concentration of 1 × 10^8 conidia/ml. No mortality (0%) due to fungi was observed in controls of either formulation. The LC_{50} was 1 × 10^5 conidia/ml in oil and 1 × 10^6 conidia/ml in water formulation (Figure 7).

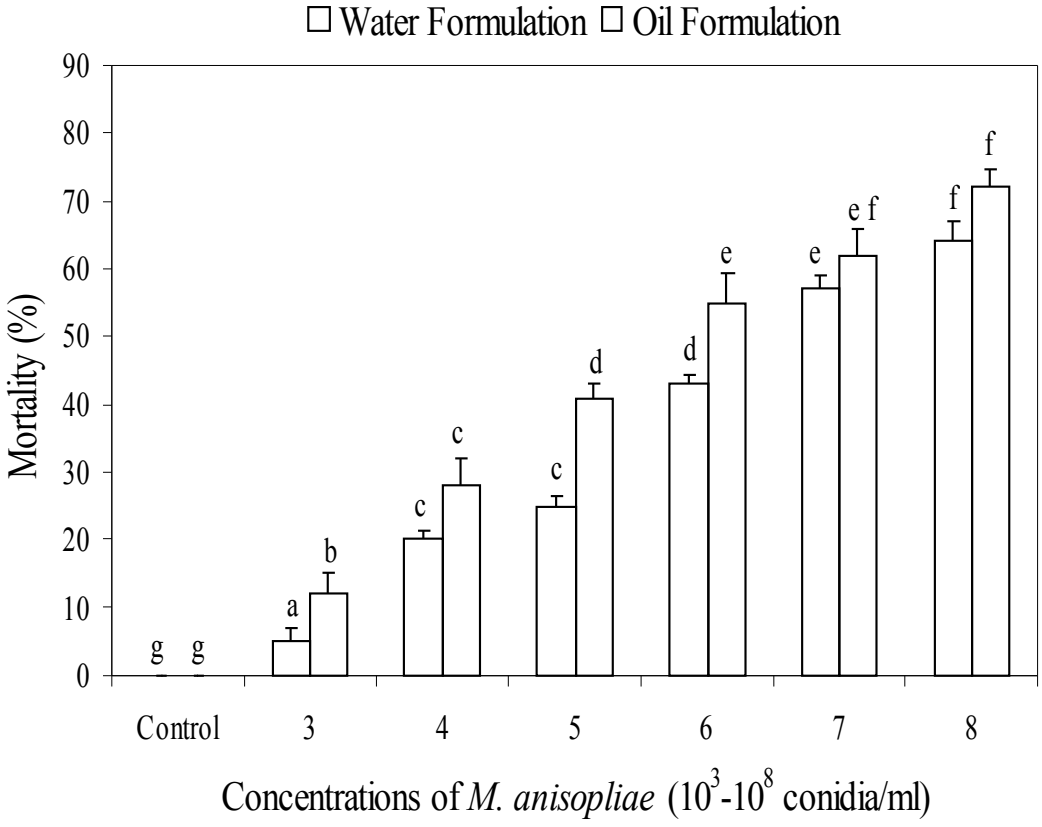


Figure 7: Mortalities (%) of *R. e.evertsi* engorged nymphs induced by increased fungal concentrations of *M. anisopliae* conidia. Controls contained no fungal conidia.

Means (±SD) of three replicates are presented. Means with the same letter are not significantly different from each other (Scheffé test, P>0.05).

4.1.2.4. *R. e. evertsi* unfed adults

Variable mortalities were obsereved when adults were exposed to varying doses of *M. anisopliae* conidia in oil, and in water formulations. A 1×10^3 conidia/ml concentration induced mortality of 11% (±0.9SE) in water and 19% (±0.7SE) in oil formulation (P<0.05) (Figure 8). A concentration of 1×10^4 conidia/ml induced mortalities of 27% (±0.9SE) in water and 39% (±1.2SE) in oil formulation (P<0.05). Mortality of 44% (±0.7SE) and 58% (±1.0SE) were recorded with the concentration of 1×10^5 conidia/ml in water and oil formulations, respectively (P<0.05). A concentration of 1×10^6 conidia/ml caused mortality of 62% (±0.3SE) in water and 70% (±0.3SE) in oil formulation (P>0.05). At concentration of 1×10^7 conidia/ml, mortality of 72% (±1.0SE) in water and 84% (±1.2SE) in oil formulation (P<0.05) were recorded. Mortality reached 83% (±1.2SE) in water and 91% (±0.7SE) in oil formulation (P<0.05) when a concentration of 1×10^8 conidia/ml was used. No mortality (0%) due to fungi was observed in controls of either formulation. The LC_{50} was 1×10^6 conidia/ml in oil and 1×10^7 conidia/ml in water formulation (figure 8).

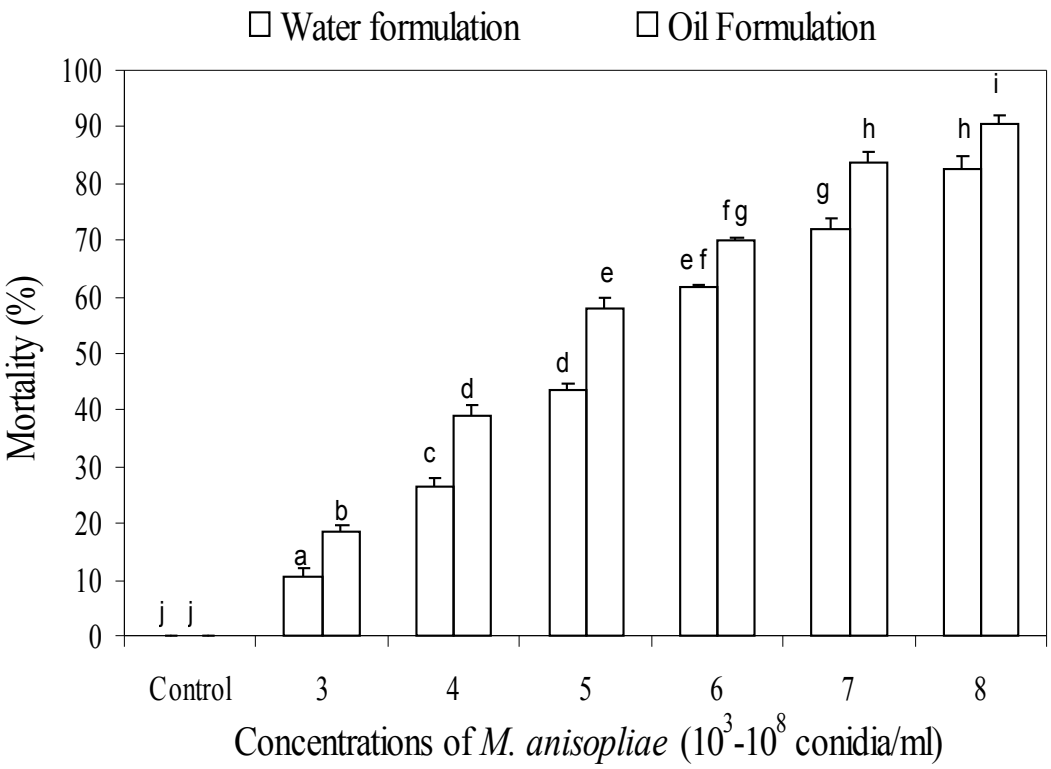


Figure 8: Mortalities (%) of *R. e.evertsi* unfed adults induced by increased fungal concentrations of *M. anisopliae* conidia. Controls contained no fungal conidia. Means (±SD) of three replicates are presented. Means with the same letter are not significantly different from each other (Scheffé test, P>0.05).

4.1.2.5. *R. e. evertsi* engorged adults

Variable mortalities were obsereved when engorged adults were exposed to varying doses of *M. anisopliae* conidia in oil, and in water formulations. A 1×10^3 conidia/ml concentration induced mortality of 11% (±2.1SE) in water and 23% (±2.3SE) in oil formulation (P<0.01) (Figure 9). A concentration of 1×10^4 conidia/ml induced mortality of 36% (±1.8SE) in water and 44% (±2.1SE) in oil formulation (P>0.05). Mortality of 47% (±1.5SE) and 61% (±2.6SE) were recorded with the concentration

of 1 × 10^5 conidia/ml in water and oil formulations, respectively (P<0.01). A concentration of 1 × 10^6 conidia/ml caused mortality of 70% (±1.8SE) in water and 79% (±2.1SE) in oil formulation (P<0.05). At concentration of 1 × 10^7 conidia/ml, mortality of 84% (±1.7SE) in water and 93% (±2.1SE) in oil formulation (P<0.05) were recorded. Mortality reached 90% (±2.3SE) in water and 97% (±0.9SE) in oil formulation (P>0.05) at a concentration of 1 × 10^8 conidia/ml. No mortality (0%) due to fungi was observed in controls of either formulation. The LC_{50} was 1 × 10^5 conidia/ml in oil and 1 × 10^6 conidia/ml in water formulation (Figure 9).

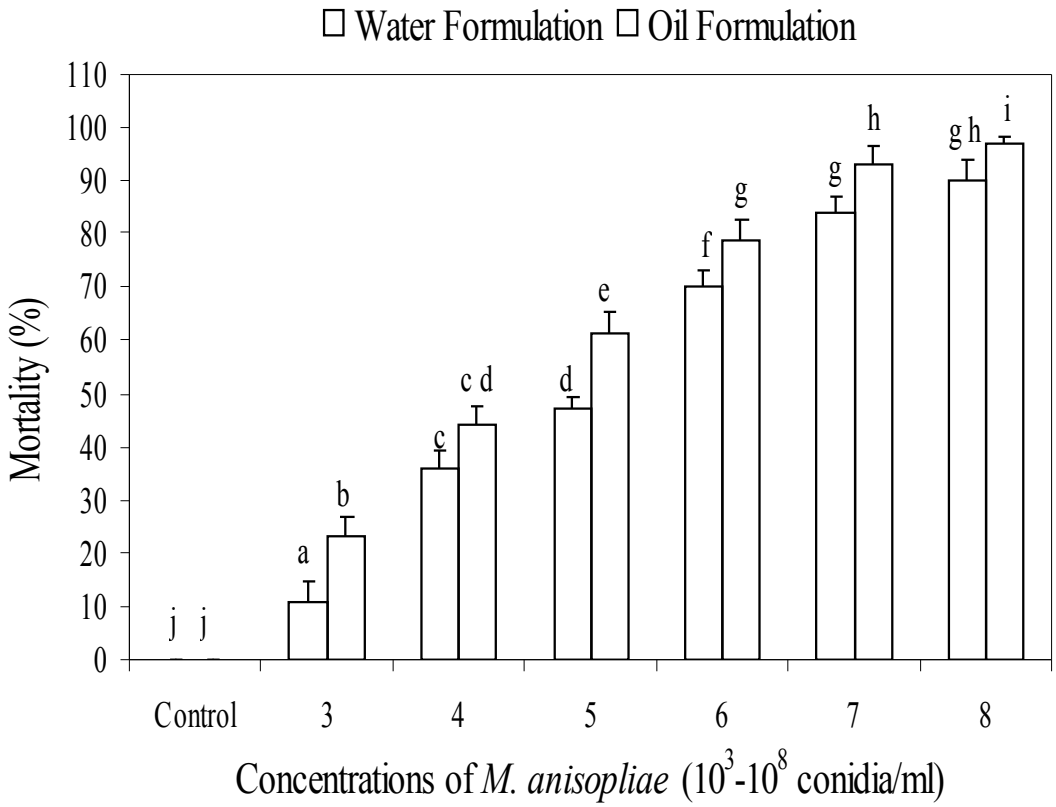


Figure 9: Mortalities (%) of *R. e.evertsi* engorged adults induced by increased fungal concentrations of *M. anisopliae* conidia. Controls contained no fungal conidia. Means (±SD) of three replicates are presented. Means with the same letter are not significantly different from each other (Scheffé test, P>0.05).

4.2. Fungal efficacy to various stages of different tick species

4.2.1. Susceptibility of eggs to *M. anisopliae* conidia

Variable susceptibility of eggs to *M. anisopliae* (1×10^8 conidia/ml) in oil and water formulations was observed. Fungal infection of *Amblyomma hebraeum* eggs was 35% (±1.0 SE) in water and 53% (±0.6 SE) in oil formulation ($P<0.01$) (Figure 10). In *Amblyomma variegatum* eggs, infection was 30% (±1.0SE) in water and 60% (±1.5SE) in oil formulation ($P<0.001$) (Figure 7). In eggs of *Rhipicephalus appendiculatus*, infection was 36% (±0.6SE) in water and 65% (±1.2SE) in oil formulation ($P<0.001$) (Figure 7). In eggs of *R. e. evertsi*, infection was 77% (±0.5SE) in water and 87% (±0.6) in oil formulation ($P<0.05$) (Figure 10). No infection (0%) due to fungi was observed in controls of either formulation. Furthermore, infected egg did not hatch into larvae.

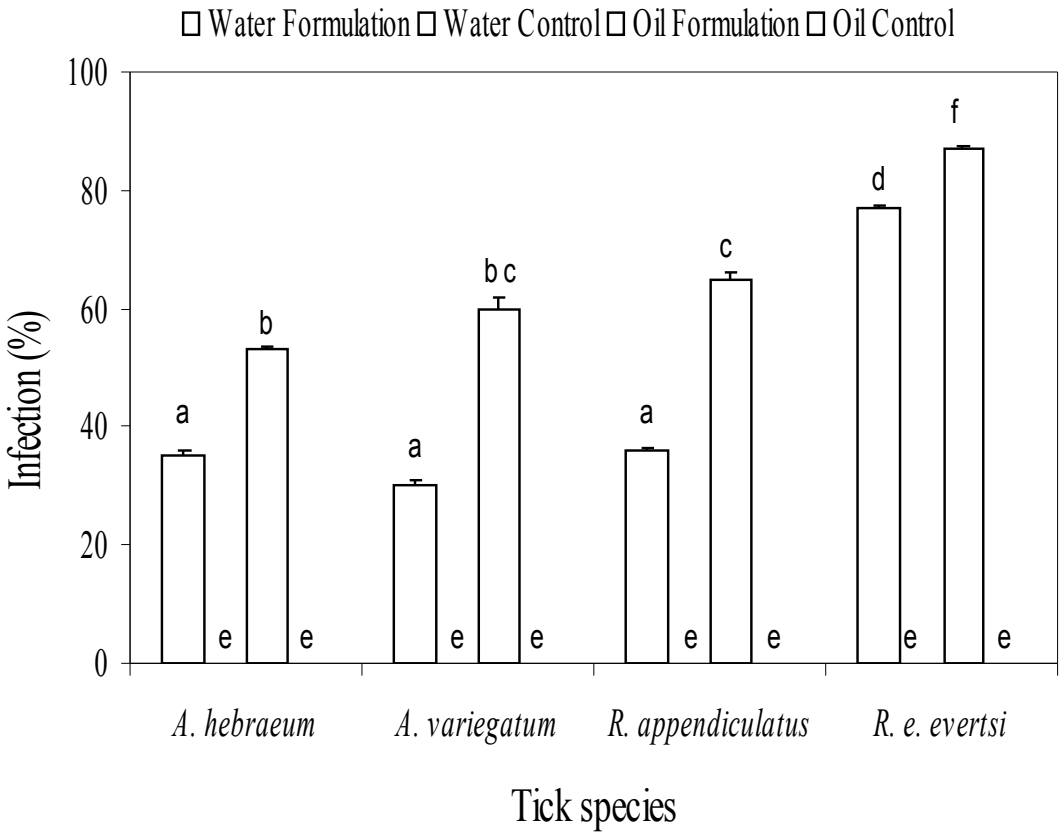


Figure 10: Infection (%) induced by *M. anisopliae* conidia in eggs of *A. hebraeum, A. variegatum, R. appendiculatus,* and *R. e. evertsi*. Controls contained no fungal conidia. Means (±SE) of three replicates are presented. Means with the same letter are not significantly different from each other (Scheffé test, P>0.05). No infection (0%) due to fungi was observed in controls of either formulation for either tick species.

4.2.2. Susceptibility of unfed larvae to *M. anisopliae* conidia

Variable susceptibility of unfed larvae to *M. anisopliae* (1×10^8 conidia/ml) in oil and water formulations was observed. Mortality due to fungi in *R. appendiculatus* larvae was 87% (±0.6SE) in water and 96% (±2.1SE) in oil formulation (P<0.05)

(Figure 11). In *R.e eversti* larvae, mortality was 95% (±5.0SE) in water and 100% (±0.0SE) in oil formulation (P>0.05) (Figure 11). No mortality (0%) due to fungi was observed in controls of either formulation.

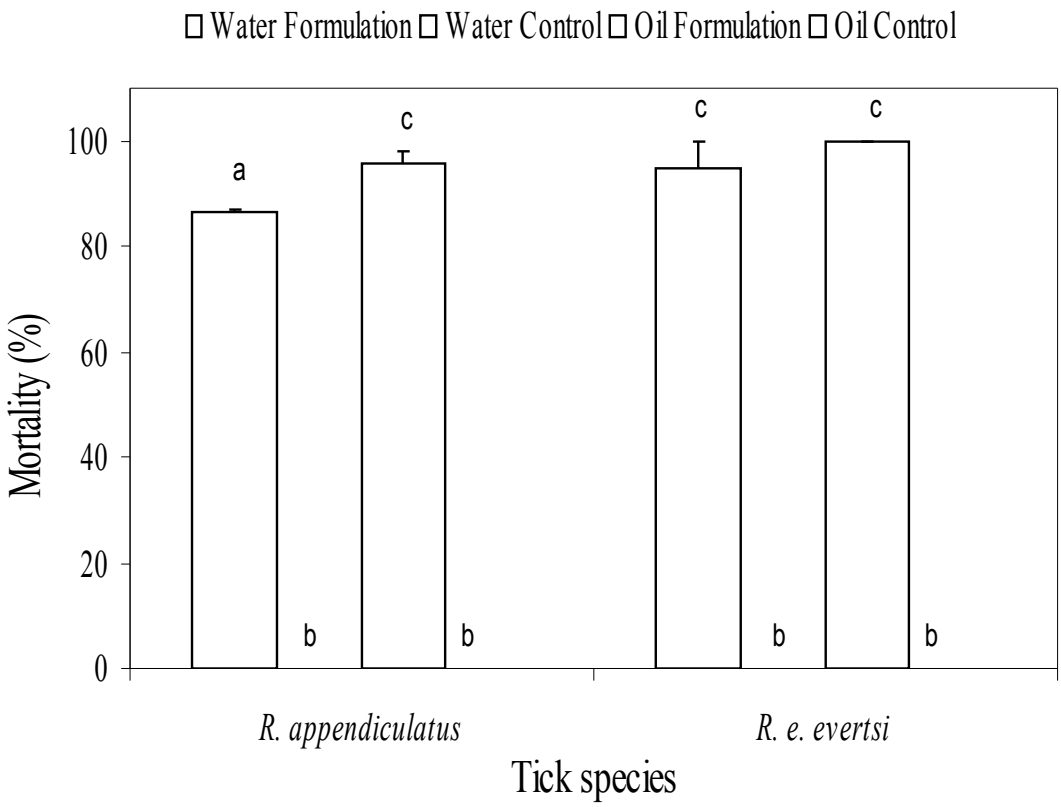


Figure 11: Mortality (%) induced by *M. anisopliae* conidia in unfed larvae of *R. appendiculatus,* and *R. e. evertsi*. Controls contained no fungal conidia. Means (±SE) of three replicates are presented. Means with the same letter are not significantly different from each other (Scheffé test, P>0.05). No mortality (0%) due to fungi was observed in controls of either formulation for either tick species.

4.2.3. Susceptibility of unfed nymphs to *M. anisopliae* conidia

Variable susceptibility of unfed nymphs to *M. anisopliae* (1×10^8 conidia/ml) in oil and water formulation was observed. Mortality in nymphs of *A. variegatum* was 32%

(±1.0SE) in water and 98% (±0.5SE) in oil (P<0.001) (Figure 12). In nymphs of *R. appendiculatus*, mortality was 19% (±0.8SE) in water and 71% (±4.2SE) in oil formulation (P<0.001) (Figure 12). No infection (0%) due to fungi was observed in controls of either formulation.

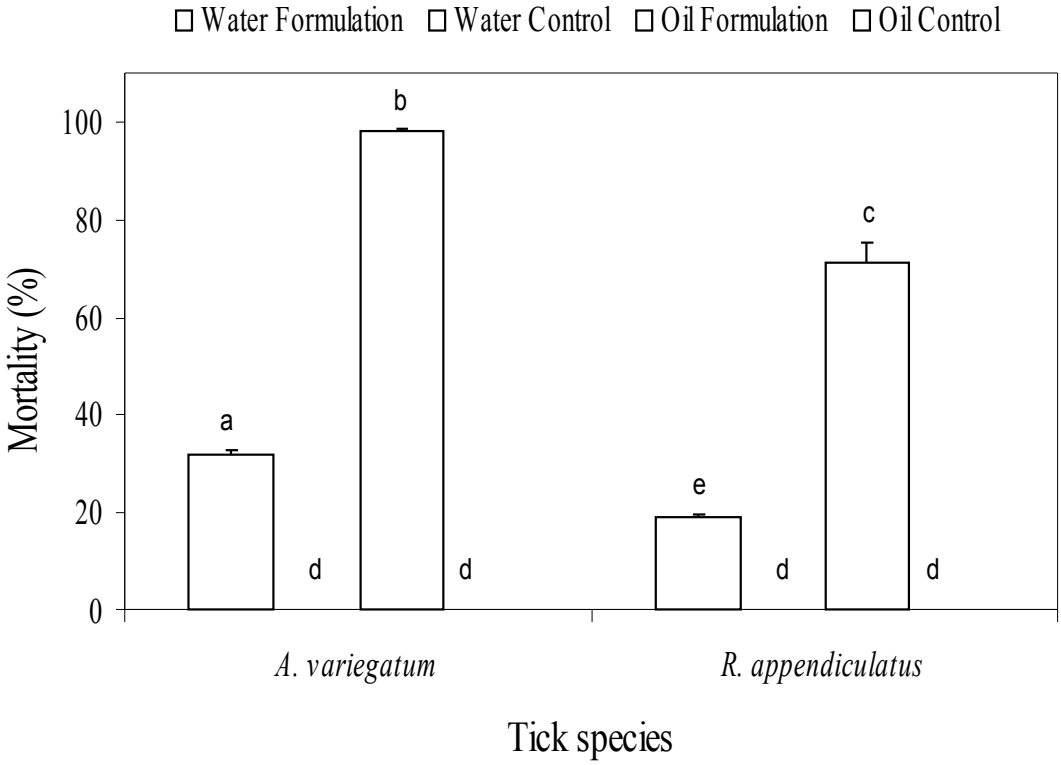


Figure 12: Mortality (%) induced by *M. anisopliae* conidia in unfed nymphs of *A. variegatum* and *R. appendiculatus*. Controls contained no fungal conidia. Means (±SE) of three replicates are presented. Means with the same letter are not significantly different from each other (Scheffé test, P>0.05). No mortality (0%) due to fungi was observed in controls of either formulation for either tick species.

4.2.5. Susceptibility of unfed adult ticks to *M. anisopliae* conidia

Variable susceptibility of adults to *M. anisopliae* (1×10^8 conidia/ml) in oil and water formulation was observed. Mortality of *R. appendiculatus* adult in water was 16% (±0.8SE) and 73% (±2.0SE) in oil formulation (P<0.001) (Figure 13). In *R. e. evertsi*, mortality was 83% (±1.2SE) in water and 91% (±0.7SE) in oil formulation (P<0.05) (Figure 13). No infection (0%) due to fungi was observed in controls of either formulation.

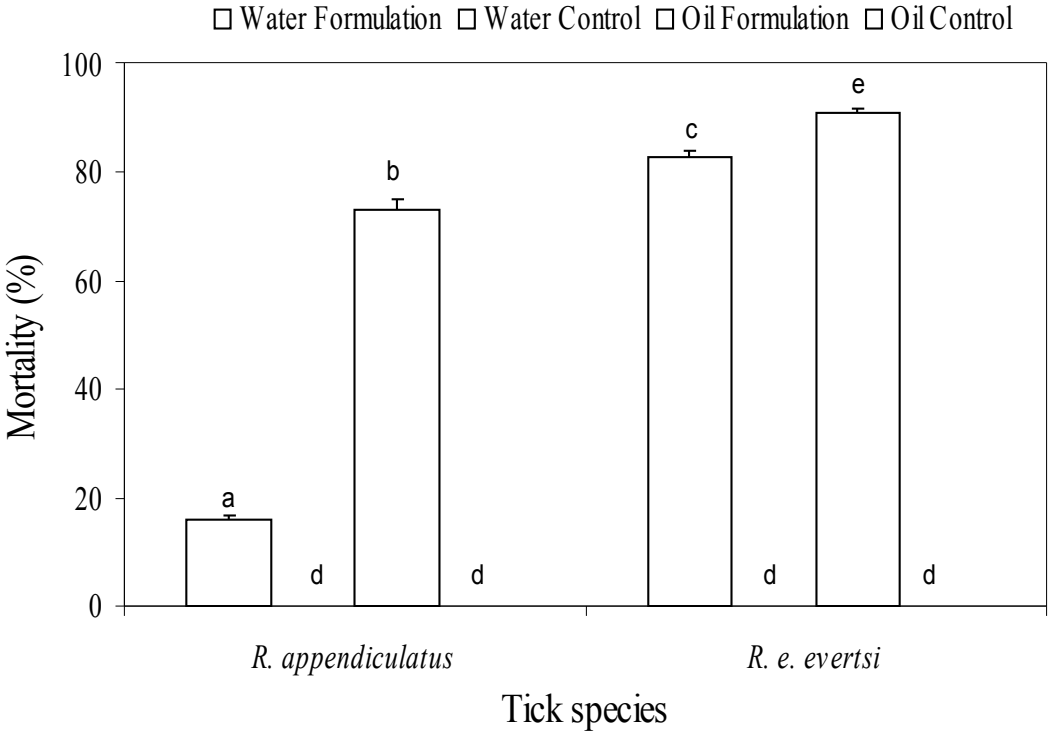


Figure 13: Mortality (%) induced by *M. anisopliae* conidia in unfed adults of *R. appendiculatus* and *R. e. evertsi*. Controls contained no fungal conidia. Means (±SE) of three replicates are presented. Means with the same letter are not significantly different from each other (Scheffé test, P>0.05). No mortalities (0%) due to fungi were observed in controls of ether formulation for either tick species.

4.3. Protection of fungal spores from UV radiation damage

4.3.1. Effects of UV protectants and formulations on the ability of *M. anisopliae* conidia to form colonies before exposure to UV radiation

Addition of sunscreens did not affect the viability of *M. anisopliae* conidia in water, or in oil formulations. When 1% sunscreen was added to formulations, germination rate was 99% (±0.7 SE), 97% (±2.4 SE) and 93% (±1.7 SE) among conidia formulated in water, protected with Everysun in water or protected with E45 in water ($P>0.05$), respectively (Figure 14). The germination rate was 95% (±2.8 SE), 94% (±3.0 SE) and 91% (±2.0 SE) in conidia formulated in oil, protected with 1% Everysun or 1% E45 ($P>0.05$), respectively. Furthermore, when 3% sunscreen was added to formulations, germination rate was 99% (±1.6 SE) and 96% (±3.3 SE) among conidia protected with Everysun or E45 in water ($P>0.05$) respectively (Figure 11). Germination rate was 92% (±3.2 SE) and 87% (±2.1 SE) in conidia protected with 3% Everysun or 3% E45 formulated in oil ($P>0.05$), respectively (Figure 14).

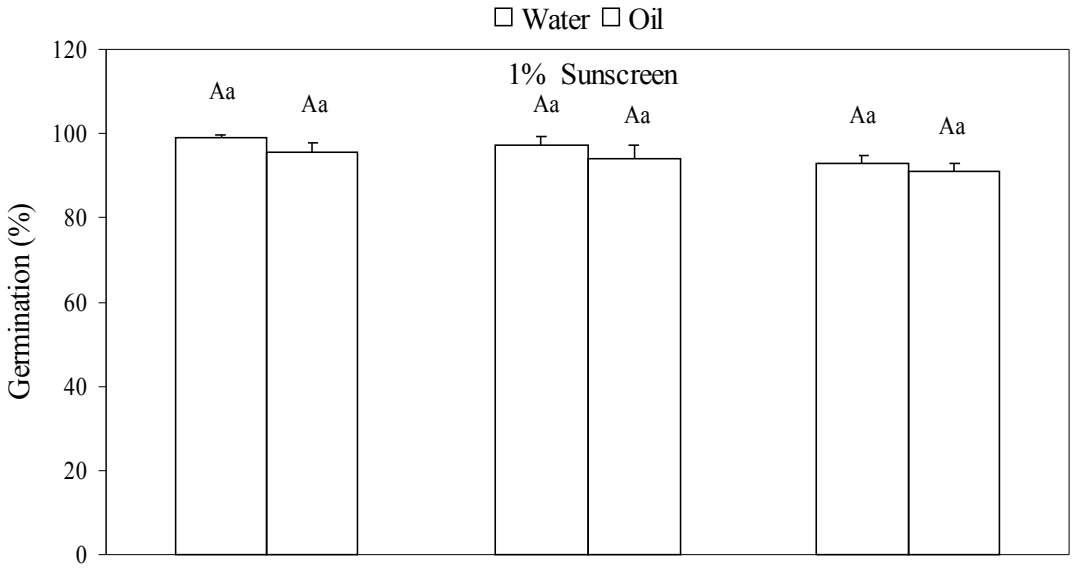

☐ Water ☐ Oil
1% Sunscreen
Germination (%)
120
100
80
60
40
20
0
Aa
Aa
Aa
Aa
Aa
Aa

Figure 14: Germination (%) of *M. anisopliae* conidia in water and oil formulations after addition of 1% and 3% sunscreens. Controls contained no sunscreens. Means (±SE) of nine replicates are presented. Means with the same lower case letter are not significantly different from each other among treatments in the same sunscreen concentration, whereas means with the same upper case letter are not significantly different from each other among treatments in different sunscreen concentration (Scheffé test, P>0.05).

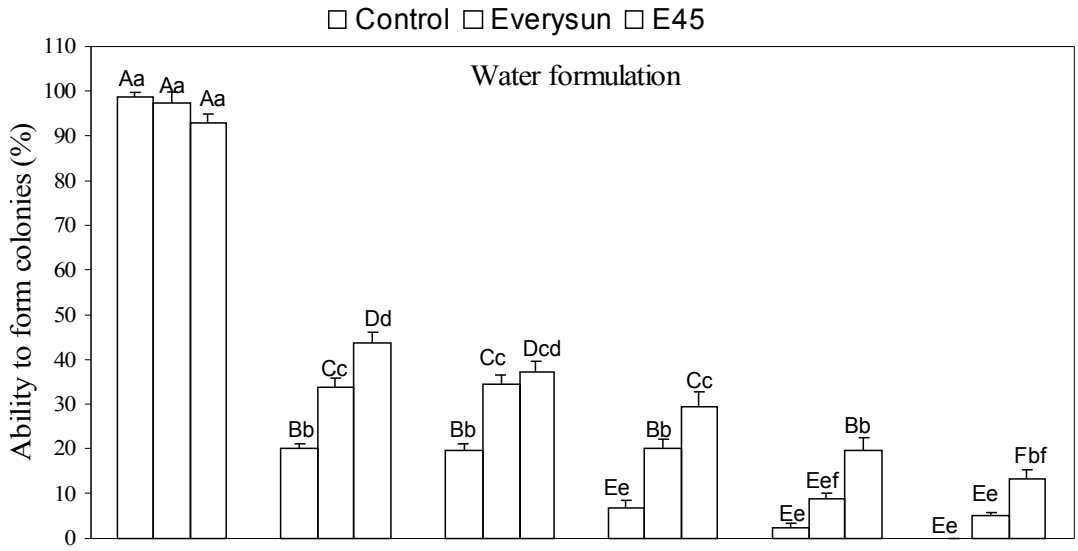
4.3.2. Effects of UV protectants and formulations on the ability of *M. anisopliae* conidia to form colonies after exposure to UV radiation

4.3.2.1. *M. anisopliae* conidia protection from UV radiation using 1% sunscreen

Survival of conidia (ability to form colonies) was observed to vary when sunscreens were included in formulations, and when exposed to UV radiation for varying periods of time. The ability of conidia to form colonies after 1 h of UV irradiation was reduced by 79%, 63% and 49% (P<0.01) when formulated in water with no protectant, in water with Everysun, and or in water with E45, respectively. Moreover, the ability of conidia to form colonies after 1 h of irradiation when formulated in oil was reduced by 47% in unprotected conidia, 35%, and 24% in conidia protected with Everysun and E45 (P<0.05), respectively (Figure 15).

The ability of conidia to germinate and form colonies decreased faster in unprotected conidia in the 2 formulations tested as the exposure to UV radiation increased in comparison to those protected with sunscreens. After 5 h of exposure to UV radiation, none of the conidia formulated in water were able to colonize, while 5% (±0.8SE) of the conidia protected with Everysun, and 13% (±2.2SE) of the conidia

protected with E45 germinated and formed colonies ($P<0.05$). The ability to form colonies after 5 h of exposure to UV radiation in conidia formulated in oil was 4% (±2.0SE) in unprotected conidia, 11% (±1.6 SE) in conidia protected with Everysun, and 21% (±3.7 SE) in conidia protected with E45 ($P<0.05$) (Figure 15).


☐ Control ☐ Everysun ☐ E45
Water formulation
Ability to form colonies (%)
110
100
90
80
70
60
50
40
30
20
10
0
Aa
Aa
Aa
Bb
Cc
Dd
Bb
Cc
Dcd
Ee
Bb
Cc
Ee
Eef
Bb
Ee
Ee
Fbf

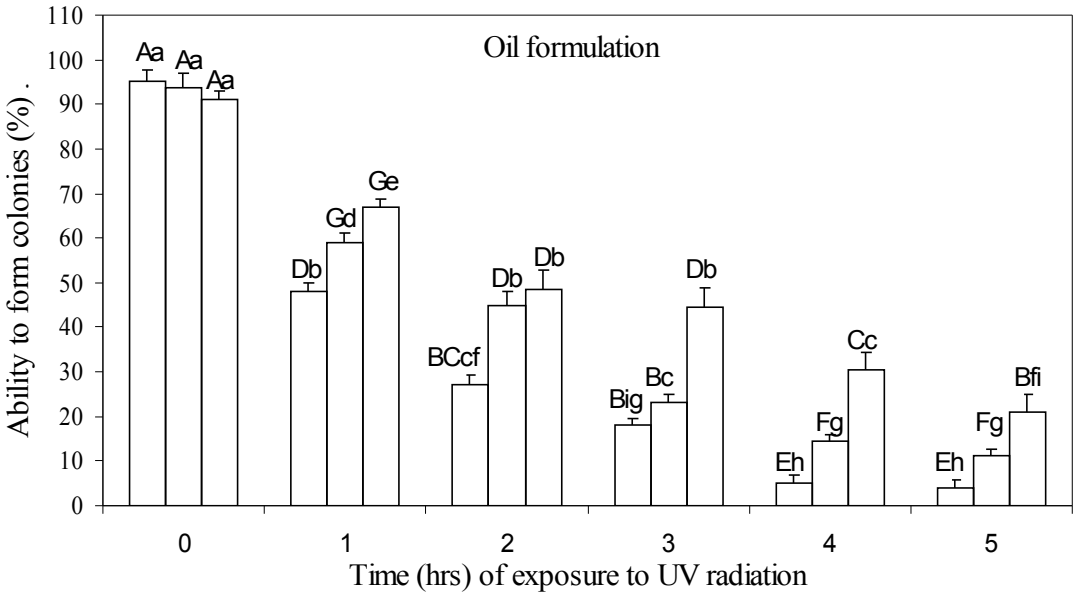


Figure 15: The ability of *M. anisopliae* conidia protected with E45 and Everysun to form colonies after exposure to UV radiation for periods of 0-5 hrs in water and oil formulations after **addition of 1% sunscreen**. Controls contained no sunscreens. Means (±SE) of nine replicates are presented. Means with the same lower case letter are not significantly different from each other among treatments in the same formulation, whereas means with the same upper case letter are not significantly different from each other among treatments in different formulations (Scheffé test, $P>0.05$).

4.3.2.2. *M. anisopliae* conidia protection from UV radiation using 3% sunscreen

Survival of conidia (ability to form colonies) was observed to vary when sunscreens were included in formulations, and when exposed to UV radiation for varying periods of time. The ability of conidia to form colonies after 1 h of UV irradiated was reduced by 79%, 36% and 14% ($P<0.01$) when formulated in water with no protectant, in water with Everysun or in water with E45 respectively. Moreover, the ability of conidia to form colonies after 1 h of irradiation when formulated in oil was

reduced by 47% in unprotected conidia, 3%, and 4% in conidia protected with Everysun and E45, respectively ($P<0.001$) (Figure 16).

The ability of conidia to germinate and form colonies decreased faster in unprotected conidia in the 2 formulations tested as the exposure to UV radiation increased in comparison to those protected with sunscreens. After 5 h of exposure to UV radiation, none of the conidia formulated in water were able to colonize, while 13% (±2.8 SE) of the conidia protected with Everysun, and 24% (±1.8 SE) of the conidia protected with E45 germinated and formed colonies ($P<0.01$). The ability to form colonies after 5 h of exposure to UV radiation in conidia formulated in oil was 4% (±2.0 SE) in unprotected conidia, 29% (±2.2 SE) in conidia protected with Everysun, and 40% (±2.4 SE) in conidia protected with E45 ($P<0.001$) (Figure 16).

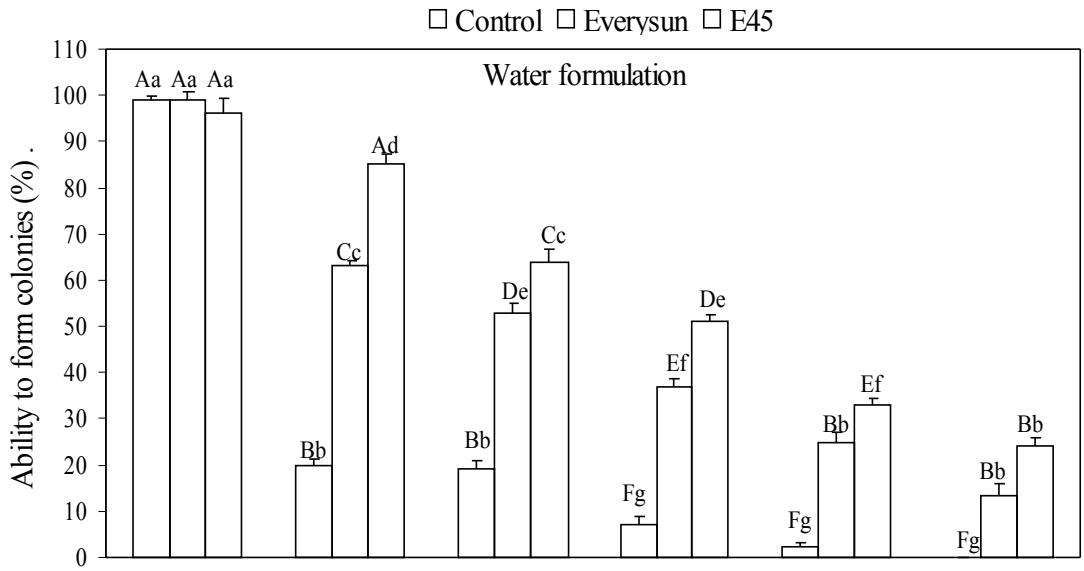

Control
Everysun
E45
Water formulation
Ability to form colonies (%) .
110
100
90
80
70
60
50
40
30
20
10
0
Aa Aa Aa
Bb
Cc
Ad
Bb
De
Cc
Fg
Ef
De
Fg
Bb
Ef
Fg
Bb
Bb

Figure 16: The ability of *M. anisopliae* conidia protected with E45 and Everysun to form colonies after exposure to UV radiation for periods of 0-5 hrs in water and oil formulations after **addition of 3% sunscreen**. Controls contained no sunscreens. Means (±SE) of nine replicates are presented. Means with the same lower case letter are not significantly different from each other among treatments in the same formulation, whereas means with the same upper case letter are not significantly different from each other among treatments in different formulations (Scheffé test, P>0.05).

4.4. Influence of protectants on the pathogenicity of conidia to various developmental stages of *R. e. evertsi*

4.4.1. *R. e. evertsi* eggs

Infection of eggs was found not to vary significantly between controls and treatments in the same formulation, but they varied between formulations. Unprotected conidia (control) formulated in water induced 77% (±0.5SE) infection to *R. e. evertsi* eggs as compared to 75% (±1.8SE), and 72% (±1.9SE) (P>0.05) infection caused by conidia formulated in water and protected with 3% Everysun and 3% E45, respectively. Conidia formulated in oil alone caused 87% (±0.6SE) infection, 86% (±2.3SE) and 84% (±2.3SE) infections was induced by conidia protected with 3% Everysun and 3% E45 formulated in oil, respectively (Figure 17).

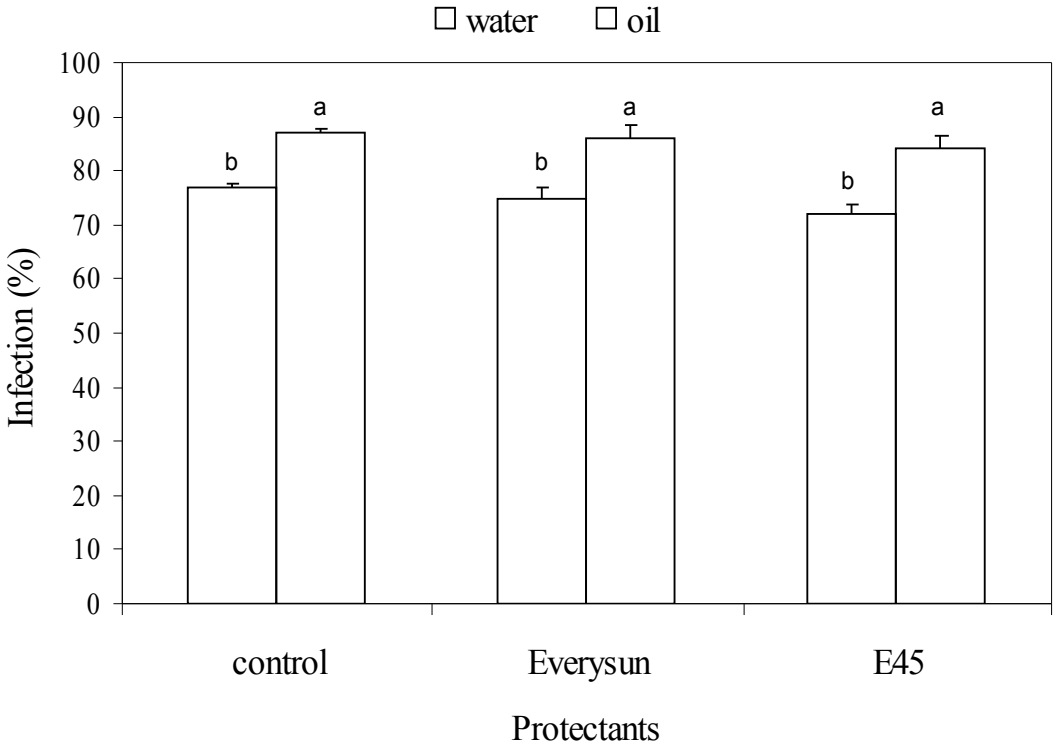


Figure 17: Ability of *M. anisopliae* conidia formulated in water and oil and protected with Everysun and E45 to infect eggs of *R. e. evertsi*. Controls contained no sunscreen. Means (±SE) of 3 replicates are presented. Means with the same letter are not significantly different from each other (Scheffé test, P>0.05).

4.4.2. *R. e. evertsi* unfed larvae

Larval mortality was found not to vary significantly in most of the treatments and controls tested. Unprotected conidia (control) formulated in water induced 95% (±5.0SE) mortality to *R. e. evertsi* larvae compared to 88% (±2.0SE), and 83% (±1.5SE) (P>0.05) mortality induced by conidia formulated in water and protected with 3% Everysun and 3% E45, respectively. Conidia formulated in oil alone caused 100% (±0.0SE) mortality, and 94% (±1.7SE) and 91% (±1.2SE) mortality was

induced by conidia protected with 3% Everysun and 3% E45 formulated in oil, respectively (Figure 18).

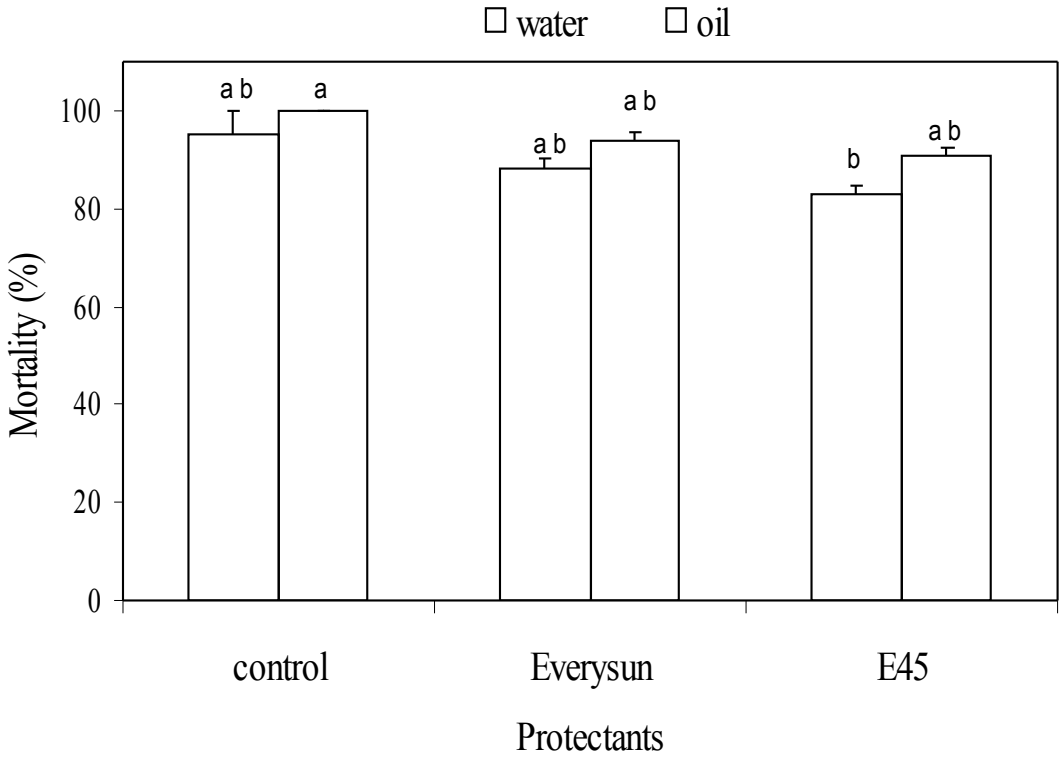


Figure 18: Ability of *M. anisopliae* conidia formulated in water and oil and protected with Everysun and E45 to induce mortality in *R. e. evertsi* unfed larvae. Controls contained no sunscreen. Means (±SE) of 3 replicates are presented. Means with the same letter are not significantly different from each other (Scheffé test, P>0.05).

4.4.3. *R. e. evertsi* engorged nymphs

Nymphal mortality was found not to vary significantly between controls and treatments in the same formulation, but they varied between formulations. Unprotected conidia (control) formulated in water induced 64% (±1.8SE) mortality to *R. e. evertsi* engorged nymphs compared to 60% (±1.5SE), and 58% (±2.1SE)

(P>0.05) mortality induced by conidia formulated in water and protected with 3% Everysun and 3% E45, respectively. Conidia formulated in oil alone caused 72% (±1.5SE) mortality, and 69% (±2.1SE) and 66% (±2.1SE) infection was caused by conidia protected with 3% Everysun and 3% E45 formulated in oil, respectively (Figure 19).

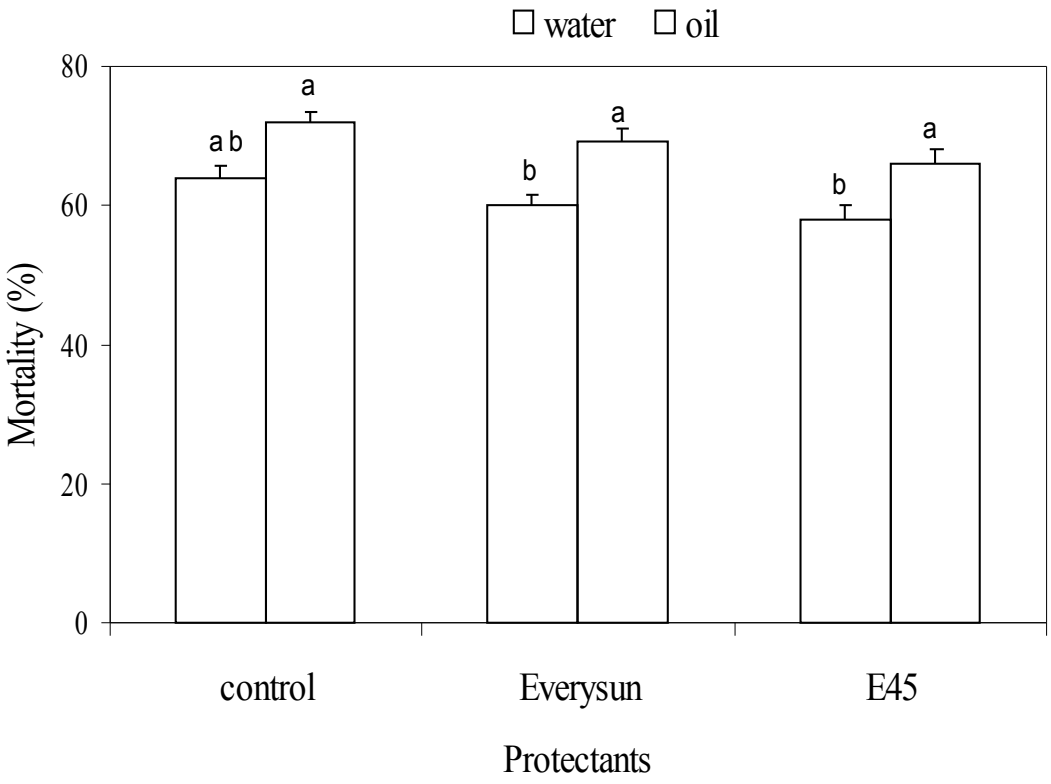


Figure 19: Ability of *M. anisopliae* conidia formulated in water and oil and protected with Everysun and E45 to induce mortality in *R. e. evertsi* engorged nymphs. Controls contained no sunscreen. Means (±SE) of 3 replicates are presented. Means with the same letter are not significantly different from each other (Scheffé test, P>0.05).

4.4.4. *R. e. evertsi* unfed adults

Adult mortality was found not to vary significantly between controls and treatments in the same formulation, but they varied between formulations. Unprotected conidia (control) formulated in water induced 83% (±1.2SE) mortality to *R. e. evertsi* unfed adults compared to 83% (±4.7SE), and 81% (±2.6SE) ($P>0.05$) mortality induced by conidia formulated in water and protected with 3% Everysun and 3% E45, respectively. Conidia formulated in oil alone caused 91% (±0.7SE) mortality, and 92% (±2.6SE) and 90% (±2.1SE) infection was caused by conidia protected with 3% Everysun and 3% E45 formulated in oil, respectively (Figure 20).

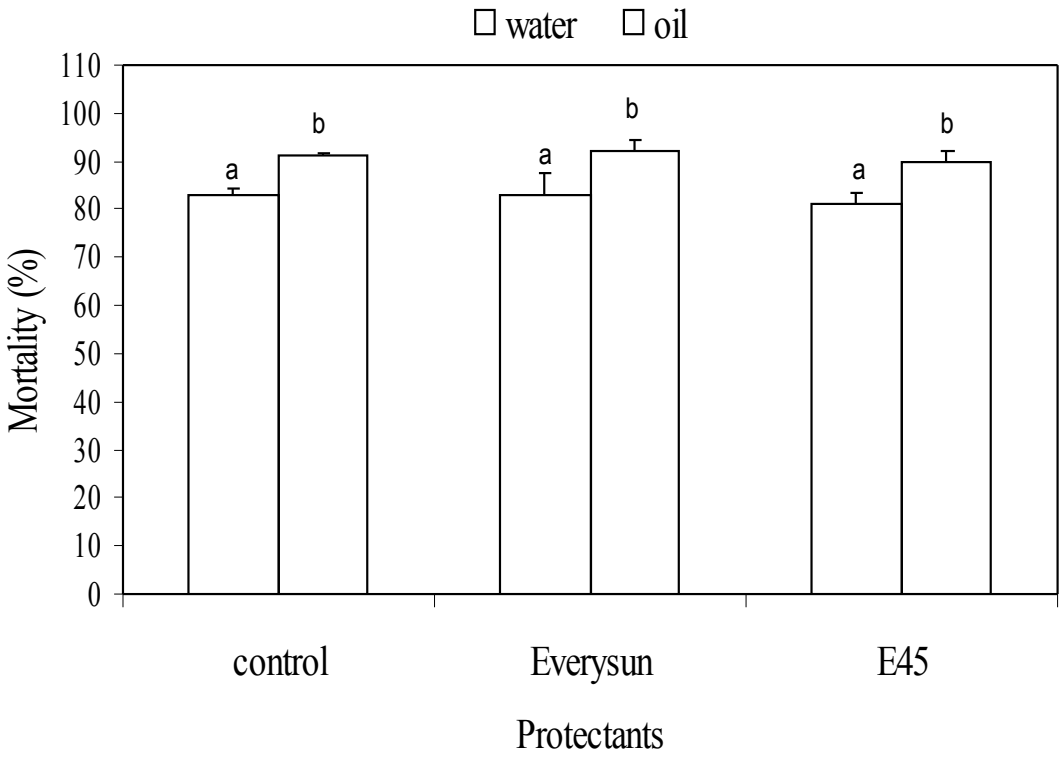


Figure 20: Ability of *M. anisopliae* conidia formulated in water and oil and protected

with Everysun and E45 to induce mortality in *R. e. evertsi* unfed adults. Controls contained no sunscreen. Means (±SE) of 3 replicates are presented. Means with the same letter are not significantly different from each other (Scheffé test, P>0.05).

CHAPTER 5: DISCUSSION

In this study, *M. anisopliae* was found to be highly pathogenic to various developmental stages of *A. hebraeum*, *A. variegatum, R. apendiculatus*, and *R. e. evertsi.* Similar to this study, Gindin *et al*. (2001) also observed variation in pathogenicity to different developmental stages of different ticks. High pathogenicity of *M. anisopliae* to various developmental stages of *R. appendiculatus* and *R. sanguineus* under laboratory and field conditions was reported preciously (Mwangi *et al.,* 1995; Kaaya *et al*., 1996; Samish *et al*., 2001). Results of this study demonstrated that *M. anisopliae* is highly pathogenic to ticks, and surface mycosis observed on treated ticks, but not on controls, indicated that fungi was the cause of death.

All developmental stages of *R. e. evertsi* were observed to be more susceptible to the entomopathogenic fungi than other ticks, both in water and oil formulations. The differences in techniques used (immersing the adults and nymphs in conidia suspension or placing eggs and larvae on filter paper soaked with conidia) may have also affected the observed differences. The ability of an isolate to kill several species of ticks at several developmental stages is particularly useful where more than one tick species may occur on livestock (Latif, 2003), as is the case in Southern Africa.

In this study, unfed stages appeared to be more susceptible than fed stages. The high susceptibility of unfed larvae and adults from several tick species to *M. anisopliae* was reported earlier (Kaaya and Hassan 2000, Samish *et al.,* 2001, Gindin *et al.,* 2003). Furthermore, in this study, a concentration of 10^8 conidia/ml of *M. anisopliae* caused mortalities of 95% in water and 100% in oil formulation to *R. e. evertsi* unfed larvae and similary; Monteiro *et al.* (1998) reported mortality of 95% in unfed larvae of *R. sanguineus* 20 days PI. Furthermore, Samish *et al.* (2001) reported that *R. sanguineus* unfed larvae and unfed nymphs were more susceptible to fungal infection than engorged ones.

In this study, the action of the fungi *M. anisopliae* on engorged females of *R. e. evertsi* was evaluated and a high mortality rate was observed, with 97 and 90% mortality in oil and water formulation respectively, using a suspension of 10^8 conidia/ml. *M. anisopliae* has been reported to have caused 100% mortality to engorged adult female *B. microplus* (Polar *et al.,* 2005). This observation suggests that blood in the engorged ticks do not inhibit fungal activity in ticks.

In this study, conidia formulated in oil were found to induce higher mortalities to different developmental stages of ticks than conidia suspended in water, and this seems to indicate that formulation to which conidia are suspended is critical to the performance of a myco-acaricide. In a similar study, conidia of *M. anisopliae* and of *B. bassiana* in oil formulation induced, under laboratory and field conditions, higher mortalities in *R. appendiculatus* and of *A. variegatum* ticks than conidia formulated in water (Kaaya and Hassan, 2000; Maranga *et al.,* 2005). Furthermore, Delgado *et*

al. (1997) noted no significant differences in efficacy between oil and clay/water formulations of *B. bassiana* conidia applied on grasshoppers in open field plots. Jenkins and Thomas (1996) also reported that aerial conidia of *M. anisopliae* var. *accridum* in oil were more effective against grasshoppers than submerged conidia in water suspension. Oil formulations have also been reported to induce higher mortalities than water formulations in desert locusts (Bateman *et al.,* 1993).

The reason for the better performance of oil formulation may be due to the fact that oil blends better with the tick cuticle since the cuticle is lipophilic and hydrophobic (Bateman *et al.,* 1993, Maranga *et al.,* 2005). The most commonly postulated mode of action is that oil droplets adhere more strongly to the lipophilic insect cuticle than droplets of water. Also, upon contacting the cuticle, oil spread rapidly and presumably carries conidia to areas of the body that are protected from unfavorable ambient environmental conditions. However, many of the reported differences between oil and aqueous formulations are not large, especially in terms of mortality induced by doses comparable to field rates (10^7-10^9 conidia ml^{-1}), and improved efficacy due to oil formulation has not been clearly demonstrated in the field (Butt *et al*., 1998).

Moore *et al.* (1997) stated that water is likely to be available to conidia from the cuticle in locust. However, the structure of the integument of the tick makes it highly impermeable, restricting water loss from the body (Evans, 1992; Polar *et al.,* 2005), thus, water for germination of conidia may not be as readily available and the use of oil formulation is prefered. On the cattle surface however, water for germination may

be readily available as the RH on the cattle skin is high (80-100%) (Polar *et al.,* 2005), and the use of oil may not be necessary. Furthermore, the high degree of sclerotization in the integument of ticks (Evans, 1992) may also make penetration and colonization relatively difficult.

Mortality of different developmental stages of *R. appendiculatus* and *R. e. evertsi* was positively related to *M. anisopliae* conidia concentration. A concentration of 10^3 conidia/ml had a low effect on tick mortality; whereas a concentration of 10^5 conidia/ml induced nearly 50% mortality in most of the developmental stages, and spore concentration of 10^8 conidia/ml caused optimum mortality in all stages of *R. appendiculatus* and *R. e. evertsi*. Furthermore, oil formulation was more superior in inducing tick mortality than water formulation in the concentrations tested. High susceptibility of engorged larval and adult *I. scapularis* to increasing fungal concentration has been reported ealier by Zhioua *et al*. (1997) and Benjamin *et al.* (2002).

The trend of increasing mortality or other biological effects, up to a point, with increasing concentrations of entomopathogenic fungi generally occurs in biological assays of tick and insects (Frazzon *et al*., 2000; Scholte *et al*., 2003). The range of concentrations used in bioassays is often insect specific. The concentration of inoculum used in this study was closely similar to concentrations of inoculums used in bioassays with mosquitoes (10^5-10^8 conidia/ml) (Scholte *et al*., 2003), and to bioassays of *Tenebrio molitor* Linnaeus larvae and adult *Schistocera gregaria* Forskal (10^2-10^5 conidia/ml) (Polar *et al.,* 2005).

In this study, a concentration of 1×10^8 conidia/ml in oil formulation induced mortalities of 73% in unfed udults of *R. appendiculatus,* 91% in unfed adults of *R. e. evertsi*, and 97% in fed adults of R. e. evertsi. In a a comparable study by Benjamin *et al.* (2002), a high spore concentration (4×10^9 spores/ml) was required to induce almost 100% mortality among unfed adult *I. scapularis*. In a another comparable laboratory study, a concentration of only 10^7 spores/ml induced 100% mortality among engorged adult *I. scapularis* (Zhioua *et al.,* 1997; Benjamin *et al.,* 2002). Similarly, the mortality rates of unfed and engorged adult *R. appendiculatus* Neumann with *M. anisopliae* at 10^6 spores/ml were 35% and 81%, respectively (Mwangi *et al.,* 1995). It therefore appears plausible that spore density must reach a certain threshold to induce death in a host insect (Zhioua *et al.,* 1997).

The high concentrations of conidia necessary for optimum pathogenicity in ticks brings into question whether entomopatogenic fungal isolates are, by nature, readily pathogenic to ticks or if the mortality observed is created by the artificial conditions of bioassays (Polar *et al*., 2005). In literature, there have been no reports of natural epizootics in tick populations. Ticks may be physiologically and/or structurally tolerant to entomopathogenic fungi and high concentrations are required to produce rapid mortality. It is therefore likely that mortality in ticks resulting from high fungal inoculum concentrations may be primarily due to the damage to the cuticle caused by simultaneous germination of a large number of conidia in the high humidity conditions of the bioassays (Polar *et al.,* 2005).

High mortality in ticks should be interpreted with caution. Placing ticks in the same container may have caused cross infection between infected and non-infected ticks. This may be true especially in cases of unfed stages which are highly mobile and their movement may have caused a transfer of inoculum from one tick to another. The level of cross infection would be different if ticks were placed in an open environment i.e. in the field. Therefore, infection may be more pronounced if an entomopathogenic fungus was applied to combat ticks on a heavily infested animal or environment, as this is likely to increase the level of contacts between infected and non-infected ticks, thereby increasing fungal infection and mortality. *M. anisopliae* has been shown to cause secondary infection in subsequently emerging stages and has been shown to prevent egg lying by engorged *R. sanguineus* females (Samish *et al*., 2001). This indicates that fungal infection may be transmitted from an infected to a non-infected host both among ticks at the same stage of development and also among ticks at different stages of development. These characteristics may increase the efficacy of fungi as anti-tick agents (Samish *et al*., 2001).

It is possible that the speed of fungal infection may depend on the ability of the pathogen to penetrate the host directly through the cuticle. In this study, *M. anisopliae* isolate was found to infect several species and stages of ticks, complete its full life cycle, and form conidiophores with conidia on surface of tick cadavers. This observation is very important, because the emergence of new generation conidia on a dead host ensures the persistence of an infectious inoculum in the ecological niche of tick populations, and hence the potential of transmission to other ticks (Gindin *et al.,*

2000). To further asses the bio-control potential of *M. anisopliae*, it will therefore be important to determine the level of cross infection among ticks.

The use of natural enemies, such as entomopathogenic fungi, is generally perceived to be ecologically preferable to chemical treatment for controlling pests (Benjamin *et al.,* 2002). Biological pesticides based on entomopathogenic fungi have several advantages in comparison to chemicals for the control of pests. Biological control agents are natural, more environmentally friendly, potentially less expensive and more effective than chemical pesticides, and problems with resistance are less likely to occur (Whipps and Lumsden, 2001; Polar *et al.,* 2005). Another advantage of fungi is their ability to grow and spread in the environment through infected ticks present in soil, and also its capacity to cause epizooties due to its wide spectrum (Bittencourt *et al*., 1999).

However, biological control carries its own risks like; inconsistent level of control and slow speed of kill are of concern (Whipps and Lumsden, 2001; Polar *et al.,* 2005), and the potential for damage to non-target organisms (Simberloff and Stiling, 1996). Non-target organisms may be less of concern when native organisms are used for biological control (Goettel and Hajeck, 2001). *M. anisopliae* occurs naturally in most of Southern Africa, but it has a broad host range, with insect species from seven orders known to be affected (Alves *et al*., 1998). Further study of the possible non target effects of *M. anisopliae* and means of reducing these effects is therefore needed if the ecological risks are to be weighed against the expected benefits of tick population control.

Only few field experiments to kill ticks with fungi have been performed as yet (Kaaya *et al.,* 1996; Correia *et al*., 1998; Kaaya and Hassan, 2000) and their value as cormmercial tick control agents are still to be proven (Gindin *et al*., 2000). With proper bioassay techiniques, it is possible to select highly virulent fungi isolates, either specific or not, and with desirable characteristics for use as insecticides (Bittencourt *et al*., 1999). Results of this study suggest that; due to its acaricidal activity, *M. anisopliae* could be used in bio-control programs to control populations of different tick species and/or different stages of ticks. Therefore, further investigation is required to identify broad spectrum isolates, which are pathogenic to all developmental stages of several tick genera yet not pathogenic to non-targets under field conditions (Polar *et al.,* 2005).

The viability of the conidia was not reduced by any of the formulations tested with or without the sunscreens. Pathogenicity of the entomopathogenic fungus, *M. anisopliae* to eggs and instars of *R. e. evertsi* was also not affected by addition of chemical sunscreens to the formulations ($P>0.05$). This implies that olive oil and chemical sunscreens (Everysun and E45) can probably be incorporated in fungal formulations without affecting the conidial ability to germinate on tick cuticle and induce mortality in the host.

In this study, it was observed that oil formulation (without sunscreens) improved survival rates of conidia following UV exposure for different durations as compared to water formulation. The ability of the conidia to form colonies was consistently

higher in oil than in water formulation at all durations of UV exposure. Improved protection by oil may be due to UV absorption properties of oil (Moore *et al.,* 1993). Since the hydrophobic conidia in aqueous suspensions have high moisture content and are metabolically active, they are more likely to suffer DNA damage than conidia in oil formulation (Moore *et al.,* 1996).

The addition of sunscreens significantly improved the ability of conidia to form colonies in each formulation after UV treatment compared to the control groups without sunscreen. This indicates that the efficacy of a formulation in protecting conidia from UV damage increases several fold with addition of a sunscreen. Moore *et al.* (1996) reported a significant difference in the ability of *Metarhizium flavoviride* Gams to germinate after addition of different types of chemical sunscreens. However, their investigations used different irradiation energy levels rather than exposure times.

In addition to causing conidia mortality, exposure to UV radiation inhibits the germination rate of *Metarhizium* spp (Moore *et al.,* 1993; Hunt *et al.,* 1994; Alves *et al.,* 1998). Furthermore, in addition to inducing inactivation, UV radiation has been demonstrated to delay the germination of surviving conidia (Moore *et al.,* 1993; Braga *et al.,* 2001). The delay in germination following exposure to simulated sunlight may be the result of damage to proteins and nucleic acids leading to slower growth rates, a defence response of the conidia, or the result of time and energy being devoted to repair mechanisms (Moore *et al.,* 1993). The reduction in inoculum due to conidial inactivation and the delay in germination are expected to reduce the efficiency of these organisms as bio-insecticides in situations with strong solar

irradiation. Among these situations are those in which the strains are exposed during inoculation and/or germination to irradiance and doses above those occurring in their original habitats and to which they are not fully adapted (Moore *et al.,* 1993).

It is possible that some conidia would be capable of germination after incubation times longer than those that were assessed during this study. The difference in germination rates among different growth periods may indicate that conidia can repair the damage that occurred when they were exposed for short periods, such as 5 h of UV exposure. The extra time taken to germinate may allow repair in conidia to take place. Molecular repair processes acts on nucleic acids, especially DNA, and hence allow survival (Alves *et al.,* 1998). It may also mean that a rapid defense mechanism develops that results in delayed germination and ensure conidia survival as explained by Moore *et al.* (1996).

When fungal suspension is applied to animals that are confined in a well shaded area, most droplets will impact on the animal/insect and exposure to UV radiation may be minimal. The effect of radiation would be insignificant and the addition of chemical sunscreen may be unnecessary (Moore *et al.,* 1993). However, if animal resting or sleeping sites were treated, much of the efficacy of the mycopesticide would depend on secondary uptake of spore by the animal from vegetation. In that case, the conidia could be exposed to solar radiation for hours and addition of chemical sunscreens (Moore *et al.,* 1993), such as E45 and Everysun might be valuable.

Metarhizium aerial conidia are hydrophobic, but when the conidia are mixed with adjuvant oils prior to addition of water, there will be a high affinity between both of them (Alves *et al.,* 1998). Most conidia can be encapsulated by emulsifiable oil and will be better protected against the deleterious effects of sunlight. The use of adjuvant oils involves vegetable oil encapsulation technology so that the active ingredient is enclosed in a protective shell of oil which efficiently sticks and spreads the pesticide on the target (Alves *et al*., 1998).

In field situation, water droplets do not persist for long periods (Moore *et al.,* 1996). Water droplets would evaporate quickly and oil droplets are rapidly absorbed onto plant cuticles (Inglis *et al.,* 1995). In addition, conidia in aqueous suspensions will have high moisture content and be metabolically active. These are more likely to be vulnerable to DNA damage by UV radiation than dried conidia in oil formulation (Moore *et al.,* 1996). Moore *et al*. (1993) demonstrated a benefit of oil formulations to protect conidia in petri dishes against simulated sunlight. However, this effect was not noted in the field and it was suggested that decreased protection on leaves was attributed to absorption of oil by the leaf tissue (Inglis *et al.,* 1995).

Chemical sunscreens may provide some protection, as may using older conidia (Moore *et al.,* 1993) but both options have disadvantages. Hunt *et al.* (1994) found little beneficial effects with chemical sunscreens. It is likely that the major benefits of sunscreens would be seen with shaded conidia exposed to reflected UV light. Moore *et al.* (1996) found that exposure of conidia to sunlight demonstrated a clear interaction with temperature. Spores in direct sunlight would probably be rapidly

deactivated, even with sunscreens, and even a doubling of exposure time have shown that most conidia were deactivated within one day of sunlight exposure (Moore *et al.*, 1996). Conidia survive longer than this in the field (Lomer *et al.,* 1993), because many are shielded from sunlight and sunscreens may extend the survival of spores, especially those in direct sunlight. Furthermore, isolates vary in their susceptibility to UV light and selection or genetic engineering may result in increased UV resistance for a mycopesticide (Ignoffo and Garcia, 1994), and genetic selection may be a valuable line of research (Moore *et al.,* 1996).

The two commercial sunscreens tested (Everysun and E45) differ tremendously not only in their level of sun protection factor (SPF) but also in their chemical composition. Nevertheless in all tests, besides to some extend, there was no difference in the protection level of these two sunscreens against UV radiation. This may suggest the relative low importance of the level of SPF in the sunscreens, and/or relative low importance of the wide range of chemical composition in the sunscreens, in the tested concentrations. Results obtained in this study indicate that chemical sunscreens can improve UV radiation tolerances of conidia during short periods of exposure i.e. up to 5 h of exposure. Oil provides some protection against UV light, and results may differ from situations in which water formulation is used.

This study has also shown that the sunscreens, Everysun or E45 do not affect the ability of the conidia to germinate nor their pathogenicity to various developmental stages of *R. e. evertsi*. Similarly Shah *et al.* (1998), under field conditions, obtained 98% and 96% mortality of *Kraussella amabile* after spraying conidia of *M.*

flavoviride in formulations with and without the sunscreen oxybenzone (2%), demonstrating that protecting conidia with sunscreen does not affect pathogenicity. Their results, and of this study, suggest that the sunscreens can be incorporated into conidia formulations, to improve their survival in the field, without reducing or interfering with their pathogenicity to target arthropods.

CHAPTER 6: CONCLUSIONS

Infection in eggs and mortalities in instars of *R. e. evertsi* increased with increasing spore concentrations in water, and oil formulation. Furthermore, various stages of *A. hebraeum, A. variegatum, R. appendiculatus* and *R. e. evertsi* were observed to be susceptible to infection with the entomopathogenic fungus *M. anisopliae* in both water and oil formulations. Conidia in oil formulation induced higher mortalities in various developmental stages of different tick species than water formulation. This study has therefore indicated that *M. anisopliae* has a potential as a myco pesticide to control different developmental stages and species of tick populations. Further studies that include the selection and testing of many fungal isolates in the laboratory and under field conditions can provide essential information on the efficacy of various fungal species as potential biological control agents.

Results of this study have also shown that olive oil and sunscreens may extend the survival of conidia in UV regions, and they do not reduce the ability of *M. anisopliae* conidia to germinate, or infect ticks and induce mortalities in different stages of *R. e. evertsi*. However, the overall efficacy of UV protectants under field conditions will depend on several additional factors such as, target, season, geographical location,

and time of application. Furthermore, fungial isolates may vary in their susceptibility to UV radiation and selection or genetic engineering may result in increased UV resistance of a mycopesticide.

CHAPTER 7: REFERENCES

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APPENDIX TABLES

1. Efficacy testing of different fungal concentrations in water and oil formulation to various developmental stages of ticks

1.1. Efficacy of larvae of *R. appendiculatus* to different fungal concentrations

Table 1. Infections (%) of *R. appendiculatus* eggs with different concentrations of *M. anisopliae* conidia in waterand oil formulations. Means of 3 replicates are presented.

	Water formulation			**Oil formulation**		
Concentration (conidia/ml)	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**
Control	0	0	0	0	0	0
$1x10^3$	13	0.4	1.3	24	1.5	4.4
$1x10^4$	30	0.8	2.3	40	0.8	2.5
$1x10^5$	42	1.8	5.3	58	1.5	4.6
$1x10^6$	54	1.2	3.5	68	0.7	2.2
$1x10^7$	69	0.8	2.5	84	1.5	4.5
$1x10^8$	87	0.6	1.8	96	2.1	6.2

1.2. Efficacy of various stages of *R. e. evertsi* to varying fungal concentrations

1.2.1. *R. e. evertsi* eggs

Table 2. Infections (%) of *R. e. evertsi* eggs with different concentrations of *M. anisopliae* conidia formulated in water and water. Means of 3 replicates are presented.

	Water formulation			**Oil formulation**		
Concentration (conidia/ml)	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**
Control	0	0	0	0	0	0
$1x10^3$	8	0.3	1.0	17	0.3	0.8
$1x10^4$	25	0.5	1.5	35	0.4	1.2
$1x10^5$	38	0.7	2.3	52	0.5	1.7
$1x10^6$	55	0.6	1.8	65	0.4	1.4
$1x10^7$	64	0.6	2.0	77	0.7	2.2
$1x10^8$	77	0.5	1.7	87	0.6	1.9

1.2.2. *R. e. evertsi* larvae

Table 3. Mortalities (%) of *R. e. evertsi* unfed larvae due to different concentrations of *M. anisopliae* conidia formulated in oil and water. Means of 3 replicates are presented.

	Water formulation			**Oil formulation**		
Concentration (conidia/ml)	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**
Control	0	0	0	0	0	0
$1x10^3$	21	1.5	2.6	39	2.1	3.6
$1x10^4$	42	0.7	1.2	52	0.9	1.5
$1x10^5$	54	1.2	2.1	67	0.9	1.5
$1x10^6$	65	1.5	2.5	76	1.0	1.7
$1x10^7$	74	2.9	4.9	90	1.5	2.5
$1x10^8$	95	5.0	9.5	100	0.0	0

1.2.3. *R. e. evertsi* engorged nymphs

Table 4. Mortalities (%) of *R. e. evertsi* fed nymphs due to different concentrations of *M. anisopliae* conidia formulated in oil and water. Means of 3 replicates are presented.

	Water formulation			**Oil formulation**		
Concentration (conidia/ml)	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**
Control	0	0	0	0	0	0
$1x10^3$	5	1.2	2.1	12	1.8	3.1
$1x10^4$	20	0.9	1.5	28	2.3	4.0
$1x10^5$	25	0.9	1.5	41	1.2	2.1
$1x10^6$	43	0.9	1.5	55	2.5	4.4
$1x10^7$	57	1.2	2.1	62	2.2	3.8
$1x10^8$	64	1.8	3.1	72	1.5	2.6

1.2.4. *R. e. evertsi* adults

Table 5. Mortalities (%) of *R. e. evertsi* unfed adults due to different concentrations of *M. anisopliae* conidia formulated in oil and water. Means of 3 replicates are presented.

	Water formulation			**Oil formulation**		
Concentration (conidia/ml)	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**
Control	0	0	0	0	0	0
$1x10^3$	11	0.9	1.5	19	0.7	1.2
$1x10^4$	27	0.9	1.5	39	1.2	2.1
$1x10^5$	44	0.7	1.2	58	1.0	1.7
$1x10^6$	62	0.3	0.6	70	0.3	0.6
$1x10^7$	72	1.0	1.7	84	1.2	2.1
$1x10^8$	83	1.2	2.1	91	0.7	1.2

1.2.5. *R. e. evertsi* engorged adults

Table 6. Mortalities (%) of *R. e. evertsi* fed adults due to different concentrations of *M. anisopliae* conidia formulated in oil and water. Means of 3 replicates are presented.

	Water formulation			**Oil formulation**		
Concentration (conidia/ml)	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**
Control	0	0	0	0	0	0
$1x10^3$	11	2.1	3.6	23	2.3	4.0
$1x10^4$	36	1.8	3.1	44	2.1	3.6
$1x10^5$	47	1.5	2.5	61	2.6	4.5
$1x10^6$	70	1.8	3.1	79	2.1	3.6
$1x10^7$	84	1.7	3.0	93	2.1	3.6
$1x10^8$	90	2.3	4.0	97	0.9	1.5

2. Testing fungal efficacy in water and oil formulation to various developmental stages of various tick species

2.1. Susceptibility of eggs of various ticks to *M. anisopliae* conidia

Table 7. Infection (%) induced by *M. anisopliae* conidia in eggs of *A. hebraeum A. variegatum, R. appendiculatus* and *R. e. evertsi.* Means of 3 replicates are presented.

Tick species	Formulation	Mean	S Error	S Dev
A. hebreum	Water	35	1.0	1.7
	Oil	53	0.6	1.0
A. variegatum	Water	30	1.0	1.7
	Oil	60	1.5	2.6
R. appendiculatus	Water	36	0.6	1.0
	Oil	65	1.2	2.0
R. e. evertsi	Water	77	0.5	1.7
	Oil	87	0.6	1.9

2.2. Susceptibility of larvae of various ticks to *M. anisopliae* conidia

Table 8. Mortality (%) induced by *M. anisopliae* in larvae of *R. appendiculatus* and *R. e. evertsi.* Means of 3 replicates are presented.

Tick species	Formulation	Mean	S Error	S Dev
R. appendiculatus	Water	87	0.6	1.8
	Oil	96	2.1	6.2
R. e. evertsi	Water	95	5.0	9.5
	Oil	100	0.0	0.0

2.3. Susceptibility of nymphs of various ticks to *M. anisopliae* conidia

Table 9. Mortality (%) induced by *M. anisopliae* in unfed nymphs of *A. variegatum* and *R. appendiculatus.* Means of 3 replicates are presented.

Tick species	Formulation	Mean	S Error	S Dev
A. variegatum	Water	32	1.0	2.5
	Oil	98	0.5	1.2
R. appendiculatus	Water	19	0.8	2.0
	Oil	71	4.2	5.2

2.4. Susceptibility of adults to *M. anisopliae* conidia

Table 10. Mortality (%) induced by *M. anisopliae* in unfed adults of *R. appendiculatus* and *R. e. evertsi.* Means of 3 replicates are presented.

Tick species	Formulation	Mean	S Error	S Dev
R. appendiculatus	Water	16	0.8	3.2
	Oil	73	2.0	7.6
R. e. evertsi	Water	83	1.2	2.1
	Oil	91	0.7	1.2

3. Protection of fungal spores from UV radiation damage

3.1. Effects of UV protectants and formulations on *M. anisopliae* conidia ability to germinate

Table 11A. Viability (%) of *M. anisopliae* conidia after addition of 1% chemical sunscreens in water and oil formulations. Means of 9 replicates are presented.

	Control			Everysun			E45		
Formulation	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**
Water	99	0.7	2.2	97	2.4	7.1	93	1.7	5.0
Oil	95	2.8	8.3	94	3.0	8.9	91	2.0	6.0

Table 11B. Viability (%) of *M. anisopliae* conidia after addition of 3% chemical sunscreens in water and oil formulations. Means of 9 replicates are presented.

	Control			Everysun			E45		
Formulation	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**
Water	99	0.7	2.2	99	1.6	4.9	96	3.3	9.9
Oil	95	2.8	8.3	90	3.2	9.7	87	2.1	6.2

3.2. Effects of UV protectants and formulations on the ability of *M. anisopliae* conidia to form colonies after exposure to UV radiation

3.2.1. *M. anisopliae* conidia protection from UV radiation using 1% sunscreen

Table 12A. The ability of *M. anisopliae* conidia protected with E45 and Everysun to form colonies after exposure to UV radiation for periods of 0-5 hrs in water formulation after addition of 1% sunscreen. Means of 9 replicates are presented.

	Control			**Everysun**			**E45**		
UV exposure (hrs)	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**
0	99	0.7	2.2	97	2.4	7.1	93	1.7	5.0
1	20	1.2	3.5	34	1.8	5.5	44	2.2	6.5
2	19	1.8	5.3	34	2.3	6.8	37	2.5	7.5
3	7	2.0	6.1	20	2.0	6.1	29	4.0	11.9
4	2	1.2	3.6	9	1.1	3.3	19	3.1	9.2
5	0	0.0	0.0	5	0.8	2.5	13	2.2	6.6

Table 12B. The ability of *M. anisopliae* conidia protected with E45 and Everysun to form colonies after exposure to UV radiation for periods of 0-5 hrs in oil formulation after addition of 1% sunscreen. Means of 9 replicates are presented.

	Control			**Everysun**			**E45**		
UV exposure (hrs)	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**
0	95	2.8	8.3	94	3.0	8.9	91	2.0	6.0
1	48	1.7	5.1	59	2.2	6.6	67	1.9	5.8
2	27	2.1	6.2	45	2.6	7.9	48	4.4	13.2
3	18	1.7	5.0	23	1.7	5.0	44	4.3	12.9
4	5	1.9	5.6	14	1.5	4.6	31	3.9	11.8
5	4	2.0	6.0	11	1.6	4.9	21	3.7	11.1

3.2.2. *M. anisopliae* conidia protection from UV radiation using 3% sunscreen

Table 13A. The ability of *M. anisopliae* conidia protected with E45 and Everysun to form colonies after exposure to UV radiation for periods of 0-5 hrs in water formulation after addition of 3% sunscreen. Means of 9 replicates are presented.

	Control			**Everysun**			**E45**		
UV exposure (hrs)	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**
0	99	0.7	2.2	99	1.6	4.9	96	3.3	9.9
1	20	1.2	3.5	63	1.2	3.6	85	2.2	6.6
2	19	1.8	5.3	53	1.9	5.7	64	2.6	7.8
3	7	2.0	6.1	37	1.7	5.0	51	1.6	4.9
4	2	1.2	3.6	25	2.0	6.1	33	1.4	4.3
5	0	0.0	0.0	13	2.8	8.3	24	1.8	5.3

Table 13B. The ability of *M. anisopliae* conidia protected with E45 and Everysun to form colonies after exposure to UV radiation for periods of 0-5 hrs in oil formulation after addition of 3% sunscreen. Means of 9 replicates are presented.

	Control			**Everysun**			**E45**		
UV exposure (hrs)	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**	**Mean**	**S Error**	**S Dev**
0	95	2.8	8.3	92	3.2	9.7	87	2.1	6.2
1	48	1.7	5.1	89	3.3	9.8	83	2.6	7.9
2	27	2.1	6.2	70	6.3	19.0	76	1.8	5.3
3	18	1.7	5.0	55	3.0	9.0	60	1.9	5.6
4	5	1.9	5.6	39	3.8	11.4	54	1.3	3.9
5	4	2.0	6.0	29	2.2	6.5	40	2.4	7.1

3.3. Influence of protectants on the virulence of conidia to various developmental stages of *R. e. evertsi*

3.3.1. *R. e. evertsi* eggs

Table 14. Ability of *M. anisopliae* conidia, protected with 3% Everysun and E45 to infect eggs of *R. e. evertsi*. Means of 3 replicates are presented.

Protectants	Formulation	Mean (%)	S Error	S Dev
Control	Water	77	0.5	1.7
	Oil	87	0.6	1.9
Everysun	Water	75	1.8	3.1
	Oil	86	2.3	4.0
E45	Water	72	1.9	3.2
	Oil	84	2.3	4.0

3.3.2. *R. e. evertsi* larvae

Table 15. Ability of *M. anisopliae* conidia, protected with 3% Everysun and E45 to induce mortality in *R. e. evertsi* larvae. Means of 3 replicates are presented.

Protectants	Formulation	Mean	S Error	S Dev
Control	Water	95	5.0	9.5
	Oil	100	0.0	0.0
Everysun	Water	88	2.0	3.5
	Oil	94	1.7	3.0
E45	Water	83	1.5	2.6
	Oil	91	1.2	2.0

3.3.3. *R. e. evertsi* engorged nymphs

Table 16. Ability of *M. anisopliae* conidia, protected with 3% Everysun and E45 to induce mortality in *R. e. evertsi* fed nymphs. Means of 3 replicates are presented.

Protectants	Formulation	Mean	S Error	S Dev
Control	Water	64	1.8	3.1
	Oil	72	1.5	2.6

Everysun	Water	60	1.5	2.6
	Oil	69	2.1	3.6
E45	Water	58	2.1	3.6
	Oil	66	2.1	3.6

3.3.4. *R. e. evertsi* unfed adults

Table 19. Ability of *M. anisopliae* conidia, protected with 3% Everysun and E45 to induce mortality in *R. e. evertsi* unfed adults. Means of 3 replicates are presented.

Protectants	**Formulation**	**Mean**	**S Error**	**S Dev**
Control	Water	83	1.2	2.1
	Oil	91	0.7	1.2
Everysun	Water	83	4.7	4.6
	Oil	92	2.6	8.1
E45	Water	81	2.6	3.6
	Oil	90	2.1	4.6