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Austin Goldsmith

*University of Arkansas, Fayetteville*

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The Discovery of *Metarhizium anisopliae* (Metchnikoff) Sorokin Isolates from Arkansas and  
their Pathogenicity to *Amblyomma americanum* L.

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science in Entomology

by

Austin Goldsmith  
University of Central Arkansas  
Bachelor of Science in Biology, 2017

May 2020  
University of Arkansas

This thesis is approved for recommendation to the Graduate Council

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## Abstract

The lone star tick *Amblyomma americanum* L. is the most abundant tick in Arkansas and has been implicated as a vector of many important disease-causing pathogens. Many species of entomopathogenic fungi have been isolated from several species of ticks, with some of these fungi being utilized for tick biocontrol. However, few studies have assessed the pathogenicity of entomopathogenic fungi to *A. americanum*. The objectives of this study were to: isolate and identify native Arkansas isolates of entomopathogenic fungi from wild *A. americanum* ticks exposed to soil and to compare the pathogenicity of one isolate (*Metarhizium anisopliae* (Metchnikoff) Sorokin isolate Savoy P10N1) to that of the mycoinsecticide Met52® (*M. brunneum* Petch strain F52) on adult, nymphal and larval *A. americanum*. Ticks exposed to soil from Savoy, Arkansas were infected with *M. anisopliae* with infection percentages of 3.3% (N = 60), 1.6% (N = 60) and 1.5% (N = 200) for adult females, adult males and nymphal ticks respectively. Only nymphal ticks exposed to soil from West Fork, Arkansas (2.4% of nymphs; N = 210) were infected with *M. anisopliae*. Eight isolates of *M. anisopliae* were cultured from infected *A. americanum* ticks. Two of these isolates (Savoy P10N1 and West Fork P9N2) were confirmed as *M. anisopliae* by genetic sequencing and a GenBank Blast analysis. Adults and nymphs were more susceptible to Met52® than to Savoy P10N1. Adults treated with Met52® died at twice the rate as adults treated with Savoy P10N1. Nymphs treated with Met52® died at the same rate as nymphs treated with Savoy P10N1 after  $88.4 \pm 6.8\%$  of nymphs died at two days post-inoculation (days PI). Adult mortality for Met52® was  $25.3 \pm 8.3\%$  and 100% at 14 and 77 days PI respectively compared to  $<1\%$  and  $25.4 \pm 8.7\%$  for Savoy P10N1. Nymphal mortality for Met52® at 14 and 77 days PI was  $89.8 \pm 6.0\%$  and 100% while mortality for Savoy P10N1 was  $5.3 \pm 3.3\%$  and  $33.1 \pm 14.4\%$ . Assessments on larval mortalities for all treatments were

inconclusive due to unknown variables. Findings from this study will give a better understanding of the entomopathogenic fungi to be evaluated for the control of *A. americanum* in future studies.

## **Acknowledgements**

I am first grateful to God through his son Jesus for the grace and faithfulness he has shown throughout the entire learning experience of this master's program. Despite all the challenges and hardships I have endured over the last two years, the Lord has used this program to allow me to mature and grow in ways I could not have imagined. I'd like to thank my advisor, Kelly Loftin for his constant input of wisdom and assistance throughout the entirety of the project, for introducing me to the realm of medical/veterinary entomology and for the countless conversations we had together (Your office was never too far away!). Also, I'd like to thank Don Steinkraus for having the crazy idea of doing this project in the first place, for introducing me to the fields of biocontrol and insect pathology and being a tremendous help during the writing of this thesis. Furthermore, I want to thank Allen Szalanski for helping me with the molecular diagnostics aspect of this project as well as his for his helpful suggestions on the writing. I'd like to thank the University of Arkansas Division of Agriculture and the Cooperative Extension Office for the funding of this project.

I'd like to thank numerous colleagues that have been a tremendous help throughout the last two years of my masters. To fellow grad students: Dylan Clearly, for showing me how to do the DNA work on the fungal pathogens; to Hillary Fisher for her constant encouragement and help; to Rosalee Knipp for providing me necessary resources and assistance that helped my research and writing; and Austin Jones for always being available to talk and laugh alongside with. To my lab members, Elizabeth Smith and fellow grad student Haylee Campbell for their assistance and input in several different aspects of the project. To Dr. David Dussourd and Dr. Sally Entrekin for introducing me to entomology and paving the path for me pursue the field in the first place.

I'd also like to thank several people in the Crawley-Warren Plant Health Clinic. To Dev Crippin for helping me find necessary supplies when I needed them and his helpful suggestions. To Keiddy Romero for letting me to use her fume hood for pouring agar plates and for allowing me to use her incubators for storing my cultures of entomopathogenic fungi. To Sherrie Smith for allowing me to use her phase contrast microscope to get pictures of my *Metarhizium anisopliae* conidia for my thesis. To Virgil Piazza for his help in constructing the dry ice traps used for the tick collections and the components for the humidity chambers used to store ticks and cultures of entomopathogenic fungi used for my tick bioassays. A huge thanks to the statisticians in the agricultural statistics department, Dr. Gbur and Kevin Thompson, for providing the necessary help for the statistical analysis of my project where it was needed.

I would also like to mention other members of the scientific community for playing an integral role in providing necessary resources and services that allowed me to conduct the research and writing for my thesis. To Lisa Coburn and the Oklahoma State Tick Rearing Facility in Stillwater, Oklahoma for providing the ticks used in the bioassay trials. To Dr. Louella Castrillo for receiving my *Metarhizium* cultures and depositing them into USDA, Agricultural Research Service Collection of Entomopathogenic Fungi (ARSEF) in Ithaca, New York. Also, to Dr. Stephan Jaronski for providing correspondence and additional resources regarding the Met52® label.

I am also thankful for my family. For my parents Max and Susan for always supporting me in whatever I wanted to set my mind to, for challenging me to get my thesis completed as soon as I could, and for always opening up their home to me (I am extra grateful they live less than 30 minutes away from the University of Arkansas!). In addition, I owe them greatly for letting them use one of their Dell PCs to make my final edits to this this thesis when my

MacBook got stolen (Thanks mom!)—especially with the onset of the COVID-19 pandemic! Thanks to my brother Tyler who has gone before me in completing his masters and for his suggestions that have helped me write this thesis.

I like to extend a huge thanks to various friends from Chi Alpha Campus Ministries for supporting me in me in my walk with God and taking in interest in what I do. To Jossie Santamaria for being a big help in getting my time management in order. I'd also like to thank several other friends in the U.S. and overseas for believing in me and encouraging me to continue in my calling. To the current and former members of my discipleship group: Clarke Vennerbeck, Cannon Hackett, Garret Achre, Garret Crump, Jeremy Thomas, Isaac Conorro, Ibsa Jalata, and Josh Williams for all the wild and crazy adventures, moments of laughter, deep conversations we've had together, and being there for me in much of the ups and downs of life. To Louis Csak for imparting a lot of his spiritual knowledge and experience to me, for the countless phone conversations and random hangout times we had together.

Last (and most certainly not least), I like to thank my roommate Tony Cosgrove. I truly can't think of anyone else who has believed in me more, encouraged me more, challenged me more, and who has sacrificed more of his time and effort to lead me in a closer walk with God and his people. I appreciate the countless conversations we've had together, the various crazy adventures we've undertaken, the many meals we've concocted together (especially the sous vide steak), for showing me how to roast coffee, the countless ideas you've shared with me and several other matters that would take a whole book to describe.

## **Dedication**

To my parents Susan and Max and my brother Tyler for their encouragement and motivation;  
also, to my roommate Tony Cosgrove for showing me the bigger picture beyond myself.



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## CHAPTER 1: Introduction

Ticks (Acari: Ixodida) and tick-borne pathogens are a growing concern in the United States and around the world due to their medical, veterinary, economic and cultural impacts. Arkansas in particular has seen rapid urban development which has resulted in fragmented tick habitats, which have put humans and their pets at a greater risk for tick bites and tick-borne illness. The lone star tick *Amblyomma americanum* L. is the most abundant tick in Arkansas and has risen in importance as a vector of several disease-causing pathogens. Although chemical acaricides have shown to be largely effective for controlling ticks and mitigating the transmission tick-borne pathogens, rising concerns of environmental toxicity and the resistance of ticks to acaricides have prompted the search for alternative control options. Entomopathogenic fungi such as *Metarhizium anisopliae* sensu lato (s.l.) (Metchnikoff) Sorokin and *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin have been found to infect ticks in nature and have shown to be effective agents for their control.

The topic of this thesis is about the isolation of the tick biological control agent *M. anisopliae* from wild *Amblyomma americanum* ticks in Arkansas and its assessment as a potential biological control agents of *A. americanum* compared to the tick bioinsecticide Met52® (*M. brunneum* Petch strain F52). Each article in this thesis was compiled into a complete work since each chapter contributes to the underlying goal of finding effective biological control agents for *A. americanum*. In addition, the findings of the second research article (Chapter 4) are dependent on the findings of the first research article (Chapter 3) in this thesis. The literature review (Chapter 2) summarizes relevant literature and background information pertinent to this study and introduces the underlying goals of this thesis. Chapter 3 talks about the isolation of native Arkansas strains of *M. anisopliae* from *A. americanum*, the identification of these fungi,

the implications of these results and suggestions of improvements for future studies. In Chapter 4, the pathogenicity of one of the Arkansas isolates of *M. anisopliae* to different life stages of *A. americanum* (e.g. adults, nymphs, and larvae) was compared to that of the bioinsecticide Met52® with the future implications these results and suggested improvements for future studies being discussed. Chapter 5 summarizes the overall findings of chapters 3 and 4, gives the conclusions for the entire thesis, and provides suggestions for future research.

## **CHAPTER 2: Literature Review**

### **Introduction**

Ticks are well known for their role in vectoring pathogens to animals and humans (Dantes-Torres et al. 2012; Sonenshine and Roe, 2013; Nicholson et al. 2018; Paules et al. 2018). Collectively, ticks harbor and transmit more pathogens than any other group of blood-feeding arthropods (Dantes-Torres 2012; Sonenshine and Roe 2013; Brites-Neto 2015; Nicholson et al. 2018). They are one of the most important vectors of disease-causing pathogens to domestic animals worldwide and are second only to mosquitoes in terms of their impact on public health. Aside from the role they play in pathogen transmission, saliva from tick bites is also known to induce toxicosis, allergic reactions and even paralyses which can range from mild to life threatening in their hosts (Sonenshine and Roe, 2013; Nicholson et al. 2018). Serious infestations of ticks to domesticated animals can lead to infection of bite wounds and can even lead to death by severe blood loss (Sonenshine and Roe, 2013; Nicholson et al. 2018). High tick populations on livestock can reduce weight gain, milk production and even lead to the abortion of newborn livestock (Sonenshine and Roe 2013; Nicholson et al. 2018). Economically, losses from tick-borne diseases, severe infestations, and costs due to tick prevention and control is estimated to be in the billions of U.S. dollars (Sonenshine and Roe, 2013; Nicholson et al. 2018). In addition, ticks can negatively affect recreational outdoor activities and can be a huge nuisance for homeowners (Sonenshine and Roe 2013; Nicholson et al. 2018).

### **Taxonomy**

As a clade, ticks (Subclass Acari: Suborder Ixodida) are a diverse group of arachnids, consisting of almost 900 species (Dantes-Torres et al. 2012; Sonenshine and Roe 2013; Nicholson et al. 2018). All species of ticks are obligate blood-sucking ectoparasites of



vertebrates, including amphibians, reptiles, birds and mammals (Sonenshine and Roe 2013; Nicholson et al. 2018). Currently, they have been grouped into three families: the Argasidae (soft ticks), the Ixodidae (hard ticks) and a family with only one species found only in Namibia, Tanzania, and South Africa (Family Nuttalliellidae: *Nuttalliella namaqua* Bedford) (Nicholson et al. 2018). The Argasidae (consisting of about 190 species in four genera) are distinguished as having soft, leathery cuticle and mouth parts that are not visible when viewed dorsally, and coxae without spurs (Nicholson et al. 2018).

The Ixodidae (707 species in 15 genera) are distinguished by a hardened, dorsal cuticular plate (i.e. scutum), spurred coxae, and prominent mouthparts that are visible when viewed dorsally (Nicholson et al. 2013). In addition, male and female ixodid ticks are sexually dimorphic. In females the scutum extends down the anterior half of the dorsal side of the body, while in males the scutum covers most of the dorsal side of the body. The Ixodidae are further grouped into two main divisions based on morphological characteristics: the Prostriata and the Metastriata (Sonenshine and Roe 2013; Nicholson et al. 2018). The Prostriata, represented by 244 species in the genus *Ixodes* are characterized by a prominent anal groove found anterior to the anus and extending to posterior body margin (Nicholson et al. 2018). The other 459 species of ticks grouped in 14 different genera within the Metastriata have a small slit-like anal groove found posterior to the anus which doesn't extend to the posterior body margin (Nicholson et al. 2018). The single species in the family Nuttalliellidae *N. namaqua* shares features found in both argasid and ixodid ticks with a couple morphological features unique to the species (Sonenshine and Roe 2013; Nicholson et al. 2018).

## Life Cycle

The stages of a generalized life cycle for ticks consist of eggs, larvae, nymphs and adults (Ostfeld et al 2006; Sonenshine and Roe 2013; Nicholson et al. 2018). All instars of ticks (except for the adults in some argasid and *Ixodes* species) feed on the blood of vertebrates and will stay attached to their respective host for hours, days or weeks (Sonenshine and Roe 2013; Nicholson et al. 2018). Ixodid ticks go through only one nymphal instar before molting to adults whereas argasid ticks go through two or more instars. Tick life cycles are categorized by the number of hosts a species will feed on in its lifetime (Trout 2010; Sonenshine and Roe 2013; Nicholson et al. 2018; CDC 2017).

In a one-host tick life cycle, all parasitic stages feed on, molt and even mate on one individual host. In contrast, a tick with a two-host life cycle, one instar (either a larva or nymph) leaves its host, seeks another, and feeds on a second host. (Trout 2010). In three-host ticks, each of the three instars will search for, attach, and feed on a different host (Trout 2010; Sonenshine and Roe 2013; Nicholson et al. 2018). Ticks in the Argasidae have a “multi-host” life cycle in which each nymphal instar (two or more) will feed on a different host, usually of the same species (CDC 2017). After each blood meal, larvae and nymphs will drop off to the ground and move to a safe location to molt. Mating for most species (except for the Argasidae and some *Ixodes* species) occurs on the host (Sonenshine and Roe 2013; Nicholson et al. 2018). Female ticks will detach from their vertebrate host after engorgement, find a place to hide and oviposit a mass of eggs. This mass of eggs, then hatches into hundreds (Argasidae) or thousands (Ixodidae) of six-legged larvae. Depending on the family, ticks can live anywhere from 1-3 years (Ixodidae) or as many 20 years (Argasidae) (Sonenshine and Roe 2013).

## **Behavior and Ecology**

### Host Associations

Ticks are known to live in various types of habitats around the world. Most species live in forested areas, grasslands, and brushy areas, while other species dwell in microhabitats such as sand, cracks and crevices, underneath stones, caves, animal nests, and under the leaf litter (Ostfeld et al. 2006; Nicholson et al. 2018). Based on their host associations, the behavior pattern of ticks is considered to be either nidicolous or non-nidicolous. Nidicolous ticks, which include most of the Argasidae and several *Ixodes* species are highly host-specific and typically reside in the same dwelling as their respective host (e.g. burrows, caves, or dens) (Ostfeld et al. 2006; Sonenshine and Roe 2013; Nicholson et al. 2018). Typically, the feeding patterns of nidicolous ticks coincides with their host patterns and is hardly affected by changes in seasonal and weather patterns. Non-nidicolous ticks on the other hand live in open habitats such as forests, brushy areas, savannahs, meadows, and even some arid to semi-arid habitats. Feeding, development and reproductive patterns of these ticks are regulated by seasonal conditions such as the ambient temperature, day length and incident solar energy in temperate to subpolar regions (Nicholson et al. 2018). In more tropical regions, the rainy-to-dry season transitions regulate tick activity.

### Host-Seeking Behavior

Non-nidicolous ticks actively seek vertebrate hosts and can be further characterized by their host seeking patterns. Ticks that ambush their host will climb on to low-lying vegetation such as grass or weeds and wait for a host to brush past them (Nicholson et al. 2018). When a host approaches, ticks are stimulated by host cues to extend their forelegs wide open and grasp onto the host in a behavior known as questing. Hunter ticks on the other hand will emerge from their dwelling in the presence of attractive host odors, move quickly towards their host and attach

to them. Ticks must recognize important host cues in order to search for, attach and feed on their respective vertebrate host. Such attractant cues include chemical cues, odors, heat, visual stimuli and vibrations. In most cases, odors are the most important cues needed for ticks to find and feed on a host. The most important animal attractants for ticks include carbon dioxide, host breath, and components of animal waste including ammonia. In addition, components of sweat such as butyric acid and lactic acid also attract ticks. The tarsi of tick forelegs each contain Haller's organ, which is an apparatus of sensillae that respond to heat, carbon dioxide and other host attractants (Sonenshine and Roe. 2013; Nicholson et al. 2018). The sensillae on the Haller's organ allow for the successful host finding activity as well as host attachment of ticks. While most tick species (about 85% of the Argasidae and some *Ixodes* species) are host specific, several other species are considered generalists (Nicholson et al. 2018).

### **Important Ticks of Arkansas**

In Arkansas, the most important species of ticks belong to ixodid genera except for a few species in the Argasidae (e.g. *Argas persicus* (Oken) and some *Onithodoros* species) that feed on poultry (Lancaster 1973; Trout 2010). In the Ixodidae, the genera, *Amblyomma*, *Dermacentor*, *Ixodes*, *Rhipicephalus*, and *Haemaphysalis*, are present in Arkansas. Within these five genera, the most important tick species in Arkansas in terms of their abundance and impact include the lone star tick, *Amblyomma americanum* L., the American dog tick *Dermacentor variabilis* Say, the blacklegged tick *Ixodes scapularis* Say, the gulf coast tick *A. maculatum* Koch and the brown dog tick *Rhipicephalus sanguineus* Latreille. Recently, the Asian longhorned tick, *H. longicornis* Des Helmore, an invasive species from eastern Asia that was first identified in the United States from a sheep in a New Jersey farm in 2017, has been reported in Arkansas in addition to 11 other

states including Connecticut, Delaware, Kentucky, Maryland, North Carolina, New Jersey, New York, Pennsylvania, Tennessee, Virginia and West Virginia (Beard et al. 2018, CDC 2019a).

*Amblyomma americanum* L.

The lone star tick (*Amblyomma americanum* L.) is an aggressive hunter tick species found from southern Maine to Florida along the coast of the Atlantic and westward from Florida to Texas and Oklahoma, Kansas and even Nebraska (Nicholson et al. 2018; CDC 2019b; Raghavan et al. 2019). A recent study by Raghavan et al. (2019) suggests that the lone star tick is more widely distributed in the United States than previously thought and that climate change could possibly allow this range to expand further northward and westward. Other factors that play a role in the expansion of *A. americanum* include the tick's high abundance, diverse habitat range, broad host spectrum, and increased exposure to humans (Trout 2010).

The lone star tick is currently the most abundant tick species in Arkansas (Goddard and Varela-Stokes 2008, Loftin and Smith personal communication; <http://gislabualr.maps.arcgis.com/apps/webappviewer/index.html?id=7846cd984bb4440795553b669c1ee31b>). A recent Arkansas survey (2017-2018) of over 10,000 ticks collected from humans, pets, livestock and wildlife from across the state (Loftin and Smith personal communication) found that 76% of all collected ticks were *A. americanum*. This three-host tick species readily attacks a wide diversity of both small and large animals such as birds, rodents, humans, livestock, and pets (Trout 2010; White and Gaff 2018; Nicholson et al 2019). Typically, adults parasitize mammals that are medium to large in size while nymphs parasitize small to medium-sized mammals and even some ground dwelling birds (Goddard and Varela-Stokes 2008). Humans are parasitized by all free-living stages of *A. americanum*. Large populations of this tick are prevalent in areas with high populations of white-tailed deer (*Odocoileus virginianus*

Zimmerman) (White and Gaff 2018; Nicholson et al. 2018). Adults have a reddish-brown coloration, with females having a characteristic white spot on their scutum (Trout 2010). Male ticks have gold and white marking along their body margins and are smaller in size than females. The typical life cycle for wild populations of *A. americanum* often takes two years to complete (Holderman and Kaufman 2013). However, under ideal laboratory conditions, an entire life cycle can be completed in as little as 22 weeks (Troughton and Levin 2007; Holderman and Kaufman 2013). Larvae emerge from eggs in mid to late summer, feed, and develop into nymphs that overwinter (Nicholson et al. 2018). Populations of *A. americanum* adults and nymphs in the southeastern United States will start host-seeking activity during late spring (Nicholson et al. 2018). Nymphs have two seasonal peaks: one occurring between late spring and early summer (May to June) and another during late summer (August) (Goddard and Varela-Stokes 2008; Holderman and Kaufman 2013). Lesions left by the long hypostome of lone star ticks can create sores that can lead to secondary infections and even the occasional myiasis (Trout 2010). Severe infestations of this tick can lead to severe blood loss in cattle. *Amblyomma americanum* is known to vector many disease-causing pathogens to animals and humans including causal agents of human and canine ehrlichiosis (*Ehrlichia chaffeensis* Anderson et al. 1991 and *E. ewingii* Anderson et al. 1992 in humans; *E. canis* (Donatien and Lestoquard) Moshkovski in dogs), tularemia (*Francisella tularensis* McCoy and Chapin), rickettsiosis, protozoan diseases such as bobcat fever (*Cytauxzoon felis* Kier), and recently, viruses such as the Heartland virus (genus *Phlebovirus*: Family Bunyaviridae) and Bourbon virus (genus *Thogotovirus*: Family Othromyxoviridae) (Trout 2010, Holderman and Kaufman 2013; Nicholson et al. 2018). The lone star tick was once thought to vector *Borrelia lonesatri* Barbour et al. 1996, which was once implicated as the causal agent of Southern Tick Associated Rash Illness (STARI) (CDC 2018).

However, further research has shown that *B. lonestari* is most likely not the causal agent of STARI (Wormser et al. 2005; CDC 2018). In addition, bites from lone star ticks have resulted in a delayed, allergic reaction to mammalian meat in humans known as alpha gal syndrome (Commins et al. 2011; Holderman and Kaufman 2013; Nicholson et al. 2018). This syndrome is caused by an allergy to the sugar galactose- $\alpha$ -1, 3-galactose found in mammalian red meat.

### **Tick Borne Pathogens and Diseases**

Vertebrate pathogens transmitted by ticks include bacteria, viruses, protozoa and in some rare instances, filarial nematodes (Sonenshine and Roe 2013; Brites-Neto 2015; Nicholson et al. 2018). Notable pathogens vectored by ticks include bacteria such as *Borrelia burgdorferi* sensu lato (s.l.) Johnson et al. 1984 (the causal agent of Lyme disease), *Anaplasma* spp. (the causal agents of anaplasmosis), *Ehrlichia* spp. (the causal agents of ehrlichiosis), and *Rickettsia rickettsia* s.l. Brumpt (the causal agents of spotted fever rickettsiosis).

Notable viral diseases include Kyasanur Forest disease virus, Powassan encephalitis virus, Colorado tick fever virus, the Congo-Hemorrhagic fever virus and more recently discovered viruses such as the Bourbon virus and Heartland virus (Nicholson et al. 2018). Several protozoan pathogens such as *Babesia* spp., *Theileria* spp. and *C. felis*—the agents that cause babesiosis, theileriosis and bobcat fever respectively are also transmitted by ticks (Nicholson et al. 2018).

### **Factors Contributing to Pathogen Transmission**

Various factors and characteristics contribute to the successful pathogen harborage and transmission potential of ticks. To feed, ticks use their chelicerae to cut all the way into the blood vessels of a vertebrate host. As they feed ticks will excrete copious amounts of saliva and enzymes into their hosts and will stay attached to a host for long periods of time (ranging from

minutes (Argasidae) to over a week (Ixodidae)), allowing the effective transmission of whatever pathogens they may be vectoring (Trout 2010; Sonenshine and Roe 2013; Nicholson et al. 2018). In addition, ticks might also transmit pathogens harbored in secretions from their saliva, midgut or coxae (some argasid species). Several tick species such as *A. americanum* and *I. scapularis* are generalists that feed on several species of hosts, allowing them to spread their pathogens to reservoir or maintenance hosts (Trout 2010). The long lifespan of ticks (2-3 years for most ixodid ticks; 5-7 years for most argasid ticks) and the slow developmental changes they undergo in most of their internal tissues make them ideal vectors for harboring pathogens for prolonged amounts of time (Trout 2010; Sonenshine and Roe 2013; Nicholson et al. 2018). Ticks have relatively few natural predators compared to many arthropod groups and are highly protected from stressors in their environment thanks to their flattened and highly sclerotized bodies (Nicholson et al. 2018).

### **Modes of Transmission**

Ticks transmit pathogens by two main modes of transmission: vertical transmission (pathogen transmission within the vector) or horizontal transmission (vector-to-host pathogen transmission) (Trout 2010).

Pathogen transmission in ticks can be grouped into two main modes: transstadial transmission and transovarial transmission. In transstadial transmission ticks get infected with a pathogen as larvae or nymphs and then pass on the pathogens onto each successive life stage after each molt. Therefore, the pathogens harbored in ticks remain infective to ticks and their respective hosts for multiple seasons to come. In transovarial transmission, which is found in a few tick species, the female tick passes on pathogens to her eggs, allowing the next generation to readily infect a vertebrate host (Trout 2010; Nicholson et al. 2018). In some tick species (e.g.



some *Dermacentor* spp.), both transstadial and transovarial transmission can allow them to maintain pathogens for many generations.

Horizontal transmission of pathogens from ticks onto a vertebrate host is not only important for the transmission of disease-causing pathogens to susceptible hosts but also for maintaining pathogens in other hosts that are less susceptible or even immune to the pathogens. Animals that have recovered from infection to some tick-borne pathogens can harbor pathogens that can be infective for years (Nicholson et al. 2018). In addition, some vertebrate maintenance hosts of ticks that are immune to infection from tick-borne pathogens can provide additional infection pathways for ticks and also serve as competent hosts for the ticks (Nicholson et al. 2018). In these pathways, uninfected ticks can indirectly get infected with ticks vectoring pathogens when both ticks feed on a host simultaneously.

### **Chemical Control**

Traditionally, the primary means of controlling ticks have been through the application of synthetic chemical acaricides (Samish and Rehacek 1999; Samish et al. 2004; Ostfeld et al. 2006; Samish et al. 2008). To control ticks two different strategies have been applied: broadcast applications of acaricides to the environment where ticks seek hosts or direct applications of acaricides to tick hosts. Conventional acaricides used for tick control fall under several different classes of pesticides including chlorinated hydrocarbons, pyrethroids, organophosphates, carbamates formamidines, macrocyclic lactones, phenylpyrazoles, insect growth regulators, and isoxazolines (Nicholson et al. 2018). Broadcast applications of pesticides are generally very effective at reducing ticks in the field and in localized areas. Chemical pesticides have also been successfully applied to control ticks on livestock (Ostfeld et al. 2006). Treatment of ticks on livestock and pets have implemented several different methods including dips, spot-ons, pour-

ons, dusts, collars and systemic acaricides. In recent years, research has focused on the application of chemical pesticides to wildlife hosts to reduce the abundance of ticks in nature (Ostfeld et al. 2006). Some of these application methods have included the four-poster feeder device for controlling ticks on deer and bait tubes and boxes for tick control on rodents (Ostfeld et al. 2006; Stafford and Williams 2017; White and Gaff 2018; Williams et al. 2018).

### **Other Control Methods**

However, several concerns have been raised over the use of chemical acaricides, including toxic, off-target effects to wildlife, environmental pollution and the development of insecticide resistance to ticks (Samish et al. 2004; 2008; Abbas et al. 2018). For these reasons, alternatives, such as naturally derived acaricides (e.g. nootkatone) (White and Gaff 2018), habitat modification (e.g. leaf litter removal, mulch) (White and Gaff 2018), controlled burns (White and Gaff 2018), invasive shrub removal (Lubelczyk et al. 2004; Elias et al. 2006; Williams et al. 2009; Allan 2010; Williams and Ward 2010), deer removal/exclusion (Ginsberg et al. 2002; Williams et al. 2018), personal protection (Trout 2010, Holderman and Kaufman 2013; CDC 2019c) and biological control (Samish and Rehacek 1999; Samish et al. 2004; 2008; Ostfeld et al. 2006; Fernandes and Bittencourt 2008; Fernandes et al. 2012) have been explored.

### **Biological Control of Ticks**

Numerous predators and pathogens have been reported to prey on, parasitize or infect ticks in nature. Predators of ticks include many species of birds, mammals, beetles, ants, spiders and various other invertebrates (Samish and Rehacek 1999; Samish and Alekseev 2001; Samish et al. 2004; 2008; Ostfeld et al. 2006). Most predators and pathogens of ticks feed on ticks only at high abundances or in a few isolated occasions. However, some predators and parasitoids,

such as oxpeckers (Buphagidae: *Buphagus*) and wasps in the genus *Ixodiphagus* (Hymenoptera: Encyrtidae) are considered tick specialists.

### **Vertebrate Predators of Ticks**

Most vertebrate predators of ticks are considered generalists that feed on ticks opportunistically or in sporadic instances (Samish and Rehacek 1999; Samish 2000; Samish et al. 2004; 2008; Ostfeld et al. 2006). However, a few predators, such as oxpeckers, are known to feed specifically on ticks and other ectoparasites (Samish and Rehacek 1999; Samish et al. 2004; 2008). Vertebrates that prey on ticks include amphibians, reptiles, birds, and some mammals (Samish and Rehacek 1999). In addition, the grooming behavior of some mammals (e.g. deer, cattle, rodents, cats, dogs, primates) is thought to reduce their tick burdens (Samish and Rehacek 1999; Ostfeld et al. 2006). Birds are considered to be the most important vertebrate predators of ticks (Samish and Rehacek 1999). About 50 species of birds have been observed feeding on ticks and include (but are not limited to) several passerine birds, domestic fowl, cattle egrets, some ground dwelling birds, birds of prey and oxpeckers (Samish and Rehacek 1999; Samish 2000; Samish 2004; 2008; Ostfeld et al. 2006). However, the nature of most tick predation by birds (with the exception of oxpeckers) is generally considered sporadic (Samish and Rehacek 1999). Studies from Africa have demonstrated that foraging chickens (*Gallus gallus* L.) could potentially reduce tick levels, with an average of 69% removed at high densities (Samish et al. 2004; 2008). While this might make chickens an effective, low-to-no cost measure for reducing ticks on small mixed farms, chickens are highly omnivorous and will only feed on ticks depending on the tick densities in their area and the availability of other food sources (Samish et al. 2004; 2008). Wildfowl such as helmeted guinea fowl (*Numidia meleagris* L.) have been shown to significantly reduce densities of blacklegged ticks (Rehacek et al. 1999; Ostfeld et al.

2006). Despite this, the helmeted guinea fowl is not considered a tick specialist and has been found to only cause significant reductions in adult blacklegged ticks, which are only responsible for leading to a relatively smaller percentage of Lyme disease cases by pathogen transmission compared to nymphal ticks (Ostfeld et al. 2006). Several species of birds, known as cleaner birds, are thought to play an important role in reducing tick numbers in the wild (Sazima and Sazima 2010). Cleaner birds seek and consume food on the body of animals, such as ectoparasites (e.g. ticks), organic debris, host secretions, skin and/or blood (Sazima and Sazima 2010). In Africa two species of cleaner birds, the yellow-billed oxpecker (*Buphagus africanus* L.) and red-billed red oxpecker (*B. erythrorhynchus* Stanley) are specialist feeders of ectoparasites, particularly ticks (Samish and Rehacek 1999; Samish 2000; Samish et al. 2004; 2008; Ostfeld et al. 2006). In addition to ticks, oxpeckers will also feed on host secretions and wound-feed on areas of their host where they've picked off ticks. Oxpeckers are known to have a daily intake of ticks numbering in the hundreds for adult ticks and in the thousands for nymphal ticks (Samish and Rehacek 1999; Samish 2000; Samish et al. 2004; 2008; Ostfeld et al. 2006). While oxpeckers might reduce tick numbers in nature, they might only be valuable as a single component of a larger integrated tick management program since tick densities remain high in areas containing oxpecker populations (Samish et al. 2004; 2008). Also, populations of oxpeckers have dwindled and have even been extirpated in some areas in Africa due to acaricide poisoning and subsequently reduced tick numbers. However, the advent of successful reintroduction programs and the use of safer acaricides have allowed oxpecker populations to rebound in recent years.

## **Invertebrate Predators and Parasitoids**

Some arthropods have been reported to attack and feed on ticks (Samish and Rehacek 1999; Samish and Alekseev). Most are “nonspecific” predators of ticks (Samish and Alekseev 2001) and have been observed preying on ticks in sporadic observations in the field or laboratory. These predators include spiders, mites, flies, ants, beetles and a few other groups of arthropods (Samish and Rehacek 1999; Samish and Alekseev 2001). Out of those groups the most important predator groups of ticks appear to be ants, beetles and spiders (Samish and Alekseev 2001). Engorged females tend to be the most preyed upon life stage of ticks. A study conducted with the red imported fire ant *Solenopsis invicta* Buren in Baton Rouge and Pine Grove, Louisiana demonstrated predation of this ant towards *A. americanum* (Harris and Burns 1972). Furthermore, *S. invicta* might also reduce pathogen transmission and questing behavior of this tick species and related species in the *Amblyomma* genus (e.g. *A. maculatum*) (Castellanos et al. 2016; Kjeldgaard et al. 2016).

## **Tick Parasitoids**

Tick parasitoids include eight species of *Ixodiphagus* wasps that exclusively parasitize ticks (Samish and Rehacek 1999; Samish 2000; Samish et al. 2004; Ostfeld et al. 2006). However, only one species, *I. hookeri* Howard has been released for the biological control of ticks. Biological control releases of this wasp have had some success in reducing tick densities (Samish et al. 2004; 2008). However, high densities of ticks and their hosts are required for *I. hookeri* to persist after being introduced into tick-infested areas. In addition, the mass labor and potentially high cost burden required for the mass rearing of *I. hookeri* make long-term biocontrol efforts against ticks with this parasitoid unsustainable (Ostfeld et al. 2006).

## **Pathogens Controlling Ticks**

Known pathogens that have infected and killed ticks in the lab and in the field include bacterial pathogens, entomopathogenic nematodes, entomopathogenic fungi and occasionally, viruses and protozoa. (Samish and Rehacek 1999; Samish et al. 2004; 2008; Ostfeld et al. 2006). Multiple studies have shown that the most promising biocontrol agents of ticks are entomopathogenic fungi.

### Tick-Infecting Bacteria

Although several species of bacteria have been isolated from ticks in nature (Martin and Schmidtman 1998; Samish and Rehacek 1999; Samish et al. 2004; 2008) most species reported from ticks are not considered pathogenic. However, bacteria, such as *Proteus mirabilis* Hauser and *Cedecea lapagei* Grimont et al. 1981., and spore-forming bacteria such as *Bacillus thuringiensis* Berliner have shown to cause mortality against ticks in laboratory studies (Samish et al. 2004; Ostfeld et al. 2006; Samish et al. 2008). However, since ticks ingest only host blood, and the endotoxin of *B. thuringiensis* must be ingested to attack the midguts of arthropod hosts, the effectiveness of *B. thuringiensis* as a tick biocontrol agent looks very limited.

### Entomopathogenic Nematodes

Several species of entomopathogenic nematodes, particularly of the families Heterorhabditidae and Steinernematidae are known to be pathogenic to ticks (Samish and Rehacek 1999; Ostfeld et al 2006; Samish et al. 2008). The free-living juvenile stage of the nematodes enters ticks through natural openings in the host via digestive enzymes and mechanical force (Ostfeld et al. 2006; Samish et al. 2008). Ticks are killed by the nematodes by the release of pathogenic bacteria into the hemocoel of the ticks. Different life stages of ticks vary in their degree of susceptibility to entomopathogenic nematodes, with engorged females

being the most susceptible. Since engorged females and diapausing ticks reside in habitats where entomopathogenic nematodes are found, these nematodes might appear to be useful tools for controlling ticks in nature. Despite this, several factors limit their effectiveness to specific ecological roles in the environment. Entomopathogenic nematodes do not complete their life cycles in ticks and are not known to infect them in nature (Ostfeld et al. 2006; Samish et al. 2008). Moreover, environmental conditions such low humidity, low temperatures, high concentrations of manure or silt in the soil and ultraviolet radiation hamper the use of entomopathogenic nematodes for tick management.

### **Entomopathogenic Fungi**

Of all biological control agents assessed for tick control, entomopathogenic fungi are considered the most promising biocontrol agents for tick control. Over 700 species of entomopathogenic fungi have been described, some of which have been developed into botanical insecticides for controlling insect pests (Samish et al. 2004; 2008; Faria and Wraight 2007). Entomopathogenic fungi are considered to be good biocontrol agents of ticks and several other arthropod pests because of their worldwide distribution, broad host range, ability to penetrate arthropod cuticles, and ability to kill several life stages of the same host species (Samish and Rehacek 1999, Samish et al. 2004; 2008). In addition, several species and strains of entomopathogenic fungi are known to infect specific hosts (Aw and Hue 2017), reducing the likelihood of off-target effects to other non-target arthropods (Fishchoff et al. 2017).

### **General Life Cycle of Entomopathogenic Fungi**

The mode of infection for entomopathogenic fungi generally includes six stages: physical adherence of spores (conidia) to the arthropod cuticle, germination, appressoria/infection peg formation, penetration of the cuticle, hemolymph colonization, and mycelial extrusion and

sporulation (Fernandes et al. 2008; Samish et al. 2008; Aw and Hue 2017). Spores of entomopathogenic fungi adhere to the waxy cuticle of host arthropods by hydrophobic forces, electrostatic interactions, and protein interactions that occur between the epicuticle and spores (Aw and Hue 2017). Next the spores of entomopathogenic fungi germinate and form a germ tube that differentiates into structures called appressoria, which are used for penetrating the host. Appressoria and infection pegs penetrate the host cuticle with the use of mechanical forces and hydrolytic enzymes that include specific proteases, chitinases and lipases (Fernandes et al. 2008; Aw and Hue 2017). Some of these chitinases such as trypsins are host specific. After host penetration, hyphal bodies invade host internal organs and some release various mycotoxins that eventually kill the host. Proteases released inside the hemocoel can cause the lysis of internal organs such as the gut and Malpighian tubes while toxins such as oxalate or destruxins further contribute to the progression of the pathogenesis (Hänel 1982; Fernandes et al. 2008; Samish et al. 2004; 2008; Aw and Hue 2017). After an entomopathogenic fungus kills its host, mycelia break through the host cuticle and form conidiophores which eventually bear asexual spores known as conidia (Hänel 1982; Aw and Hue 2017). These conidia will then infect further arthropod hosts by direct contact or by airborne exposure. Characteristically, Hypocrealean fungi will switch from a parasitic phase to saprotrophic phase, fully colonizing the dead body of the arthropod cadaver (Roy et al. 2006).

### ***Metarhizium anisopliae* Sensu Latu (Metchnikoff) Sorokin**

*Metarhizium anisopliae* (Metchnikoff) Sorokin (Ascomycota; Hypocreales; Clavicipitaceae) along with its different varieties, strains and related species in its genus are the cause of green muscardine disease in insects and several arachnid groups such as mites and ticks



(Webster and Weber 2007; Bischoff et al. 2009; Aw and Hue 2017; Species Fungorum 2020).

The *M. anisopliae* species complex is sometimes referred to as *M. anisopliae* sensu lato (s.l.).

*Metarhizium anisopliae* was first described from Russia by Elie Metchnikoff as a pathogen of the wheat cockchafer *Anisoplia austriaca* Herbst as *Entomophora anisopliae* (Driver and Milner 2000; Zimmerman 2007). This species was later transferred to the genus *Metarhizium* by Sorokin who described the genus in 1883 (Bischoff et al. 2009). At one point, *M. anisopliae* was classified as four varieties (*M. anisopliae* var. *anisopliae*, *M. anisopliae* var. *acridum* Driver and Milner, *M. anisopliae* var. *lepidiotum* Driver and Milner, and *M. anisopliae* var. *majus* (Johnst.) Tulloch) and also included three other supposed species from China (*M. pingshaense* Chen and Guo, *M. guizhouense* Chen and Guo and *M. taii* Liang and Liu) that were considered a part of the species (Driver and Milner 2000; Bischoff et al. 2009). Recent work by Bischoff et al. (2009) used multigene, phylogenetic sequencing and morphology to reclassify the *M. anisopliae* species complex into nine species: *M. anisopliae*; *M. acridum*; *M. brunneum* Petch; *M. guizhouense*; *M. pingshaense*; *M. lepidiotae*; *M. majus*; *M. robertsii* Bischoff, Rehner and Humber and *M. globosum* Bischoff, Rehner and Humber.

When grown in culture or on an arthropod host, *M. anisopliae* s.l. and other *Metarhizium* species grow columns of characteristically green, mononucleate, lipid-rich, cylindrically shaped conidia borne on candle-shaped conidiophores or phialides (Humber 1997; Webster and Weber 2007). These entomopathogenic fungi have a worldwide distribution and have been extensively studied due to their virulence, broad host range, ease of mass production, and relative safety to the environment (Aw and Hue 2017). *Metarhizium anisopliae* s.l. has been isolated from soils of tropical and temperate regions across the globe in addition to numerous different arthropod hosts (Meyling and Eilenberg 2007; Bischoff et al. 2009). Over 200 insect species from more

than seven orders (including the Blattaria, Orthoptera, Coleoptera, Hemiptera, Diptera, Hymenoptera and Lepidoptera) as well as some groups in the Arachnida are known to be infected by *M. anisopliae* s.l. (Chandler et al. 2000; Faria and Wraight 2007; Webster and Weber 2007; Zimmerman 2007; Aw and Hue 2017). Although it is considered a generalist fungal pathogen, some varieties, strains, and species of *M. anisopliae* s.l. have a narrow host range (Samish et al. 2004; 2008; Aw and Hue 2017).

Various mycoinsecticide formulations of *M. anisopliae* s.l. strains have been marketed, sold and extensively used for the biological control of various agricultural pests on all of the six major continents (Faria and Wraight 2007; Fernandes et al. 2012). Compared to the slew of *M. anisopliae* s.l. products marketed for agricultural pests, very few of these products are marketed for controlling pests of medical/veterinary importance, including ticks and mosquitos. According to Faria and Wraight (2007) four mycoinsecticide products containing *M. anisopliae* s.l. are (or were) marketed for the control of ticks: Tick-EX® EC, Tick-EX® G, Metazam®, and Metarril® SC (Fernandes et al. 2008). Metazam® is marketed for use in Honduras while Metarril® is marketed Brazil. Since the publication of Faria and Wraight (2007), Met52® EC and Met52® granular have also gone on the market for controlling ticks in the United States. However, Tick-EX® EC and Tick-EX® G have been withdrawn from the market.

### **Met52®**

#### Active Ingredient *Metarhizium brunneum* Petch

Met52® (Novozymes Biological Inc., Salem, VA) is commercial mycoinsecticide that contains live conidia of *Metarhizium brunneum* Petch strain F52, which was previously classified as *M. anisopliae* strain F52 (Bischoff et al. 2009; Fishchoff et al. 2017). This strain was originally isolated in Austria from the codling moth *Cydia pomonella* L. (EPA, 2003; EFSA

2012; Fishchoff et al. 2017). Like most entomopathogenic fungi, arthropod pests contact spores of F52, where the spores start to germinate and grow. The fungus then penetrates the host cuticle, invades the host and ultimately kills it (Novozymes 2019). *Metarhizium brunneum* strain F52 typically kills its host in three to seven days or longer, depending on temperature. Met52® is currently formulated as an emulsifiable concentrate (EC) or a granular formulation. Both Met52® products are marketed for use the United States, Canada, and some European countries.

#### EC Formulation

The Met52® EC formulation can be applied as either a foliar application (in low or high-volume sprays) or as drench application (Novozymes 2019). This product has been approved for use in nurseries, green houses, and outdoor sites such as on urban turf, suburban turf, and outdoor agricultural sites. Met52® is approved for use of treating thrips, white flies, mites, and weevils found infesting agricultural crops (e.g. cucurbits, onions, celery, lettuce, spinach, peppers, tomatoes, grapes, strawberries, cranberries, raspberries blackberries) and ornamental plants (e.g. shrubs, vines, trees, lawn bedding plants, poinsettias, and other plants). On turf grass, Met52® is labeled to treat both *A. americanum* and *I. scapularis*.

#### Granular Formulation

The Met52® Granular formulation is labeled for application as a potting mix treatment for growing mediums used in nurseries and as a broadcast application to turf (Novozymes 2019). Approved site locations for use include the same areas that are approved for the application of Met52 EC. It is labeled to treat thrips pupae and vine weevils in most of the same agricultural crops (except cucurbits) and ornamental plants as Met52® EC. As a turf application, Met52® Granular is approved to treat the same tick species as the EC formulation.

## F52 Tick Control Trials

In various lab and field studies, *M. brunneum* strain F52 has shown to effectively reduce numbers of adult and nymphal and *I. scapularis* ticks (Bharadwaj and Stafford, 2010; 2012; Stafford and Allan 2010; Behle et al. 2013). In a series of field experiments conducted by Stafford and Allen (2010) in the years 1999, 2000, Old Lyme, Connecticut, an application of the pyrethroid bifenthrin provided 86% and 87% control against nymphal *I. scapularis* in 1999 and 2000 respectively. In the same study, follow-up field experiments in which *M. brunneum* strain F52 (*M. anisopliae* strain F52 at the time of the study) was applied to field plots in Westport and Weston, Connecticut found that F52 reduced the abundance of nymphal ticks by 55.6% and 84.6% on lawn and wood plots respectively. Bharadwaj and Stafford (2010) found that two applications of F52 (one at a low and high rate) made before seasonal *I. scapularis* nymph activity (May to early June) was not effective reducing tick activity. On the other hand, a second application made during seasonal *I. scapularis* nymphal activity (June to July) showed significant reductions in tick activity at three, and five weeks post-treatment. Both low and high applications rates were found to be effective for controlling ticks with one-third of the nymphal ticks collected at treatment sites showing signs of *M. brunneum* infection. A few studies have evaluated the EC and granular formulations of F52 against both adult and nymphal *I. scapularis* ticks (Bharadwaj and Stafford 2012; Behle et al. 2012). Bharadwaj and Stafford (2012) found a lower  $LC_{50}$  ( $5.9 \times 10^4$  conidia/cm) for the spraying the EC F52 formulation on *I. scapularis* ticks vs. the granular formulation ( $LC_{50} = 8.1 \times 10^5$  conidia/ml). In another study Behle et al. (2013) found that F52 microsclerotial granules in moistened potting soil produced high rates of conidia ( $3.05 \times 10^9$  to  $1.24 \times 10^{10}$  conidia/gram) and showed significant mortality against *I. scapularis* after ticks were exposed to the granules for 7 weeks ( $F < 16.29$ ;  $DF = 1, 4$ ;  $P < 0.0157$ ). One study by

Williams et al. (2018) conducted in Redding Connecticut from 2013-2016 designed an experiment to determine if an integrated tick control program utilizing, deer removal, fipronil-impregnated rodent bait boxes and a broadcast application of Met52® EC would reduce the abundance of questing *I. scapularis* nymphs and the prevalence of *Borrelia burgdorferi* in the questing nymphs. The results of this study showed that a combination of the fipronil-impregnated boxes and a broadcast application of Met52® EC showed the highest reduction of questing *I. scapularis* nymphs (78-95% reduction for each year) and *B. burgdorferi* infested nymphs (66%) of any treatment combination (deer removal alone, deer removal plus bait boxes plus Met52®, bait boxes plus Met52®). In Dutchess County, New York, region known for its high occurrence rate of tick-borne diseases, a 5-year study known as the Tick Project ([www.tickproject.org](http://www.tickproject.org)) is currently being implemented to assess whether tick-borne diseases can be controlled at a neighborhood-wide scale (Fischhoff 2017; Keesing and Ostfeld 2018). To make this assessment, the project uses Met52® broadcast sprays and fipronil-impregnated bait boxes for treating small mammals. Both methods are used either separately or together in yards and were chosen based on their commercial availability, tick control efficacy, and safety features.

#### Off-Target Effects

Before it was registered for use as an active ingredient of Met52® several trials were conducted by the U.S. Environmental Protection Agency to assess the possible effects of *M. brunneum* strain F52 on non-target organisms (EPA 2003). Overall F52 was found to be safe for terrestrial and aquatic vertebrates (EPA 2003; Fischhoff et al. 2017). Additional tests of F52 on honeybees, parasitic wasps, lacewings and earthworms also showed no negative effects to the tested invertebrates. However other studies have shown F52 to cause mortality in some invertebrate taxa including some species of predatory rove beetles, and predatory mites

(Fishchoff et al. 2017). As part as part an assessment for the Tick Project, a study by Fischhoff et al. (2017) evaluated possible off-target effects of F52 when Met52® was applied to non-target arthropod taxa. The findings of this study showed that reductions in the abundance of non-target arthropods as a result of broadcast applications of Met52® were highly unlikely.

### **Isolation of Entomopathogenic Fungi from Ticks in Nature**

Entomopathogenic fungi have been isolated from the leaf litter and organic layers of soil, which serve as natural harborage sites for ticks that are diapausing, not seeking hosts or not feeding (Tuininga et al. 2009; Greengarten et al. 2011). Over 20 species of fungi have been found to infect ticks in nature (Samish et al. 2004; 2008). Additionally, thirteen tick species within seven genera have been documented with natural fungal infection. The natural percentage rate of ticks infected by entomopathogenic fungi can vary substantially with infection rates ranging from <1% to over 50% (Samsinakova et al. 1974; Estrada-Peña et al. 1990; Kalsbeek et al. 1995; Zhioua et al. 1999; da Costa et al. 2001; Samish et al. 2004; 2008; Benoit et al. 2005; Tuininga et al. 2009; Greengarten et al. 2011). Depending on the region, life stage, species of tick and ecology of the collection site, the percentage of fungal-infected ticks tends to vary (Samish et al. 2004; 2008; Fernandes and Bittencourt 2008). The adult stages tend to have a higher percentage of infected individuals in nature than immature stages of ticks, with engorged females being the most readily infected stages (Samish et al. 2004; 2008). In the U.S. and across the world, several important species of entomopathogenic fungi have been found infecting ticks including *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin, *M. anisopliae* s.l., *Isaria farinosa* (Holmsk) Fries (= *Paecilomyces farinosus*), *I. fumosorosea* Wize (= *P. fumosoroseus*), and *Lecanicillium lecanii* Zare and Gams (= *Verticillium lecanii*) (Samish et al. 2008; Tuininga et al. 2009; Greengarten et al. 2011; Fernandes et al. 2012).

A study conducted by Zhioua et al. in southern New England and New York (1999) found that 4.3% of *I. scapularis* females collected were naturally infected with fungi. Another study conducted in Paracambi, Rio de Janeiro, found that out of 200 engorged female *Rhipicephalus (Boophilus) microplus* Canestrini ticks collected from soil, 49 (24.5%) were infected with *B. bassiana* while 20 (10%) were infected with *M. anisopliae* var. *anisopliae* (da Costa et al. 2001; Samish et al. 2004; 2008). Greengarten et al. (2011) identified 70 species fungi in 25 genera. Of the fungi isolated 48 species of fungi were isolated from soil samples and 27 species were isolated from *I. scapularis* ticks (5 species of fungi isolated from ticks were also isolated from soil samples). Fifteen of the isolated fungi were found to cause significant mortality to *I. scapularis* ticks in laboratory bioassay trials. In addition, two species of fungi—*Hypocrea lixii* Patouillard and *Penicillium sopii* Zalesky—caused mean tick mortalities of 71% and 58% respectively in field trials.

### **Laboratory Studies**

Most laboratory and field studies of entomopathogenic fungi have focused mainly on species of *Beauveria* and *Metarhizium* (Samish et al. 2004; 2008; Fernandes and Bittencourt, 2008). In lab studies that have tested multiple species and strains of entomopathogenic fungi, isolates of *B. bassiana* and/or *M. anisopliae* s.l. have so far shown to be the most pathogenic to ticks, eliciting the highest mortality (Gindin et al. 2001; 2002; Samish et al. 2004; 2008; Pirali-Kheirabadi et al. 2006; Abdigoudarzi et al. 2009; Fernandes et al. 2011). Tick mortality by entomopathogenic fungi varies considerably among different species of ticks, different life stages, and among different fungal genera, species, isolates and conidial formulations used. (Ostfeld et al. 2006; Samish et al. 2004; 2008).

*Amblyomma maculatum* nymphs and adults for instance were more susceptible to strains of *B. bassiana* and *M. anisopliae* than adults and nymphs of *A. americanum* (Kirkland et al. 2004a). In a similar study, nymphs and adults of *I. scapularis* and *R. sanguineus* were more susceptible to pathogenic fungi than nymphs and adults of *D. variabilis* (Kirkland et al. 2004b). Gomathinayagam et al. (2002) observed 100% mortality for *A. americanum* ticks two weeks after the ticks were treated with *B. bassiana*. However, no mortality was observed for *D. variabilis* at the same time period. Significant mortality for this species was only observed at five weeks post-treatment. These differences in mortality might be due fungistatic compounds present on the cuticle of ticks and/or competitive interactions from fungal endosymbionts (e.g. *Scopulariopsis brevicaulis* (Saccardo) Bainer (Yoder et al. 2003; 2004; 2005; 2006; 2008; Kirkland et al. 2004; Samish et al. 2004; 2008; Benoit et al. 2005). Tick eggs are relatively more susceptible to entomopathogenic fungi than insect eggs, with 100% mortality recorded in some studies (Gindin et al. 2002; Samish et al. 2004; 2008; Cafaracia et al. 2015; Luz et al. 2016). The high susceptibility of tick eggs to entomopathogenic fungi is most likely due to the composition of the host cuticle, adhesion of conidia to host cuticle, germination of conidia, formation of appressoria or infection pegs, fungal penetration, invasion of host cuticle the production of conidia, and abiotic factors such as relative humidity and temperature (Frazzon et al. 2000; Samish et al. 2001; Gindin et al. 2009; Ment et al. 2010; 2012; Fernandes et al, 2012; Cafaracia et al. 2015). Furthermore, a study by Gindin et al. (2009) found that older tick eggs were more susceptible to strains of *M. anisopliae* s.l. (e.g. *M. a.* var. *anisopliae* and *M. acridum* (= *M. a.* var. *acridum*)) than younger eggs and that cuticular extracts of eggs stimulated growth of *M. anisopliae* var. *anisopliae*. Entomopathogenic fungi have also been found to cause sublethal effects on ticks in addition to their direct mortality effects (Hornbostel et al. 2004; Ostfeld et al



2006). Some of these effects include reduced body mass and egg masses of engorged females, increased water loss, and significantly lighter molt weights of engorged larvae molting into nymphs and engorged nymphs molting into adults (Hornbostel et al. 2004; Samish et al. 2004; 2008; Yoder et al. 2006; Craddock and Needham 2011b).

### **Field Studies and Control Strategies**

Although not implemented as much as in laboratory studies, some studies have been conducted to test the efficacy of entomopathogenic fungi for the control of ticks in field conditions. In general, two approaches have been implemented for the biocontrol of ticks in the field: on-host control and off-host control (Samish et al. 2004; 2008). On-host control utilizes livestock or other animals as bait, allowing for the control of ticks that are highly concentrated in one area and that have a high potential to reproduce (i.e. engorging females). Furthermore, this strategy might be very cost effective since less fungal insecticide is sprayed over a smaller area to treat ticks (Fernandes and Bittencourt 2008; Fernandes et al. 2012). Off-host control strategies on the other hand, treat ticks in their natural environment (i.e. in the vegetation, soil and leaf litter) where they typically spend about 90% of their life cycle (Samish et al. 2004, 2008; Nicholson et al. 2018; Burtis et al. 2019). Since these conditions tend to have more favorable temperatures and relative humidities (RH) conducive for the growth of entomopathogenic fungi and tick survival, an off-host control strategy might be favorable as a tick management strategy. In addition, tick densities found in the environment can be substantially higher than those found on the host, sometimes ranging from 1000-10,000 ticks in vegetation for every tick attached cattle (Kaaya and Hassan 2000).

Tick mortality rates in field studies that use entomopathogenic fungi tend to vary more than laboratory assays, and often show lower mortality rates than lab trials. While some studies

have reported no significant reduction in tick numbers, others have reported mortality close to 100% (Samish et al. 2004; 2008; Fernandes and Bittencourt 2008; Fernandes et al. 2012). Generally, the efficacy of entomopathogenic fungi in the field depends on several different factors including, UV radiation, temperature fluctuations, changes in relative humidity, rainfall, and the specific behavior patterns of the tick species being treated. In a Kenyan study conducted by Maranga et al. (2005) tick mortality for *B. bassiana* and *M. anisopliae* was greater in the rainy season than in the dry season. Other studies (Kaaya 2000; Kaaya and Hassan 2000; 2011; Samish et al. 2004; 2008) have shown that treatment of ticks with *B. bassiana* and *M. anisopliae* months after the rainy produced high tick mortalities, whereas treating during rainy season did not substantially affect mortality. The increased mortality of ticks from both entomopathogenic fungi was most likely due to a subsequent increase in relative humidity as a result of the rainy season and higher temperatures. In addition, Bharadwaj and Stafford (2010) showed that it is important to treat *I. scapularis* nymphs during their active season (June to late July) in order to achieve significant tick reductions with an application of *M. brunneum* strain F52. The effect of combining both *B. bassiana* and *M. anisopliae* into a “cocktail” formulation of the two species resulted in a higher mortality of *A. variegatum* Fabricius ticks, than when both fungi were applied separately (Maranga et al. 2005; Samish et al. 2004; 2008). A novel concept in the biocontrol of ticks includes the use of carbon dioxide and semiochemical baited traps treated with entomopathogenic fungi (Maranga et al. 2006; Samish et al. 2008; Nchu et al. 2010). Nchu et al. (2010) tested one such trap treated with *M. anisopliae* against *A. variegatum* and found significant reductions ( $F = 66.4$ ;  $DF = 1, 4$ ;  $P = 0.01231$ ) of ticks collected from field plots ( $31.1 \pm 5.2\%$  of ticks collected from treated plots compared to  $85.6 \pm 3\%$  of ticks collected from control plots). In addition, significant mortality ( $F = 586.32$ ;  $DF = 1, 4$ ;  $P < 0.0001$ ) was

observed treated plots ( $93.8 \pm 2.3\%$ ) verses control plots ( $3.3 \pm 0.09\%$ ) for ticks collected from the field and held in lab conditions for two weeks.

Treating ticks attached to hosts such as cattle with entomopathogenic fungi present the additional obstacle of overcoming the conditions of a host's microclimate (Fernandes and Bittencourt 2008). These conditions include skin temperature, host microbiota, and the composition of chemicals secreted by the host. Furthermore, the skin temperature of warm-blooded hosts is greatly influenced by several factors including host species, age of host, body region, food, outdoor climate and host housing conditions (given that the host is domesticated) (Samish et al. 2004; 2008). Temperature in the body regions of large animal hosts such as cattle can vary substantially ranging from 30-40°C in some body regions (Polar et al. 2005a). Such temperature extremes could be detrimental to the growth, sporulation and virulence of most strains of entomopathogenic fungi, which typically require optimal temperature of 24-27°C (Samish et al. 2004; 2008). With that taken into consideration, different strains of entomopathogenic fungi tend to have virulence capacities that differ by temperature, with some able to cause mortality on ticks at above optimal temperatures (Samish et al. 2004; 2005; Polar et al. 2005a). Polar et al. (2005a) found that the *M. anisopliae* strain IMI386697 strain grew on synthetic media at 31-35°C and was more pathogenic to *R. (B.) microplus* ticks feeding on cattle than *M. anisopliae* strain ARSEF3297 under the same growth conditions. Most on-host tick control trials with entomopathogenic fungi have treated ticks infesting gerbils (Samish et al. 2008), mice (Hornbostel et al. 2005), rabbits (Kaaya et al. 1996) horses (Samish et al. 2004; 2008), and cattle (Samish et al. 2004; 2008). Results of these trials have varied quite markedly and tend to give lower mortalities than most laboratory studies. Many on-host studies record tick mortality from cattle by looking at tick reductions on the cattle host (Samish et al. 2004; 2008).

In some of these studies engorged females are taken from cattle and then reared in the lab at optimum temperatures to encourage mycoses of infected ticks (Samish et al 2004; 2008; Nchu et al 2006).

### **Formulations for the Application of Entomopathogenic Fungi**

In most laboratory studies conducted with *M. anisopliae* s.l. and *B. bassiana*, spores are typically suspended in water with a small amount of emulsifying agent (e.g. Tween 80, Tween 20, Triton-X 100, etc.) (Samish et al. 2004; 2008). In experimental field trials however, aqueous suspension of fungal conidia has resulted in low tick mortalities except in the instance of multiple applications (Fernandes et al. 2012). In order to increase the efficacy of conidial suspensions, several studies have formulated conidia in oil suspensions mixed with water and emulsifying agents (Kaaya 2000; Kaaya and Hassan 2000; Maranga et al. 2005; Kaaya et al. 2011). These oil-formulated conidial suspensions have shown to be effective at controlling several tropical and subtropical tick species, causing higher mortality in ticks than aqueous suspensions of conidia (Fernandes and Bittencourt 2008; Fernandes et al. 2012). Additionally, oil-and-water formulations might also allow for more conidia to adhere to tick cuticles and can even serve to protect conidia from damage caused by ultraviolet radiation and desiccation (Fernandes and Bittencourt 2008; Fernandes et al. 2012). The use of ultra-low volume technology to apply oil-formulated conidia has also shown to be effective for assisting in the fungal control of ticks (Fernandes and Bittencourt 2008). A few biocontrol studies conducted on the tick *D. nitens* Nuemann (= *Anocentor nitens*) have shown significant tick mortality when the ears of cattle were treated with fungal conidia formulated in polymerized cellulose gel (Reis et al. 2008; Souza et al. 2009; Fernandes et al. 2012). A study by Polar et al. (2005b) evaluated different formulations of water and oil-based agents (paraffin oil, palm oil, emulsifiable adjuvant

oils, Cropspray and Codacide oil) by using germination tests of *M. anisopliae* spores and a bioassay of the *M. anisopliae* conidia on *R. (B.) microplus* ticks. Overall, a formulation of 10% emulsifiable liquid paraffin was found to be the best formulation for *M. anisopliae*. As mentioned previously, Behle et al. (2013) tested a microsclerotial, granular formulation of *M. brunneum* strain F52 against *I. scapularis* nymphs. Results from this study showed significant tick mortality (56% and 74% in fed and unfed nymphs respectively), high rates of conidial production ( $3.05 \times 10^9$  to  $1.24 \times 10^{10}$  conidia/g) from granules placed in moistened soil, and the persistence of viable conidia after 8 weeks post-application. In addition, the microsclerotial granules started to become infective to ticks two weeks after placement into moist potting soil.

### **Factors Affecting the Virulence of Entomopathogenic Fungi**

One of the main drawbacks of using entomopathogenic fungi for the control of ticks is that they take long periods of time to cause mortality in ticks. This time interval ranges from as few as two days to several weeks or even months (Gindin et al. 2002; Samish et al 2004; 2008). The application of entomopathogenic fungi in the field requires a high concentration of conidia (e.g.  $10^8$  to  $10^9$  conidia/ml) to effectively control ticks (Fernandes et al. 2012). Efficacy of entomopathogenic fungi in killing ticks varies considerably by the fungal species and strain used, conidial formulation and the life stage of tick infected (Samish et al. 2004; 2008; Ostfeld et al. 2006; Fernandes et al. 2008; 2012). In addition, culturing and storage conditions of entomopathogenic fungal strains can either degrade, enhance or preserve virulence of the fungi (Samish et al. 2008; 2004; Fernandes et al. 2012). Improper culturing conditions such as improper culture media, pH range, temperature and multiple transfers of the fungi to synthetic media can degrade cultures of entomopathogenic fungi (Fernandes et al. 2012). Improper mass production, and prolonged storage at high humidity and temperature can also degrade the

virulence of entomopathogenic fungal strains. Maintaining fungal isolates on live arthropod hosts however can sometimes restore the degraded virulence of those isolates (Fernandes et al. 2012). Furthermore, the virulence of entomopathogenic fungal strains can be genetically manipulated to improve virulence, higher tolerance to environmental conditions and increased conidial output (Fernandes et al. 2012; Aw and Hue 2017).

### **Research Objectives**

The objectives of this thesis were to: isolate strains of entomopathogenic fungi from *A. americanum* ticks and soil in Arkansas; identify potential fungal pathogens of ticks by using morphological and molecular sequencing techniques; and to assess the pathogenicity of few of the Arkansas isolates of entomopathogenic fungi on *A. americanum* adults and nymphs in laboratory bioassays compared to the pathogenicity of the fungal insecticide Met52®. Results of these findings could lead to further exploration of entomopathogenic fungi from Arkansas as viable agents for the control of *A. americanum* and other important tick species in the state of Arkansas, potentially as part of an integrated tick management program.

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### **CHAPTER 3: Isolation of Strains of *Metarhizium anisopliae* (Metchnikoff) Sorokin from *Amblyomma americanum* L. and Soil from Northwest Arkansas**

#### **Abstract**

Arkansas has seen an increase in the number of reported tick encounters and cases of tick-borne illness due to the rapid urbanization of land across the state. The lone star tick *Amblyomma americanum* L. is the most abundant tick species found in Arkansas and is implicated in the transmission of pathogens of medical and veterinary importance. When not feeding, most non-nidicolous tick species shelter themselves in the soil and leaf litter where they become exposed and potentially infected with entomopathogenic fungi that naturally reside in the soil. The entomopathogenic fungus *Metarhizium anisopliae* (Metchnikoff) Sorokin has previously shown promise as a biological control agent of ticks. Here, the first study to isolate and identify Arkansas-derived isolates of *M. anisopliae* from field-collected *A. americanum* ticks exposed to soil is presented. From Savoy, Arkansas, 1.9% of all ticks artificially exposed to soil (N=320) were infected with *M. anisopliae* with 2.5% of adults (N=120) and 1.5% of nymphs (N=200) showing infection. Of the Savoy adults infected with *M. anisopliae*, 3.3% of adult females (N=60), and 1.7% of males (N = 60) were infected. A total of 1.9% of ticks (N = 260) from West Fork, Arkansas, consisting of 2.4% of nymphs (N = 210) were infected with *M. anisopliae*. Eleven fungal isolates (eight *M. anisopliae* and three of unknown species) were cultured from infected ticks exposed to soil. The identities of two *M. anisopliae* isolates (named Savoy P10N1 and West Fork P9N2) were confirmed by DNA sequencing and a GenBank BLAST analysis. Savoy P10N1 (GenBank accession number MN255810) was a 100% match to *M. anisopliae* with the GenBank accession number MG844433, while West Fork P9N2 (GenBank accession number MN255811) differed from the other two isolates by two



nucleotides. The two *M. anisopliae* isolates in addition to two other *Metarhizium* isolates that had not been identified to species were deposited into the United States Department of Agriculture (USDA), Agricultural Research Service (ARS), Collection of Entomopathogenic Fungi (ARSEF): Savoy isolate P10N1 (ARSEF 14329), West Fork isolate P9N2 (14330), Savoy P10AF1 (ARSEF 14331) and Savoy P2AM1 (ARSEF 14332). The isolation and identification of *M. anisopliae* from *A. americanum* in Arkansas merits further exploration of entomopathogenic fungi to be used for development as biological agents to control *A. americanum* in Arkansas and elsewhere in the U.S. where this tick species is abundant.

### **Introduction**

Ticks (Acari: Ixodida) are a diverse group of arachnids (approximately 900 species) that are obligate, blood feeding ectoparasites of several vertebrate groups including humans, livestock, companion animals and wildlife (Dantes-Torres et al. 2012; Sonenshine and Roe 2013). Globally, ticks are important vectors of many disease-causing pathogens, including bacteria, rickettsia, viruses, protozoa, and even toxic components of their saliva. In addition, they harbor the highest diversity of vector-borne pathogens of any arthropod disease vector and their medical/veterinary impact worldwide rivals that of mosquitoes (Sonenshine and Roe 2013; Nicholson et al. 2018). In particular, the state of Arkansas has seen an increase in the number of tick encounters and cases of tick-borne illness due to the rapid urban development that has brought humans closer to tick habitats (Trout 2010).

In Arkansas and throughout much of the southeastern United States, the most encountered tick species is the lone star tick *Amblyomma americanum* L. (Childs and Paddock 2003; Goddard and Varela-Stokes 2008; Trout 2010; Loftin and Smith personal communication; <http://gislabualr.maps.arcgis.com/apps/webappviewer/index.html?id=7846cd984bb4440795553b>

669c1ee31b). This species feeds on a wide variety of domesticated and wild animals including livestock, humans, companion animals and even birds (Childs and Paddock 2003; Holderman and Kaufman 2013). However, the white-tailed deer (*Odocoileus virginianus* Zimmermann) is considered to be a key host of *A. americanum* since it is a food source for all three host-seeking life stages of this tick species and is thought to harbor many of the pathogens vectored by *A. americanum* (Childs and Paddock 2003; Paddock and Yabsley 2007; Holderman and Kaufman 2013). Furthermore, growing populations of white-tailed deer have also led to an increase in the densities of *A. americanum* and are implicated in the distribution of *A. americanum* to other habitats and regions throughout the U.S (Paddock and Yabsley 2007).

*Amblyomma americanum* is known to vector many pathogens including the causal agents of human ehrlichiosis (*Ehrlichia chaffeensis* Anderson et al. 1991 and *E. ewingii* Anderson et al. 1992), tularemia (*Francisella tularensis* McCoy and Chapin), and some *Rickettsia* species (Childs and Paddock; Trout 2010; Holderman and Kaufman 2013; Loftin and Hopkins 2014; Nicholson et al. 2018). This tick species was once implicated in vectoring *Borrelia lonestari* Barbour et al. 1996 the supposed causal agent of southern tick associated rash illness (STARI) (CDC 2018). However, further research has shown that the cause of STARI is still unknown (Wormser et al. 2005). Recently, *A. americanum* has been implicated as the vector for several emerging viral tick-borne pathogens such as the Heartland virus (Phenuiviridae: *Banyangvirus*) and the Bourbon virus (Orthomyxoviridae: *Thogotovirus*) (Nicholson et al. 2018). In addition, components of *A. americanum* saliva have been known to induce an allergic reaction to mammalian meat red meat known as alpha-gal syndrome (Commins et al. 2011; Nicholson et al. 2018). Using acarological survey data and environmental data Ragahavn et al. (2019) modeled the potential geographic extent of *A. americanum* under present and future environmental

conditions and found that this species has been expanded its range farther northward and westward than was previously known. Furthermore, the range of *A. americanum* is likely to expand due to climate change.

Historically, most tick control measures have implemented the use of synthetic chemical acaricides, which provide a quick knockdown of ticks as well as long-term residual control (e.g. over 3 weeks' time) (Samish et al. 2004; 2008; Stafford and Williams 2017; White and Gaff 2018). However, concerns of increasing acaricide resistance in several species of ticks (Abbas et al. 2014) and concerns of possible negative environmental effects (Kunz and Kemp 1994) have led to the search for alternative tick control measures. These methods include habitat modification (Meyer et al. 1982; Presley and Hair 1988), deer exclusion/deer culling (White and Gaff 2018; Williams et al. 2018) the use of botanical acaricides (White and Gaff 2018), on-host tick control on wildlife (e.g. 4-poster system on deer, bait-boxes and bait tubes on mice) (Ostfeld et al. 2006; Stafford and Williams 2017; Williams et al. 2018), and the use of natural enemies and pathogens (e.g. tick predators, parasitoids, and pathogens) (Samish and Rehacek 1999; Samish and Alekseev 2001; Samish 2000; Samish et al. 2004; 2008; Ostfeld et al. 2006; Fernandes et al. 2012).

Tick predators and parasitoids are mostly effective at high tick densities with some predators feeding on ticks when other food sources are in limited supply (Samish and Rehacek 1999; Samish et al. 2004; 2008). Tick pathogens such as bacteria, entomopathogenic nematodes and entomopathogenic fungi have been studied as potential tick control agents. Of the three tick pathogens mentioned, entomopathogenic fungi are thus far considered to be the most effective biocontrol agents of ticks. These fungi are known to infect all life stages of ticks and penetrate directly through the integument of various arthropod groups (Samish et al. 2004; 2008).

Entomopathogenic fungi are known to occur naturally in the leaf litter and soil where ticks spend 90% of their life cycle (Samish et al 2004; 2008; Tuininga et al. 2009; Burtis et al. 2019). A few studies in Europe, South America, Africa, and the North America have examined the natural associations of ticks with entomopathogenic fungi and have isolated a number of well-known fungal pathogens from ticks (Samsinakova et al. 1974; Estrada-Peña 1990; Kalsbeek et al. 1995; Mwangi et al. 1995; Zhiou et al. 1999; Samish and Rehacek 1999; da Costa et al. 2002; Samish et al. 2004; 2008; Tuininga et al. 2009; Greengarten et al. 2011; Fernandes et al 2012). Of these fungi, strains of *Metarhizium anisopliae* sensu lato (s.l.) (Metchnikoff) Sorokin and *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin are thus far, the most extensively studied and also, the most effective biocontrol agents used against ticks. To date, few studies have reported on the isolation of *M. anisopliae* s.l. from ticks (da Costa et al. 2002; Benoit et al. 2005; Tuininga et al. 2009). Furthermore, much less is known about entomopathogenic fungi isolated from *A. americanum*, and their potential for use as a biocontrol agent for this tick species.

The objectives of this study were to isolate entomopathogenic fungi using soil exposure methods with *A. americanum* ticks and to identify fungal pathogens from ticks using morphological and molecular techniques.

## **Methods**

### **Tick Collections**

Ticks were collected from two locations: the University of Arkansas Agricultural Experiment Station, Savoy Research Complex, Beef Cattle Research Area in Savoy, Arkansas (36.128° latitude, -94.331° longitude) on April 4, 2018, and on May 18<sup>th</sup>, 2018, and West Fork, Arkansas (35.960° latitude, -94.151° longitude) on June 7<sup>th</sup>, 2018 (Figure 2.1). Locations ticks were collected from were 24.7 km apart from each other (Figure 2.1). Collection sites at both

locations were within forested areas or on the edge of forested areas close to open pastureland. Ticks were collected with carbon dioxide traps consisting of 1.9 L coolers (Igloo Products Corp. Katy, TX) filled with 0.5 kg of dry ice and placed on 1m<sup>2</sup> white cloth (Figure 2.2). Carbon dioxide traps were placed 130 meters apart at 10 different collection sites around each location and were left exposed to the environment for approximately two hours. In addition, drag samples were collected at both locations by dragging with 58 × 114 cm drag cloths (Bioquip Products Inc., Rancho Dominguez, CA) in 5-10 transects for 130 m. At both sampling locations in Savoy and West Fork, ten soil samples were collected from the top 5 mm of soil, 5 meters from each carbon dioxide trap. GPS coordinates for the location of each carbon dioxide trap at Savoy and West Fork were recorded with the Compass app on an iPhone 7 Plus (Apple Incorporated, Cupertino CA). Cloths containing ticks collected by carbon dioxide were placed in 7.6 L plastic bags and placed in a refrigerator at 4°C along with bags of soil samples. Ticks were held in the storage anywhere from 1 to 42 days (Savoy) or 1 to 7 days (West Fork) days while soil samples were held in storage from 5-7 days. Ticks collected by dragging were placed in plastic vials containing a small blade of grass and stored at room temperature (29-28° C).

#### Tick Identification and Sorting

Ticks from both sampling locations were sorted by location, species, sex, and life stage. Sorted ticks were placed in plastic vials along with a piece of grass to retain moisture and to keep them from dehydrating (Loftin personal communication). Ticks were held at room temperature for 1-7 days (the approximate time it took to sort ticks). Identification of ticks to species were made using Lancaster (1972) and an online tick identification key from Georgia Southern University (Bischof n.d.). Ticks that were found dead when sorted were preserved in glass vials with 70% ethanol.

## Soil Exposure Assays

Soil exposure assays were used for collecting entomopathogenic fungi by the forced contact of *A. americanum* ticks to soil samples collected from Savoy and West Fork using modified methods similar to Zimmerman (1986), Meyling (2007) and Tuininga et al. (2009). Only *A. americanum* ticks were exposed to soil since they comprised 99% (N=2802) of all ticks collected from both Arkansas locations (Table 2.1). Ticks were placed in three sets of ten petri dishes, each filled with 8-16 grams of moist soil from one of the ten collection sites from Savoy or West Fork (Figure 2.3). The three sets of soil samples were either given adult females (4 or 6 ticks per sample), adult males (1 or 6 ticks per sample) or nymphal ticks (20-21 ticks per sample) depending on the availability of live, wild-caught ticks. Ticks placed in each soil sample were pooled together from all collection sites from either Savoy or West Fork before exposing them to soil samples. Soil samples were then moistened with 6-7 ml of deionized water, covered with filter paper, and wrapped in Parafilm® (Bemis Company, Inc., Neenah, WI) and medical tape to prevent ticks from escaping. Each set of soil samples with ticks were wrapped in aluminum foil and stored in an incubator at  $28.7 \pm 0.7^{\circ}\text{C}$  and  $61.4 \pm 5.6\%$  relative humidity (RH). Ticks from each soil sample were checked once a week for mortality for two weeks. Dead ticks that were found were washed in two separate baths of deionized water and placed in separate petri dishes moistened with 1-2 ml of deionized water. Dead ticks were photographed under a Nikon® SMZ-1 or Nikon® SMZ1000 microscope (Nikon Corporation, Tokyo, Japan). After two weeks of exposure to the soil all ticks were removed from petri dishes containing soil. Ticks that had died were checked weekly for fungal growth once a week for a period of two weeks.

## Fungal Isolations

Ticks from Savoy and West Fork that were identified as having potential isolates of entomopathogenic fungi (i.e. fungi were seen emerging out from within the tick's leg joints, mouthparts and idiosoma) were inoculated on plates of Sabouraud dextrose agar (SDA 64 g/L) supplemented with yeast extract (2 g/L) and gentamicin sulfate (10 mg/L) on June 21, June 28, and July 20, 2018 (Goettel and Ingles 1997; Ingles et al. 2012). Fungal isolates were scraped from infected ticks with a sterile, platinum inoculating loop and immersed in 1 ml of solution of sterile deionized water with 0.05% Tween 80 and penicillin/streptomycin (1.5 mg/L penicillin G: 2.5mg/L streptomycin). Spore suspensions from all fungal isolates were vortexed thoroughly and 0.2 ml of spore suspension was added to five 100×15mm SDA plates for each isolate. Spore suspensions were spread across each plate with a sterile glass spreading rod. Plates were wrapped in aluminum foil and placed in an incubator at  $26.8\pm0.6^{\circ}\text{C}$  and  $58\%\pm5.2$  RH.

## Fungal Identification: Morphology

Conidia from fungal isolates grown on SDA plates were scraped with a sterile needle into a drop of lactophenol solution (Humber 1997). A coverslip was placed over each fungal isolate and sealed with red fingernail polish (L.A. Colors Cosmetics, Ontario, CA). Fungal isolates were viewed under a Nikon® Eclipse E600 phase contrast microscope (Nikon Corporation, Tokyo Japan) at 200X and 400X magnification and at two different phases corresponding to each power of magnification. Fungal identification was performed by examining conidia and conidiophore structures using Humber (1997). Conidia identified as *Metarhizium* species from cultures were additionally examined and measured at 630X magnification using a Zeiss® Axio Imager A1 with AxioCam 1C and Zen 2 Lite software (Carl Zeiss MicroImaging, Göttingen, Germany).

### Molecular Identification Using DNA Sequencing

Samples from two different cultures isolated from nymphal *A. americanum* ticks (one from Savoy and one from West Fork) were taken to the Insect Genetics Lab, University of Arkansas (Fayetteville, AR) for genetic analysis. DNA was extracted with DNeasy® (Qiagen Sciences, Germantown, MD) and resuspended in 10mM Tris: EDTA (pH 8.0). Both isolates were previously identified as *Metarhizium* species based on the morphology of their conidia and conidiophore structures (Humber 1997). Extracted samples of DNA were maintained at -20°C. A section of the nuclear ribosomal rRNA gene internal transcribed spacer (ITS) region was amplified with PCR using primers ITS4 and ITS5 (White et al. 1990) following Taylor et al. (1996). Samples were purified and concentrated with centrifugal devices from VWR™ (Radnor, PA) and sent off to Eurofins Genomics (Huntsville, AL) for direct sequencing in both directions. Sequences were aligned and annotated with Geneious v6.16 (Kearse et al. 2012). A BLAST search was used for species level identification. Sequences of both fungal isolates were deposited into GenBank and assigned unique accession numbers.

### Depositing Fungal Isolates into USDA ARSEF

Four fungal isolates that were morphologically identified as *Metarhizium* species (including the two isolates that were sequenced) were sent to the United States Department of Agriculture (USDA), Agricultural Research Service (ARS), Collection of Entomopathogenic Fungi (ARSEF) (USDA ARS 2019). Fungal cultures were then deposited to the ARSEF collection and given a unique accession number.



## Results

### Tick Collections

A total of 2802 ticks were collected from both Savoy and West Fork (Table 2.1). On April 5, 2018 and May 18, 2018, 555 and 1746 ticks (all *A. americanum*) were collected from Savoy on the first and second date respectively, for a total of 2301 ticks. On June 7, 2018 a total of 501 ticks that were collected from West Fork comprised primarily of *A. americanum* (96.8%). In addition, 14 (2.8 %) *Dermacentor variabilis* Say and two (0.4%) unidentified *Ixodes* nymphs were found at West Fork. At both locations, 99.4% of the ticks found (N=2802) were *A. americanum*, the majority of which were nymphs (81.2%). Adults comprised only 18.3% (N = 2802) of ticks collected from both locations.

### Soil Exposure Assay

All life stages of *A. americanum* ticks exposed to soil samples from Savoy were found with fungi (Tables 2.2 and 2.3) with the highest rate of fungal presence reported for adult male ticks (14 ticks; 23.3%; N = 60) followed by nymphs (41 ticks; 20.5%; N = 200) and adult females (8 ticks; 13.3%; N = 60). A total of 18.3% of adult ticks (22 ticks; N = 120) were found with fungal growth (Tables 2.2 and 2.3). Two adult female ticks (N = 60; 3.3%) and three nymphal ticks (N = 200; 1.5%) exposed to soil from one collection site in Savoy (plot 10; 36.128 latitude, -94.333 longitude) were infected with *Metarhizium* (Tables 2.2 and 2.3; Figure 2.4). Three weeks after ticks were exposed to soil an additional male tick (N = 60; 1.7%) from Savoy plot 2 (36.129 latitude, -94.322 longitude) was also found infected with *Metarhizium*. Overall, the percentage of soil-exposed ticks from Savoy infected with *Metarhizium* was 1.9% (N = 320) with 2.5% of these ticks being adults (N = 120; Tables 2.2 and 2.3).

Of the ticks exposed to soil from West Fork, 40.0% of adult males (4 males, N = 10), 37.5% of adult females (15 females; N = 40), 38.0% of all adult ticks (19 ticks; N = 50) and 20.5% of nymphs (43 nymphs; N = 210) were found with fungi (Tables 2.2 and 2.3). The overall percentage of infected *A. americanum* ticks exposed to soil from West Fork was the same as Savoy, with 1.9% of soil-exposed ticks (5 ticks; N = 260) being infected. However, unlike *A. americanum* ticks exposed to the soil samples from Savoy, *Metarhizium* infection was found from only nymphal ticks exposed to soil from three sites: plot 1 (35.962° latitude, -94.150° longitude), plot 2 (35.963° latitude, -94.151° longitude), and plot 9 (35.957° latitude, -94.151° longitude). The overall infection rate of nymphs with *Metarhizium* was 2.4% (5 nymphs; N = 210; Tables 2.2 and 2.3). In addition to ticks infected with *Metarhizium*, some adult and nymphal ticks were observed to be infected with unknown fungi.

#### Fungal Isolations

A total of twelve fungal isolates from ticks were inoculated onto SDA plates (Table 2.4). Of these fungal isolates, cultures from eleven isolates were isolated (all plates from one isolate were lost due to contamination from a black, fast-growing, saprophytic mold). Eight fungal isolates (five from Savoy, three from West Fork) were later identified as species of *Metarhizium*.

#### Morphological Identifications

Specimens of conidia and conidiophores from all eleven fungal cultures were examined (Table 2.4). Eight fungal isolates on SDA plates were identified as species of *Metarhizium*. Mycelia and conidia of the *Metarhizium* isolates varied somewhat in appearance (Figure 2.5). Fungal colonies isolated from ticks and soil from Savoy had mycelial growth that was light orange to pale yellow in appearance, had a wrinkled look, contained dark green spores borne on white mycelia, and had conidia measuring 6-7 µm by 2-3 µm (Figures 2.5 and 2.6). The

*Metarhizium* colonies from the West Fork isolate were found to be light yellow and somewhat smoother in appearance than the other *Metarhizium* isolates (Figure 2.5). The phialides borne on white mycelia in these colonies bore conidia that were a mixture of dark green and olive-green and measured 7.5-9  $\mu\text{m}$  by 3-4  $\mu\text{m}$ . The *Metarhizium* conidia from all isolates were rod-shaped and bound together in palisades. The other three fungal isolates were not identified although they were thought to be species of *Isaria* (= *Paecilomyces*). The mycelia of these fungi were white, fluffy in appearance to the naked eye and had conidia borne in spore balls. At 200-400X magnification, specimens of these fungi had ball-shaped conidia bound on phialides that were pear-shaped and pointed on the apex.

#### Molecular Identifications

Examined DNA sequences from a *Metarhizium* isolate from Savoy (designated Savoy P10N1 after Savoy collection plot 10, infected nymph number 1) and a *Metarhizium* isolate from West Fork (designated West Fork P9N2 after West Fork collection plot 9, infected nymph number 2) were positively identified as *M. anisopliae* based on a GenBank BLAST analysis. Savoy P10N1 was a 100% match to the *M. anisopliae* sequence with the GenBank accession number MG844433, while the isolate from West Fork differed from the other two sequences by two nucleotides. Sequence data for both isolates were sent off to GenBank and given the following accession numbers: Savoy P10N1 was given the GenBank accession number MN255810 while the West Fork P9N2 was given the GenBank accession number MN255811.

#### Deposition of *Metarhizium* isolates into ARSEF

Four fungal isolates that were identified as *Metarhizium* species from their morphology (including the two isolates that were confirmed as *M. anisopliae*) were submitted to the USDA ARSEF fungal collection, deposited and each given a unique accession number. The following

isolates included: Savoy P10N1; West Fork P9N2; an isolate from Savoy collection plot 10, infected adult female *A. americanum* tick number 1 (Savoy P10AF1); and an isolate from Savoy collection plot 2, infected adult male tick number 1 (Savoy P2AM1) (Table 2.5). The accession numbers given for each isolate were 14329 for Savoy P10N1, 14330 for West Fork P9N2, 14331 for Savoy P10AF1 and 14332 for Savoy P2AM1 (Louela Castrillo personal communication).

## Discussion

This is the first study to report on the isolation of entomopathogenic fungi from ticks in Arkansas and one of the first recorded instances of isolating entomopathogenic fungi infecting *A. americanum*. In particular, the confirmed presence of the generalist fungal pathogen, *M. anisopliae*, which has been used for the biological control of ticks worldwide, merits further exploration of the micro-fauna of potentially pathogenic fungi from local Arkansan soil samples and tick collections. Although fungi other than *M. anisopliae* were observed in this study, the identities of these fungi were not confirmed. Future research on isolating, identifying, and culturing pathogens other than *M. anisopliae* would be worthwhile.

*Metarhizium* species (including *M. anisopliae*) were isolated from ticks exposed to soil from both Savoy and West Fork, Arkansas. At both locations, the adult females were found to have the highest infection rate of any life stage with *Metarhizium* while nymphs had the lowest (Tables 2.2 and 2.3). Studies that have reported on ticks naturally infected with entomopathogenic fungi have found that adult ticks (particularly engorged females) have a higher infection rate to pathogenic fungi than nymphal or larval ticks (Kalsbeek et al. 1995; Zhiou et al. 1999; Samish et al. 2004; 2008). However, the percentages of ticks infected with *Metarhizium* species among all life stages exposed to soil hardly differed from one another (< 2%). In addition, the overall percentages of soil-exposed *A. americanum* with *Metarhizium* for

both Savoy and West Fork was the same (i.e. 1.9%). While ticks used in this study were caught in the wild, infection with entomopathogenic fungi from soil was induced by forced contact with the soil samples in laboratory conditions. The findings in this study might not give an accurate representation of true infection rates for all life stages infected since the sample sizes used for adult females and males (4-6 per soil sample for females and 1-6 for males) were lower than those used for nymphs (20-21 per soil sample). The low sample size was due to the fact that fewer adults were collected than nymphs (Table 2.1) and because many of the ticks collected (which were held in refrigeration or room temperature anywhere from 1-42 days at < 30% RH) died from desiccation before they could be exposed to moist soil. Future studies should allow for recently collected ticks to be stored in a humidity chamber with a relative humidity of > 90% (Winston and Bates 1960; Troughton and Levin 2007; Levin and Schumacher 2016). In addition, the actual number of ticks that were infected by entomopathogenic fungi was unknown, likely due to the ticks drowning in waterlogged soil (6-7ml of water was used to moisten soil samples), and the fact that many of the ticks that had died were found infected with fungi (either saprophytic or entomopathogenic) that were not identified. As a measure to minimize growth of contaminant organisms such as saprophytic fungi and bacteria from dead ticks, future studies should use surface sterilization of field collected ticks exposed to moist soil immediately after ticks have died from fungal infection (da Costa et al. 2002; Tuininga et al. 2009). Future trials could also implement the sterilized soil as a control in order to compare infection rates from different locations (Tuininga et al. 2009).

All ticks and soil collections were collected from the mid-spring to early summer, a period of time when adult and nymphal *A. americanum* ticks are most active and abundant in the southeastern United States (Childs and Paddock 2003; Goddard and Varela-Stokes 2008; Trout

2010; Nicholson et al. 2018). Depending on the season, the infection rate of entomopathogenic fungi on ticks tends to vary (Samsinakova et al 1974; Kalsbeek et al. 1995; Samish et al. 2004; 2008). In this study the overall infection rate of *A. americanum* ticks with fungi (other fungi and *Metarhizium* species) ranged from 19.7-24.2% (Tables 2.2 and 2.3) from ticks collected in the spring to early summer (early April to early June). However, the overall infection rate of ticks with species of *Metarhizium* from either Savoy or West Fork was <2% at the same time period. Samsinakova et al. (1974) reported that a significantly higher percentage of adult *Ixodes ricinis* L. ticks collected from forested areas in Moravia, Czech Republic forest were infected with fungi in the summer (50%) than in the winter (7.5%). Kalsbeek et al. (1995) found significantly higher proportion of engorged females collected from Grib Skov, Denmark were infected during the fall (32.2%) than during the spring (6.1%). Overall, the prevalence of infected ticks collected in the fall was 30.3% compared to 10% infected in the spring. Future studies conducted in Arkansas could look at the fungal infection prevalence and species composition of fungi found in *A. americanum* ticks in different seasons.

Despite being one of the most pathogenic fungi on ticks, and also one of the most extensively studied pathogens for their control, *M. anisopliae* has rarely been isolated from naturally infected ticks (da Costa 2002; Samish et al. 2004; 2008; Benoit et al. 2005; Tuininga et al. 2009). However, one study conducted in Brazil (da Costa et al. 2002) has previously documented the isolation of *M. anisopliae* from engorged female *Rhipicephalus (Boophilus) microplus* Canestrini in addition to *B. bassiana*, which has been identified and isolated from ticks in other studies (Samsinakova et al. 1974; Kalsbeek et al. 1995; Tuininga et al. 2009). Out of 200 engorged female *R. (B.) microplus* ticks, 69 colonies (71%) of *B. bassiana* and 20 colonies (29%) of *M. anisopliae* were isolated from ticks. In addition, Benoit et al. (2005) was able to

isolate *M. anisopliae* internally and externally from *I. scapularis* (24 % of ticks internally, 9% of ticks externally) and *R. sanguineus* (7% of ticks externally, 0.5% of ticks externally). However, these fungal infection percentages reported by Benoit et al. are not comparable, and do not accurately represent the actual estimations of ticks in the wild (i.e. *I. scapularis* ticks in this study came from wild caught population while *R. sanguineus* ticks came from lab-reared tick colonies). In a study conducted by Tuininga et al. (2009) at the Fordham University, Louis Calder biological field station in Armonk Westchester Co., NY, entomopathogenic fungi were cultured from 25% of wild-caught *I. scapularis* Say nymphs (N = 64), which included the genera *Beauveria*, *Metarhizium*, *Isaria* (= *Paecilomyces*) and *Lecanicillium* in addition to five unidentified species of fungi.

A common method used for isolating entomopathogenic fungi from soil is by the insect baiting method, which involves exposing highly susceptible arthropod hosts (typically larvae of the greater wax moth *Galleria mellonella* L. or the yellow mealworm *Tenebrio molitor* L.) to soil samples that might contain entomopathogenic fungi (Zimmermann 1986; Meyling 2007; Tuininga et al. 2009; Bharadwaj and Stafford 2011). In a study conducted by Tuininga et al. (2009) soil exposure assays with *G. mellonella* were used successfully to isolate colonies of pathogenic fungi belonging to the genera, *Beauveria*, *Metarhizium*, *Isaria* (= *Paecilomyces*) as well as other entomopathogenic fungi. This current study utilized a novel approach of the insect baiting method by substituting *G. mellonella* larvae with field collected *A. americanum* ticks. Since ticks naturally associate with the soil and leaf litter (Samish 2004; 2008; Burtis et al. 2019), their use in the arthropod soil baiting method might be advantageous for pinpointing entomopathogenic fungi that have a specific virulence to a particular tick species—especially *A. americanum*. However, in order for the soil-baiting method to be effective for isolating

entomopathogenic fungi using ticks, dead ticks would first need to be surface sterilized immediately after they die in order to eliminate the possibility of contaminant fungal growth or naturally occurring fungal infection with entomopathogenic fungi. Furthermore, it must be mentioned that a strain of entomopathogenic fungus isolated from a specific arthropod host does not necessarily mean that a fungal strain is more virulent to one host than fungi isolated from other hosts (Fernandes et al. 2006; Samish et al. 2008). Further research should explore standardization and application of the soil baiting method with ticks as a means of surveying for and isolating potential entomopathogenic fungi that can be developed for tick management. The effectiveness of the tick soil-baiting method to isolate cultures of entomopathogenic fungi could then be compared to the *G. mellonella* method and the direct isolation of entomopathogenic fungi from naturally infected, wild-caught ticks.

Of significant importance to this study is the successful isolation and identification of *M. anisopliae* from Arkansas ticks and soil. Strains of the *M. anisopliae* species complex (i.e. *M. anisopliae* s.l.) have been some of the most extensively studied biological control agents of ticks and also some of most effective fungi implemented for tick management programs in the United States and across the globe (Samish et al. 2004; 2008; Ostfeld et al. 2006; Fernandes and Bittencourt 2008; Fernandes et al. 2012). *Metarhizium anisopliae* s.l. are saprotrophic fungi that are widely distributed in soils in forests and in disturbed habitats such as agricultural fields (Meyling and Eilenburg 2007). A previous review conducted by Faria et al. (2007) found that 58 out of 170 mycoinsecticide products that had been developed (33.9%) were *M. anisopliae* s.l. Of those products, four were marketed for the control of ticks (Chapter 2). Currently the bio-insecticide Met52® (Novozymes Biological Inc. Salem, VA), which contains conidia of the fungus *M. brunneum* Petch strain F52 (= *M. anisopliae* strain F52) (Bischoff et al. 2009), is the



only mycoinsecticide marketed for the control of ticks in the United States (Braverman 2019; Stefan Jaronski, personal communication). Previous lab and field studies conducted with this mycoinsecticide in the U.S. have shown it to be effective against the blacklegged tick *I. scapularis* (Bharadwaj and Stafford 2010; 2012; Stafford and Allen 2010; Behle et al. 2013; Williams et al. 2018). Strain F52 is not a native *Metarhizium* strain from the United States as it was originally isolated in Austria from an infected codling moth (*Cydia pomonella* L.) (EPA 2003; EFSA 2012; Fischhoff et al. 2017). For the management of ticks in Arkansas and across the U.S., further discovery of local strains of entomopathogenic fungi that are comparable, if not more efficacious than *M. brunneum* strain F52 would be particularly useful for development of mycoinsecticides that show specific virulence to *A. americanum*. Such mycoinsecticides could potentially be an invaluable part of any integrated tick control program.

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## Tables and Figures

Table 3.1 Numbers of ticks collected from both Savoy and West Fork, Arkansas (Washington Co.) by location and collection date.

Tick Species and Life Stage	Location		
	Savoy		West Fork
	Collection Date		Collection Date
	April 5, 2018	May 18, 2018	June 7, 2018
<i>Amblyomma americanum</i> adults	269	135	108
<i>Amblyomma americanum</i> nymphs	286	1611	377
Total <i>Amblyomma americanum</i>	555	1746	485
<i>Dermacentor variabilis</i> adults	0	0	14
<i>Ixodes</i> spp. Nymphs	0	0	2
Total Ticks	555	1746	501

Table 3.2 The number of wild caught *Amblyomma americanum* ticks infected with fungi after two weeks of exposure to soil from northwest Arkansas. Both ticks and soil samples were collected from Savoy and West Fork Arkansas (Washington Co.) during the summer of 2018.

Location	Sex/Life Stage	No. Ticks <sup>1</sup> Exposed	No. Dead	No. with Fungi <sup>2</sup>	No. Infected <i>M.a.</i> <sup>3</sup>
Savoy	Adult Female	60	10	8	2
	Adult Male	60	15	14	1
	Total Adults	120	25	22	3
	Nymph	200	60	41	3
	Total	320	85	63	6
West Fork	Adult Female	40	15	15	0
	Adult Male	10	4	4	0
	Total Adults	50	19	19	0
	Nymph	210	67	43	5
	Total	260	86	62	5

<sup>1</sup>Ticks were exposed to samples of moistened soil from Savoy and West Fork, Arkansas.

<sup>2</sup> Total number of ticks found with *Metarhizium anisopliae* and other unidentified fungi.

<sup>3</sup> Total number of ticks found with *Metarhizium anisopliae*.



Table 3.3 Percent of wild caught *Amblyomma americanum* ticks infected with fungi after two weeks of exposure to soil from northwest Arkansas. Both ticks and soil samples were collected from Savoy and West Fork Arkansas (Washington Co.) during the summer of 2018.

Location	Sex/Life	No. Ticks <sup>1</sup>	% Mortality	% With Fungi <sup>2</sup>	% <i>M.a.</i> <sup>3</sup>
	Stage	Exposed			
Savoy	Adult Female	60	11.7	13.3	3.3
	Adult Male	60	25.0	23.3	1.7
	Total Adults	120	20.8	18.3	2.5
	Nymph	200	30.0	20.5	1.5
	Total	320	26.7	19.7	1.9
West Fork					
	Adult Female	40	32.5	37.5	0
	Adult Male	10	40	40.0	0
	Total Adults	50	38.0	38.0	0
	Nymph	210	31.9	20.5	2.4
	Total	260	32.3	24.2	1.9

<sup>1</sup>Ticks were exposed to samples of moistened soil from Savoy and West Fork, Arkansas.

<sup>2</sup>Percent of ticks found with *Metarhizium anisopliae* and other fungi.

<sup>3</sup>Percent of ticks found with *Metarhizium anisopliae*.

Table 3.4 Entomopathogenic fungal cultures isolated from infected *Amblyomma americanum* ticks.

Fungi	Savoy			West Fork			Total
	Adult Female	Adult Male	Nymph	Adult Female	Adult Male	Nymph	
<i>Metarhizium</i> spp.	2	1	3	0	0	3	8
<i>M. anisopliae</i>	0	0	1	0	0	1	3
Other	2	1	0	0	0	0	3

Table 3.5 *Metarhizium* cultures sent off to the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) Collection of Entomopathogenic Fungi (ARSEF).

Isolate Locality <sup>1</sup>	Plot No.	Life Stage <sup>2</sup>	Tick ID No.	GenBank Accession No.	ARSEF Accession No.
Savoy	10	Nymphs	1	MN255810	14329
Savoy	10	Adult female	1	–	14331
Savoy	2	Adult male	1	–	14332
West Fork	9	Nymph	2	MN255811	14330

<sup>1</sup>Localities from which the isolates came from were in Washington County, Arkansas.

<sup>2</sup>All hosts were *Amblyomma americanum* ticks.

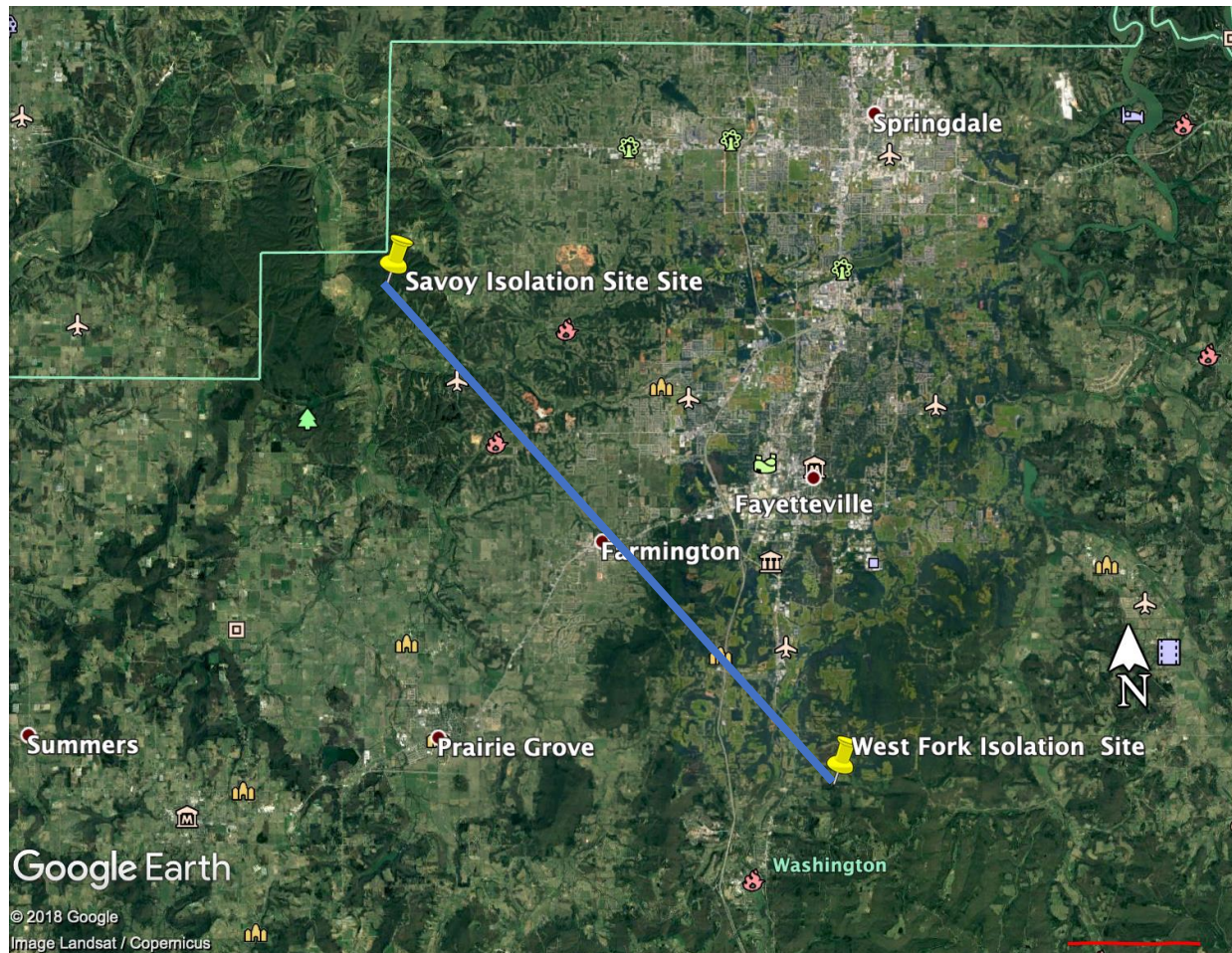


Figure 3.1 Collection locations of for *Amblyomma americanum* ticks in northwest Arkansas. Red line is approximately equal to 5 km. Blue line is approximately equal to 25 km. Photo by Google and Landsat/Copernicus.





Figure 3.3 Carbon-dioxide trap used to collect wild *Amblyomma americanum* ticks for soil exposure.



Figure 3.2 Soil samples from a northwest Arkansas collection site used for baiting assays with wild caught *A. americanum* ticks. Filter paper used to cover ticks not pictured.

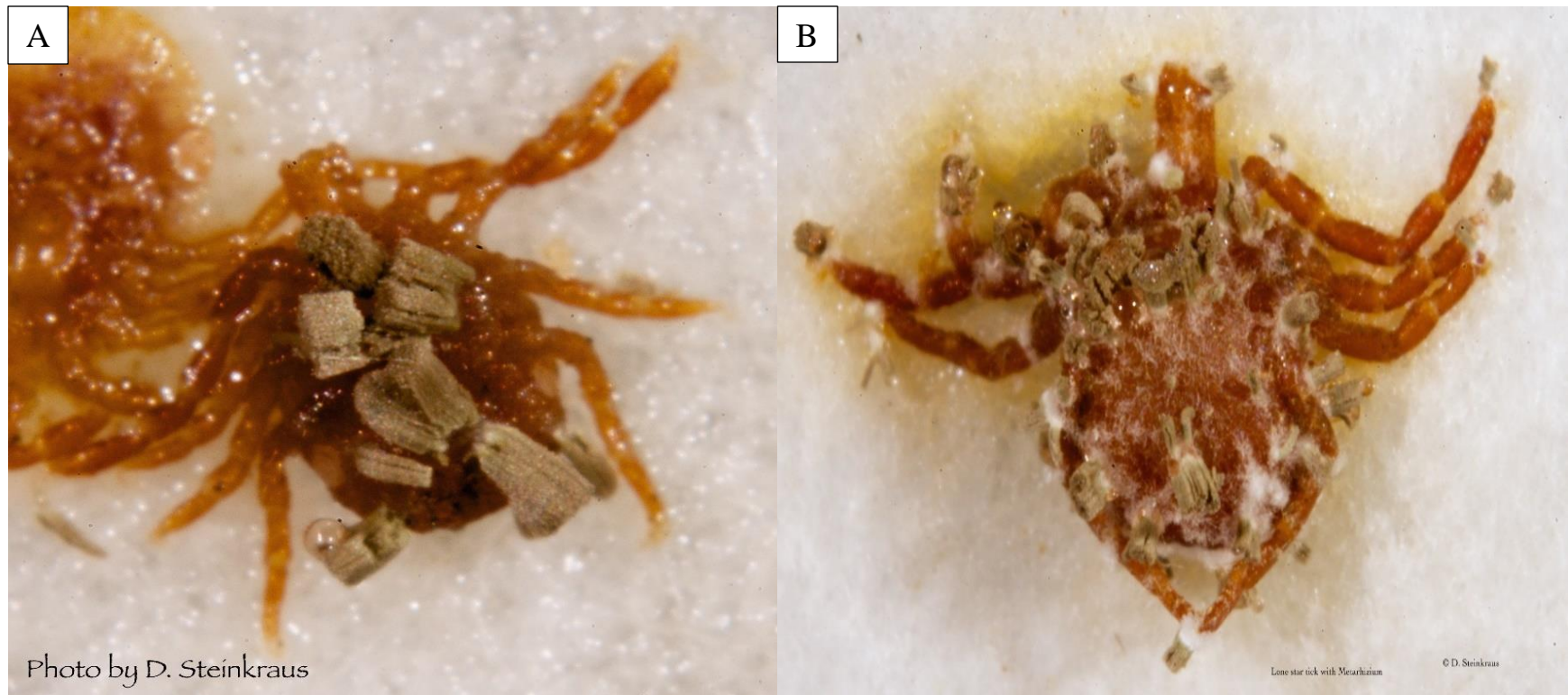


Figure 3.4 *Amblyomma americanum* nymph (A) and adult female (B) infected with *Metarhizium anisopliae* three weeks after exposure to soil samples from northwest Arkansas (Washington Co.). The diagnostically green sporulation of *Metarhizium* is shown. Photos A and B by Donald Steinkraus.



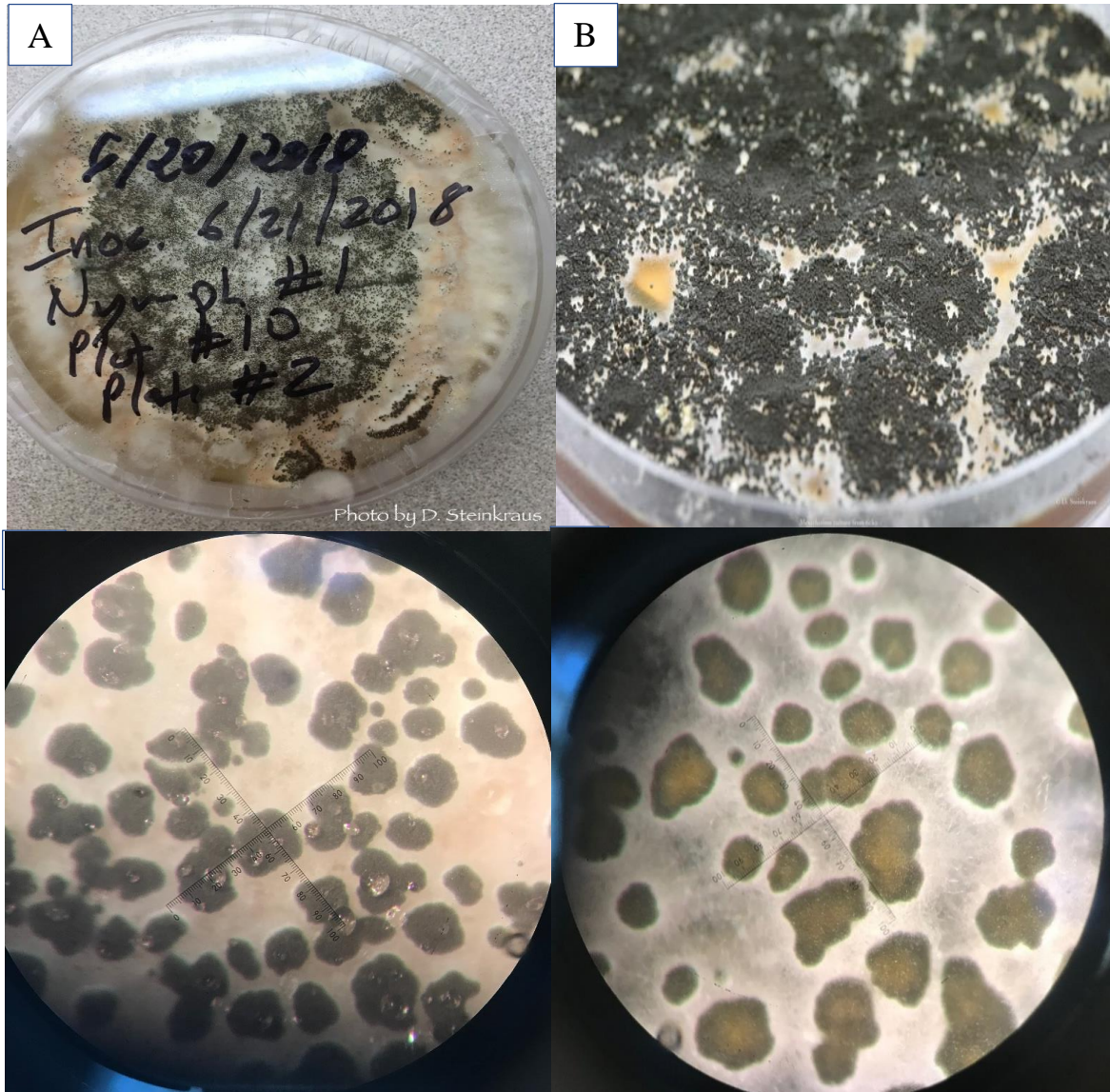


Figure 3.5 Cultures of *Metarhizium* isolated from infected *Amblyomma americanum* ticks from Savoy (A and D) and West Fork (B and C) Bottom photos taken at 80X magnification. Photos A and B by Donald Steinkraus.

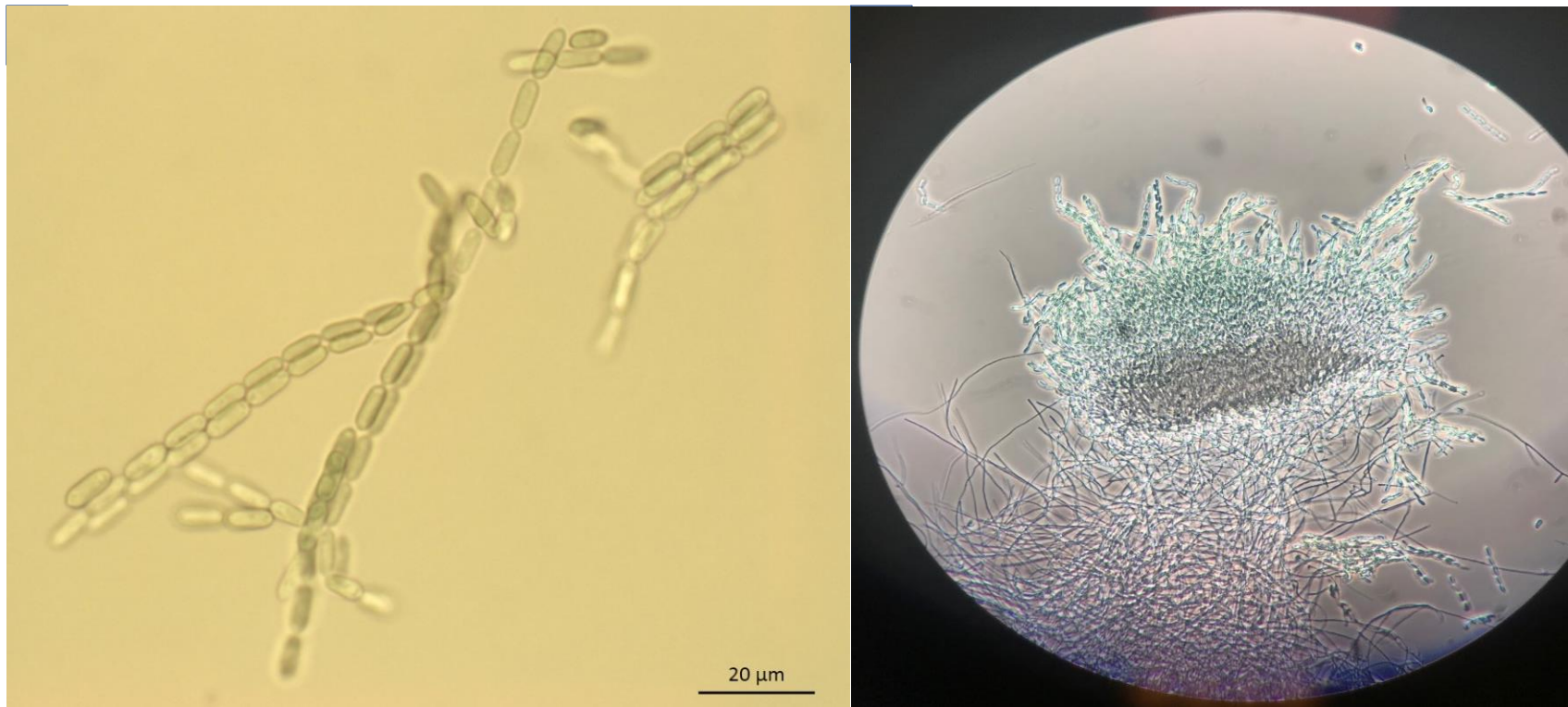


Figure 3.6 Photomicrograph of *Metarhizium* conidia chains unbound by phialides (A) and bound to phialides (B).

## **CHAPTER 4: Laboratory Bioassays of a Native Arkansas Isolate of *Metarhizium anisopliae* (Metchnikoff) Sorokin for the Control of *Amblyomma americanum* L.**

### **Abstract**

The rise of tick encounters and the incidence of tick-borne diseases in the United States (particularly in the state of Arkansas) necessitates the use of alternative tick control strategies. The lone star tick *Amblyomma americanum* L. is the most abundant tick in Arkansas and is implicated as a major pathogen vector of humans, livestock and companion animals. This study presents a laboratory bioassay evaluating the effectiveness of a native Arkansas isolate of *Metarhizium anisopliae* (Metchnikoff) Sorokin (named Savoy P10N1) compared to the commercial mycoinsecticide Met52® (*M. brunneum* Petch strain F52) for the control of adults, nymphs and larvae of *A. americanum*. A bioassay on adult ticks with Savoy P10N1 and Met52® showed that there were significant differences observed in the mortality rates between treatments ( $F = 102.17$ ;  $DF = 1, 196$ ;  $P < 0.0001$ ). At 14 days post-inoculation (days PI) over 25% of adults treated with Met52® had died from the treatment compared to <1% mortality observed for adults treated with Savoy P10N1. The mortality rate at experiment termination (77 days PI) was 100% for adults treated with Met52® at concentration of  $1 \times 10^8$  conidia/ml while the same conidial concentration of Savoy P10N1 killed only  $25.4 \pm 8.7\%$  of adults at the same time period. Adult ticks treated with Met52® died at twice the rate compared to adults treated with Savoy P10N1 on the logit scale. There were no significant differences observed in the fungal infection rates between adult ticks that died from Met52® and Savoy P10N1 ( $F = 3.43$ ;  $DF = 1, 143$ ;  $P = 0.07$ ). In the nymphal bioassay, there was no significant difference seen in the mortality rates between treatments ( $F = 0.38$ ;  $DF = 1, 196$ ;  $P = 0.54$ ) with nymphs treated with Savoy P10N1 and Met52® dying at the same rate. Nymphs were highly susceptible to Met52® with close to 90%



mortality achieved at just 2 days PI compared to  $5.3 \pm 3.3\%$  nymphs killed at 14 days PI for Savoy P10N1. Mortality for nymphs treated with  $1 \times 10^8$  conidia/ml of Savoy P10N1 and Met52® after 77 days PI was  $33.1 \pm 14.4\%$  and 100% respectively. Unlike adults, differences in the infection rates of dead ticks were seen between treatments ( $F = 4.50$ ;  $DF = 1, 176$ ;  $P = 0.03$ ). Results from the larval bioassay were inconclusive since much of the tick mortality was determined to be from confounding variables other than the treatments. This study demonstrates efficacy of Met52® compared to an Arkansas isolate of *M. anisopliae* to *A. americanum* and necessitates further research to find strains entomopathogenic fungi that are highly pathogenic, and thus effective for the control of *A. americanum*.

## Introduction

In Arkansas, the number of tick encounters with people and the number of disease cases caused by tick-vectored pathogens have been on the rise due to the rapid urbanization of the region and increased population growth (Trout 2010). The encroachment of humans onto potential tick habitats have increased the chances that ticks will move from wildlife hosts to humans.

The lone star tick *Amblyomma americanum* L. in particular, is the most abundant tick species found in Arkansas and has increased in status from being nuisance pest to a major vector of disease-causing pathogens in the United States (Lancaster 1973; Childs and Paddock 2003; Trout 2010; Loftin and Smith personal communication; <http://gislabualr.maps.arcgis.com/apps/webappviewer/index.html?id=7846cd984bb4440795553b669c1ee31b>). A recent Arkansas survey of over 10,000 ticks collected from humans, wildlife and the environment in the state from 2017-2018 showed that 76% of the ticks collected were *A. americanum* (Loftin and Smith personal communication). Pathogens vectored by *A. americanum*

include *Ehrlichia chaffeensis* Anderson et al. 1991 and *E. ewingii* Anderson et al. 1992, *E. canis* (Donatien and Lestoquard) Moshkovski, *Franciscella tularensis* McCoy and Chapin, *Cytauxzoon felis* Kier, and some *Rickettsia* species—the causal agents of human ehrlichiosis, canine ehrlichiosis tularemia, bobcat fever and some spotted fever rickettsioses respectively (Trout 2010; Holderman and Kaufman 2013; Loftin and Hopkins 2014; Brites-Neto 2015; Abubaker 2018; Nicholson et al. 2018). In addition, an emerging, life-threatening, allergic reaction to red meat known as alpha-gal syndrome (named after the oligosaccharide galactose- $\alpha$ -1, 3-galactose found in mammalian meat) has been linked to bites from *A. americanum* (Commins et al. 2011; Nicholson et al. 2018). The range of this tick species has been found to be expanding northward and westward in the United States—likely as a result of climate change (Raghavan 2019). Densities of *A. americanum* and pathogen transmission associated with them are heavily influenced by densities of white-tailed deer (*Odocoileus virginianus* Zimmermann), a major “keystone species” for all life stages of the tick (Childs and Paddock 2003; Paddock and Yabsley 2007).

Most tick control measures have involved the application of chemical acaricides — either as broadcast applications to the environment, or as direct applications to livestock or wildlife (Ostfeld et al. 2006; Stafford and Williams 2017; Nicholson et al. 2018). Although most modern acaricides are considered to be relatively safe (Nicholson et al. 2018), growing concerns of perceived environmental toxicity by the general public and problems with acaricide resistance have led to the search for novel tick control agents and strategies (Samish et al. 2004; 2008; Ostfeld et al. 2006; Abbas et al. 2014; White and Gaff 2018). Some of these methods include natural acaricides (i.e. nootkatone) (White and Gaff 2018), habitat modification (Meyer et al. 1982; White and Gaff 2018), mulch barriers (Piesman et al. 2006), deer removal/exclusion

(White and Gaff 2018; Williams et al. 2018), personal protection (CDC 2019), treatment of wildlife with self-application devices (e.g. bait boxes for rodents, “four-poster” self-application devices for deer; (Ostfeld et al. 2006; Stafford and Williams 2017; Williams et al. 2018) and the use of natural enemies and pathogens (Samish and Rehacek 1999; Samish 2000; Samish et al. 2004; 2008; Kirkland et al 2004a; b; Ostfeld et al 2006; Fernandes and Bittencourt 2008; Craddock and Needham 2011a; b; Fernandes et al. 2012). So far, entomopathogenic fungi have been some of the most effective biological control agents used for the control of ticks (Samish et al. 2004; 2008; Fernandes et al. 2012).

Many of these entomopathogenic fungi reside in the soil and have been isolated from infected ticks collected from the wild (Samsinakova et al. 1974; Estrada-Peña 1990; Kalsbeek et al. 1995; Zhioua 1999; da Costa et al. 2002; Tuininga et al. 2009; Greengarten et al. 2011). Entomopathogenic fungi can directly penetrate arthropod cuticles and can infect and kill all stages of ticks (Samish et al. 2004; 2008). The most extensively studied (and the most pathogenic) entomopathogenic fungi for the control of ticks include strains of *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin and *Metarhizium anisopliae* sensu lato (s.l.) (Metchnikoff) Sorokin (Samish et al. 2004; 2008; Fernandes and Bittencourt 2008; Fernandes et al. 2012). Several studies conducted in the laboratory and field have demonstrated the effectiveness of *B. bassiana* and *M. anisopliae* s.l. to many species of ticks across the globe (Samish et al 2004; 2008; Fernandes and Bittencourt 2008; Fernandes et al. 2012). In the United States, the biological control of ticks has focused primarily on the blacklegged tick *Ixodes scapularis* Say (Benjamin et al. 2002; Hornbostel et al. 2004; 2005; Kirkland et al. 2004b; Bharadwaj and Stafford 2010; 2012; Stafford and Allen 2010; Behle et al. 2013; Williams et al. 2018) while a few studies have focused on *A. americanum* and the American dog tick

*Dermacentor variabilis* Say (Gomathinayagam et al. 2002; Kirkland et al. 2004a; Craddock and Needham 2011a; b).

The fungus *M. brunneum* Petch strain F52 (= *M. anisopliae* strain F52) (Bischoff et al. 2009) has been developed into the mycoinsecticide Met52® (Novozymes Biological Inc., Salem, VA) for use in United States, Canada and some European countries to control thrips, white flies, weevils, mites and ticks (Novozymes 2019). Met52® has been found to be safe for use around aquatic and terrestrial vertebrates and has shown few off-target effects to non-target invertebrates such as honeybees, lacewings, earthworms, parasitic wasps and some soil-dwelling arthropods. (EPA 2003; EFSA 2012; Fischhoff et al. 2017). Several field and laboratory studies (Bharadwaj and Stafford, 2010; 2012; Stafford and Allen 2010; Behle et al. 2013; Williams et al. 2018) have shown *M. brunneum* F52 to be an effective control agent against *I. scapularis*.

To date there have been no studies in Arkansas that have evaluated the effectiveness of strains of *M. anisopliae* or Met52® for the control of the *A. americanum*. The objectives of this study were to assess the effectiveness of a native strain of *M. anisopliae* for the control of adults, nymphs and larvae of *A. americanum* and to compare its effectiveness to that of Met52®.

## **Methods**

### **Source of Ticks**

Adult (males and females), nymphal and larval *A. americanum* ticks were obtained from the Oklahoma State University Tick Rearing Facility (OSU, Stillwater, OK). The *A. americanum* colony at OSU dates to 1971, originating from a collection of engorged adult females collected from the Cookson Game Refuge, 5 miles east of Cookson, Oklahoma, Cherokee County (Lisa Coburn, OSU Tick Rearing Facility, personal communication). Currently, the *A. americanum* tick colony from OSU is maintained by the annual introduction of females from a natural

population of ticks in Stillwater, OK, Payne County. Ticks from the colony are maintained on sheep and rabbits.

#### Native Arkansan Fungal Isolate: Savoy P10N1

Preliminary bioassays that used two *M. anisopliae* isolates from Arkansas—Savoy P10N1 and West Fork P9N2 (Chapter 3) found that Savoy P10N1 performed slightly better than West Fork P9N2 at causing mortality in adult *A. americanum* ticks. In addition, the percent germination of Savoy P10N1 ( $98.2 \pm 0.9\%$ ) was greater than that of West Fork P9N2 ( $69.0 \pm 3.7\%$ ) and continued to kill ticks at 77 days post-inoculation (days PI) (experiment termination). Therefore, Savoy P10N1 was the only Arkansas isolate of *M. anisopliae* used in this study. This isolate was previously identified as *M. anisopliae* (Chapter 3) and was cultured from a soil-exposed, *A. americanum* nymph on June 21, 2018. This nymph came from a sample of wild *A. americanum* ticks collected from the University of Arkansas Agricultural Experiment Station, Savoy Research Complex, Beef Cattle Research Area in Savoy, Arkansas (Washington County, AR; 36.128 latitude, -94.333 longitude) along with soil samples on May 18, 2018. In order to isolate entomopathogenic fungi from soil samples and ticks, ticks were exposed to soil samples from Savoy by forced contact with soil the samples in laboratory conditions ( $28.7 \pm 0.7^\circ\text{C}$  and  $61.4 \pm 5.6\%$  relative humidity (RH)) on May 23, 2018 (Tuininga et al. 2009). This isolate was named Savoy P10N1 after the fact that it was isolated from infected nymph number 1 exposed to soil collected from Savoy collection plot number 10. DNA sequencing analysis of Savoy P10N1 (GenBank accession number MN255810) revealed that it was a 100% match to *M. anisopliae* GenBank accession number MG844433 (Chapter 3). Savoy P10N1 was deposited into the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) Collection of Entomopathogenic Fungi (ARSEF) with the accession numbers ARSEF 14329

along with the isolate West Fork P9N2 (GenBank accession number MN255811; ARSEF accession number ARSEF 14330) and two other *Metarhizium* isolates (Savoy P10AF1= ARSEF 14331 and Savoy P2AM1 = ARSEF 14332) from Arkansas that were not identified to species (USDA ARS 2019; Louela Castrillo personal communication). This isolate was sub-cultured from an infected adult *A. americanum* tick inoculated in a preliminary bioassay of adult ticks. Conidia of the *M. anisopliae* isolate were suspended into a solution containing 0.05% Tween 80 and streptomycin/penicillin solution (1.5mg/L penicillin G; 2.5mg/L streptomycin) (Chapter 3). This spore suspension was then plated onto Sabouraud dextrose agar (SDA) plates (64 g/L) supplemented with yeast extract (2g/L) and gentamicin sulfate (10mg/L). Plates were maintained at  $24.4\pm0.3^{\circ}\text{C}$  and  $90.3\pm3.2\%$  RH for 2 months and 9 days.

#### Commercial Fungal Isolate: Met52® EC

A commercial formulation of a *Metarhizium* fungal insecticide known as Met52® EC (Novozymes Biological Inc., Salem, VA) was obtained from Evergreen Growers Supply (Clackamas, OR) (Figure 3.2A). The active ingredient was “*M. anisopliae*” strain F52 (later reclassified as *M. brunneum*) (Bischoff et al. 2009) concentrated at  $2\times10^9$  colony forming units per gram (CFU/g) at 11% w/w in an emulsifiable concentrate of “paraffinic petroleum distillates” (Novozymes 2019).

#### Preparation of Inoculum

Bioassays on ticks were conducted using methods modified from Gindin et al. (2001; 2002) and Kirkland et al. (2004a; b). Conidia from Savoy P10N1 were scraped with a sterile spatula from plates of fungi grown on SDA agar (64 g/L) supplemented with yeast extract (2 g/L) and gentamicin sulfate (10 mg/L). Conidial suspensions of Savoy P10N1 and Met52® EC (1 gram of the conidial formulation) were added to 1 ml of sterile deionized water with 0.05%

Tween 80 and vortexed. Direct counts of conidia for each isolate were determined by using a Bright Line® improved Neubauer hemocytometer (Hausser Scientific, Horsham, PA). Counts were then diluted achieve a treatment concentration of  $1 \times 10^8$  conidia/ml (Figure 3.2B).

Germination plates of SDA agar (64 g/L) measuring 60×15mm with yeast extract (2 g/L) and gentamicin sulfate (10 mg/L) were inoculated with 0.1ml of  $1 \times 10^6$  conidia/ml spore suspension from each isolate, with four plates inoculated per isolate. Germination plates were wrapped in aluminum foil and stored at 23°C at 83% RH for 28 h. The number of germinated spores were counted under an Olympus BX40 phase contrast microscope in 5 fields of view at 200X magnification (Olympus Corporation, Shinjuku, Japan).

#### Bioassays on *A. americanum*

Ticks were sorted into 3 treatment groups: Savoy P10N1, Met52® and an untreated control (deionized water with 0.05% Tween 80) (Figure 3.1). Bioassays were conducted with adult, nymphal and larval *A. americanum* ticks. Ticks were inoculated by immersing them in 1 ml of  $1 \times 10^8$  conidia/ml suspensions and gently mixing them in a vortex mixer for two minutes (Figure 3.2C and D). Ticks in the control group were vortexed in 1 ml of deionized water and 0.05% Tween 80® (Figure 3.2D). Thirty adult ticks (15 males and 15 females), approximately 30 nymphal ticks and a variable number of larvae (29-230) were inoculated per treatment. Each treatment was replicated four times. Ticks and inoculum were simultaneously added onto 100×15mm petri dishes with 90 mm filter paper (Gindin et al. 2001; 2002) (Figure 3.3A). Larval or nymphal ticks that were stuck to the inoculation tubes were removed with a combination of paint brushes and/or soft fine forceps and placed into the petri dishes. Petri dishes with ticks and moist filter paper were sealed with Parafilm® (Bemis Company, Inc., Neenah, WI) and medical tape then placed in humidity chambers (Figure 3.3B). Humidity chambers for holding ticks were

constructed according to specifications from the OSU Tick Rearing Facility (Stillwater, OK) (Winston and Bates 1960; Lisa Coburn, OSU Tick Rearing Facility, personal communication). The chambers were constructed from plastic Sterilite® tubs (Sterilite Corporation, Townsend, MA) measuring 60×43×115cm (Figure 3.3C). The bottom 2.5 cm of the tub was filled with water and mixed with 64 g of potassium sulfate to give the chamber a relative humidity close to saturation (> 90 % RH) (Winston and Bates 1960). To keep the ticks situated above the waterline, a wire mesh table with 2.5 cm mesh and measuring 53×30×8 cm was placed in the plastic container (Figure 3.3C). Humidity chambers for holding ticks were maintained at  $21.9\pm0.09^{\circ}\text{C}$  and  $86.9\pm1.5\%$  RH. To prevent ticks from escaping, petri dishes containing ticks were examined in plastic bins. Ticks were examined with the aid of a chill table (BioQuip Products Incorporated, Rancho Dominguez, CA) in order to slow down their movement and to prevent escapees. Ticks were checked for mortality every 2 days for 5 weeks, then once a week for another 6 weeks. Adult and nymphal ticks that died were placed in separate petri dishes with filter paper that were gridded with 10×10 mm squares labeled with numbers corresponding to each dead tick. Larval ticks were placed on filter paper without a grid or numbers. Filter paper under the dead ticks was moistened with 1 ml of deionized water and sealed with Parafilm® (Figure 3.3B). To maintain optimal conditions of relative humidity, petri dishes with ticks were periodically remoistened with 0.2-1.0 ml of deionized water, depending on the dryness of the filter paper. Ticks were considered dead from the treatment if they had fungal mycoses and dark green conidia sporulating from them. Ticks that died were checked daily for fungal growth and sporulation and were photographed when possible.



## Statistical Analysis

Experiments were completely randomized with two treatments and four replications. An analysis of covariance (ANCOVA) of adult and nymphal mortality data—with days post-inoculation (days PI) as the covariate—was performed using the PROC GLIMMIX procedure, version 15.1 in SAS for Windows version 9.4 (SAS Institute, Cary, NC). Analyses used a logistic regression of days PI on “total dead” ticks. Sporulation of dead ticks was used as an indicator of mortality from fungal infection resulting from the treatments. All data was transformed on the logit scale, analyzed, and then reconverted to percentages. The response variable analyzed was the proportion of ticks that died on or before each day after treatment out of the number of ticks per replication (N=30 for adults and N= 29-33 for nymphs).

## **Results**

### Adult Bioassays

Since there was no mortality observed for adult ticks in the control group, this treatment was excluded from the analysis. Ticks that died from *Metarhizium* infection across all bioassays initially displayed white mycoses followed by the sporulation and production of dark green to olive green conidia (Figure 3.4). The observed percent germination for Savoy P10N1 and Met52® was  $86.3 \pm 2.2\%$  and  $0.4 \pm 6.5\%$  respectively. Significant differences in the mortality rates between treatments were observed ( $F = 102.17$ ;  $DF = 1, 196$ ;  $P < 0.0001$ ) (Figure 3.5) for the adult bioassay. The mortality rate of adults treated with Met52® was twice the rate as adults treated with Savoy P10N1 on the logit scale. No significant differences in the infection rate (i.e. sporulation rate) of dead adult ticks observed between treatments ( $F = 3.43$ ;  $DF = 1, 143$ ;  $P = 0.066$ ). At experiment termination (77 days PI) the cumulative percentage of dead adult ticks infected with *Metarhizium* was  $58.3 \pm 5.9\%$  (N =120) and  $72.4 \pm 9.7\%$  (N =29) for Met52® and

Savoy P10N1 respectively with  $61.1 \pm 4.0\%$  of dead ticks ( $N = 149$ ) being infected. The percent cumulative mortality of adults exposed to Savoy P10N1 at 14, 28, 42, 56, and 77 days PI was  $0.8 \pm 0.4\%$ ,  $1.9 \pm 0.8\%$ ,  $4.2 \pm 1.8\%$ ,  $9.0 \pm 3.7\%$ ,  $18.4 \pm 6.8\%$  and  $25.4 \pm 8.7\%$  respectively (Table 3.1). On the other hand, adults treated with Met52® exhibited  $25.3 \pm 8.3\%$ ,  $65.6 \pm 9.8\%$ ,  $91.5 \pm 3.5\%$ ,  $98.4 \pm 0.7\%$  and  $100\%$  cumulative mortality at 14, 28, 42, 56, and 70 days PI respectively. Many of the dead adults treated with Met52® ( $41.6 \pm 4.5\%$ ;  $N = 120$ ) and the Savoy isolate ( $27.6 \pm 8.3\%$ ;  $N = 29$ ), did not sporulate. Some of these dead ticks displayed white mycoses of *Metarhizium*, growth of saprophytic fungi and/or bacteria. In total,  $38.9 \pm 4.0\%$  ( $N = 149$ ) of dead adults did not sporulate. The mycelia that had grown on some of these tick cadavers were seen with condensation buildup. Throughout the adult bioassay, a few live ticks were observed with white mycelia and dark green conidia growing out of one or more of the ticks' legs.

### Nymphal Bioassays

Due to a sudden, complete die-off of nymphal ticks that was observed in one of the control replications at 63 days PI, the control group for the nymphs was also excluded from the analysis. Unlike the adult bioassay there were no significant differences observed in the nymphal mortality rates between treatments ( $F = 0.38$ ;  $DF = 1, 196$ ;  $P = 0.54$ ). At 2 days PI,  $88.4 \pm 6.8\%$  of ticks treated with Met52® were killed by the treatment (Figure 3.6). Following this massive die-off, the remaining Met52®-treated nymphs died at the same rate as ticks treated with Savoy P10N1. Also dissimilar from the adult bioassay was the fact that significant differences in the infection rates of dead nymphs was observed between treatments ( $F = 4.5$ ;  $DF = 1, 176$ ;  $P = 0.035$ ). The cumulative infection percentage of dead nymphs treated with Met52® and Savoy P10N1 were  $93.9 \pm 2.3\%$  ( $N = 115$ ) and  $94.1 \pm 4.2\%$  ( $N = 34$ ) respectively with  $94.0 \pm 2.0\%$  of all dead nymphs ( $N = 149$ ) being infected with *Metarhizium*. The percent cumulative mortality

observed for Savoy P10N1 for the nymphal assay on days 14, 28, 42, 56, 70 PI and at experiment termination was  $5.3\pm3.3\%$ ,  $8.4\pm4.9\%$ ,  $12.9\pm7.2\%$ ,  $19.4\pm10.1\%$ ,  $28.0\pm13.0\%$  and  $33.1\pm14.4\%$  respectively (Table 3.1). Mortality in the nymphs treated by Met52® on the other hand was  $92.3\pm4.7\%$ ,  $95.3\pm2.9\%$ ,  $97.2\pm1.8\%$ ,  $98.3\pm1.1\%$ , and 100% at 14, 28, 42, 56, and 70 days PI respectively (Table 3.1). In contrast to the adult bioassay, very few of the dead nymphal ticks treated with Met52® and Savoy P10N1 did not show signs of *Metarhizium* infection with only  $6.0\pm1.9\%$  of dead nymphs (N = 149) not sporulating. Of the dead ticks,  $6.1\pm2.2\%$  (N = 115) nymphs treated with Met52® and  $5.9\pm4.0\%$  (N = 34) of nymphs treated with Savoy P10N1 did not sporulate. A few of the non-sporulating ticks exhibited growth of saprophytic fungi with cream-colored or light green spores, and or a slimy bacterial film. Several of the dead ticks treated with Met52® were later colonized with saprophytic fungi, after they sporulated.

### Larval Bioassays

The same methodology used to treat adults and nymphs was unsuccessful at treating larval ticks since most of the dead ticks treated with Met52® and Savoy P10N1 did not show signs of *Metarhizium* infection. Shortly after a chill table was used to minimize larval ticks from escaping, large die-offs of ticks were seen across all treatments—particularly in Met52®. Most of the ticks found dead had turned a light brown, opaque color and were easily torn apart or smashed when handled with fine forceps. No *Metarhizium* sporulation was seen from ticks in this condition. Several ticks were seen stuck to the condensation droplets that formed on the lid of the petri dish from exposure to the chill table. Several ticks were found dead on the lid, with some found in the condensation droplets. Since it was determined that larval ticks died from factors other than the treatment, and due to the huge variation in replication sample sizes (i.e. 29-

229 larvae per replication) resulting from handling difficulties, larval mortalities were excluded from the analysis.

## Discussion

The failure of a native Arkansan, *M. anisopliae* isolate to control *A. americanum* in this study suggests that this tick is likely resistant to infection with *M. anisopliae*. The pathogenicity of entomopathogenic fungi is known to vary markedly among different species of ticks and even different life stages in the same tick species (Gomathinayagam et al. 2002; Kirkland et al. 2004; Samish et al. 2004; 2008). In a study conducted by Kirkland et al. (2004a), adult *A. americanum* ticks exposed to  $1 \times 10^8$  conidia/ml of *M. anisopliae* exhibited  $13 \pm 2.0\%$  mortality compared to  $61 \pm 17.0\%$  mortality for *A. maculatum* Koch, 28 days after treatment. Mortality of *A. americanum* nymphs infected with *M. anisopliae* was higher than that for adults, with  $21 \pm 2\%$  mortality occurring after four weeks post-inoculation compared to  $13 \pm 2.0\%$  adults. Despite the lower mortality percentages that occurred in this study more nymphs treated with the Savoy P10N1 isolate died than adults at 28 days PI (Table 3.1). The mortality rate for adults treated with Met52® killed adult ticks twice as great as the rate observed for Savoy P10N1 (Figure 3.5).

There are likely two possible mechanisms that confer resistance of *A. americanum* to *M. anisopliae* and other entomopathogenic fungi: fungistatic cuticular hydrocarbons (Kirkland et al. 2004a) and competitive interactions from internal endosymbiotic fungi (Yoder et al 2003; 2004; 2005; 2006; 2008; Benoit et al. 2005). Kirkland et al. (2004a) found that pentane extracts of hydrocarbons from the cuticle of *A. americanum* inhibited the germination and the growth of germ tubes from *B. bassiana* conidia ( $18 \pm 10\%$  germination;  $4 \pm 2 \mu\text{m}$  germ tube length), compared to the relative lack of germination inhibition of *B. bassiana* conidia grown on *A. maculatum* cuticular extracts ( $80 \pm 9\%$  germination;  $18 \pm 7 \mu\text{m}$  germ tube length) or the control

(pentane solvent only) ( $75\pm 8\%$  germination;  $14\pm 4\ \mu\text{m}$  germ tube length). It's likely that these same cuticular extracts would greatly increase the tick's survival when encountering potentially pathogenic fungi in the leaf litter. Although several soil-dwelling fungi (including some entomopathogenic that are virulent to ticks) have been found on the surface of tick integuments, (Samsinakova et al. 1974; Estrada-Peña et al. 1990; Kalsbeek et al. 1995; Zhiou et al. 1999; da Costa et al. 2002; Yoder et al. 2003; Benoit et al. 2005; Tuininga et al. 2009; Greengarten et al. 2011) the internal mycoflora of certain tick species such as *A. americanum* and *D. variabilis* seem to be solely occupied by the fungus *Scopulariopsis brevicaulus* (Saccardo) Bainer (Yoder et al. 2003). This fungus is known to cause skin mycoses in humans but does not appear to be vectored by the tick species that host it (Yoder et al. 2004; 2005). Within *A. americanum* and *D. variabilis*, the fungal mycelium of *S. brevicaulus* is established within the integument glands of these tick species, producing conidia that circulate throughout the ticks' hemolymph (Yoder et al. 2004; 2005). The absence of other internal mycoflora from within *A. americanum* and *D. variabilis* is likely due to competitive exclusion from *S. brevicaulus* (Yoder et al. 2006; 2008). This competitive interaction between *S. brevicaulus* and other entomopathogenic fungi such as *M. anisopliae* could potentially protect *A. americanum* from entomopathogenic fungi that could potentially infect them. Furthermore, *S. brevicaulus* appears mutualistic symbiote that appears to protect its tick host from desiccation imposed by infection with entomopathogenic fungi (Yoder et al. 2006; 2008). Further studies should look for ways to overcome potential mechanisms of resistance of *A. americanum* to entomopathogenic fungi.

There is also a possibility that sub-optimal holding conditions for the fungal-inoculated ticks could have played a role in the performance of the Savoy P10N1 *M. anisopliae* isolate. In this study, ticks were held at  $21.9\pm 0.09^\circ\text{C}$  and  $86.9\pm 1.5\%$  relative humidity. For most

entomopathogenic fungi including *M. anisopliae* the optimal temperature range is between 24-27°C with an optimal relative humidity >90% (Samish et al. 2004; 2008; Zimmermann et. al. 2007). Although the humidity chamber used in this study eventually reached the targeted relative humidity values, this did not occur until two weeks after the humidity chamber was given new salt solution (i.e. before the start of the experiment). In future studies, a humidity chamber should set up at least two to three weeks to allow it to calibrate to the desired temperature and relative humidity (Winston and Bates 1960) before treated ticks can be placed inside.

Several adults and a few nymphal ticks were observed to have mycelial growth with no sporulation that was observed on ticks. These ticks were not counted having been infected with pathogenic fungi, meaning that the actual number of dead infected ticks could have been underestimated in this study. During the evaluations, the filter paper of several petri dishes with ticks had to be remoistened frequently since exhaust from the chill table would dry out the filter paper whenever the half of petri dish containing the filter paper was not placed on the chill table. The added condensation from the chill table on the petri dish lids, in addition to the remoistened filter paper led to the buildup of condensation on dead ticks. This condensation appeared to inhibit the growth of fungal mycelium and sporulation from a few dead nymphs and many dead adults. Furthermore, dead ticks were observed with the growth of saprophytic fungi or slimy looking, saprophytic bacteria that also obscured or potentially prevented *Metarhizium* mycelial growth and sporulation. Contaminants likely colonized dead ticks due to the prolonged time period it took to make mortality and sporulation assessments (e.g. 15 minutes to > 2 hours sometimes spent checking petri dishes with inoculated ticks) and due to the 77-day time period for which the experiments were conducted. These additional contaminants might have also contributed to the decreased amount of *Metarhizium* sporulation observed on the dead ticks.

Additional care should take place to prevent factors such as excess moisture, temperature extremes, long mortality assessment times, and long experiment run times from affecting mortality readings from ticks. Furthermore, dead ticks showing signs of fungal mycoses without sporulation should be recorded in addition to ticks that have sporulated in order to get a more accurate estimate of ticks that died from fungal infection.

Other factors that could have affected the results of these experiments are the storage conditions under which the *M. anisopliae* isolates were held before the bioassay as well as culturing conditions. These conditions include, temperature, relative humidity, storage time and the number of subcultures onto artificial media (Fernandes et al. 2012). It is known that storing entomopathogenic fungi under conditions of high humidity and high temperatures for extended periods of time can decrease germination of conidia from entomopathogenic fungi (Fernandes et al. 2012). In addition, multiple transfers of entomopathogenic fungi onto synthetic media can potentially alter their virulence. However, the virulence of entomopathogenic fungi can sometimes be restored by passing these fungi through arthropod hosts. Although the Savoy P10N1 culture used in this bioassay was sub-cultured from an infected *A. americanum* adult, the germination was only  $86.4 \pm 2.2\%$  after inoculating conidia from this isolate onto germination plates compared to  $98.2 \pm 0.9\%$  germination from a preliminary trial. This was likely due in part from the prolonged storage (over two months) of this isolate being used in this experiment. In addition, this isolate had been sub-cultured onto SDA agar twice before inoculating ticks in the preliminary bioassay. Although storage conditions might have affected tick mortality from Savoy P10N1, it is more likely that it was the resistance of the *A. americanum* ticks that resulted in the low mortality for this isolate since the trends in mortality for the preliminary bioassay and the bioassay mentioned in this study were similar. Germination from the *M. brunneum* F52 spores in

Met52® EC formulation was extremely low (almost 0% germination) after incubation on SDA agar for 28 hours. The lack of germination of the conidia in Met52® was likely due to the dark-green, tar-like nature of the formulation itself. To obtain accurate conidial counts and germination percentages for *M. brunneum* F52, counts need to be obtained from fungi grown directly from culture inoculated with the Met52® EC formulation.

Although the Arkansas Savoy P10N1 isolate of *M. anisopliae* failed to control *A. americanum* in this current study, this does not rule out the possibility that there are highly pathogenic strains of entomopathogenic fungi that can control *A. americanum*. A few other studies conducted with *B. bassiana* have shown that some strains of this fungus hold some promise as a biocontrol agent for control of *A. americanum* (Gomathinayagam et al. 2002; Kirkland et al. 2004 a; b; Craddock and Needham 2011a; b). In a study conducted by Gomathinayagam et al. (2002) both *A. americanum* and *D. variabilis* ticks were inoculated with *B. bassiana*. *Amblyomma americanum* ticks treated with the entomopathogenic fungus exhibited 100% mortality two weeks after inoculation. In this study 100% mortality for adults treated with Met52® was not achieved until 10 weeks PI. There's also the possibility that some strains of *B. bassiana* might only be effective against *A. americanum* under specific conditions. Kirkland et al. (2004a) found that *B. bassiana* strain ATCC 90517 was highly pathogenic (60% mortality after 28 days post-inoculation) to *A. americanum* only after blastospores of *B. bassiana* were taken directly from the growth medium (e.g. Sabouraud dextrose and 0.5% yeast extract liquid media). Nevertheless, a few field studies have demonstrated the effectiveness of at least one commercially available strain of *B. bassiana* for the control of *A. americanum*. Craddock and Needham (2011a) treated *A. americanum* ticks with BotaniGard™ (*B. bassiana* strain GHA) and held the ticks in outdoor enclosures. Two weeks after treatment, a significant difference in



mortality ( $P = 0.001$ ) was seen between ticks treated with *B. bassiana* (Mean =  $23 \pm 1.0$  ticks) and the control group (Mean =  $4.5 \pm 1.3$  ticks). Exposure of *A. americanum* ticks to *B. bassiana* has also been shown to increase the water loss of this species, demonstrating that *B. bassiana* disrupts the tick's ability to intake water from the environment (Craddock and Needham 2011b). The pathogenicity of *B. bassiana* to *A. americanum* demonstrates the further need to explore the development of this entomopathogenic fungus as a biocontrol agent for this tick species.

Ticks across all life stages treated with Met52® showed mortality that eventually reached 100% at experiment termination. However, complete mortality was not achieved until 70 and 77 days PI for adults and nymphs respectively. Most of the nymphal mortality ( $88.4 \pm 6.8\%$ ) (Table 3.1; Figure 3.6) occurred 2 days after treatment. Even though most of the dead nymphal ticks displayed sporulating conidia of *M. brunneum* strain F52, sporulation of these ticks did not occur until 7-13 days after mortality was observed. Therefore, there is a good possibility that much of the nymphal tick mortality from Met52® resulted from the green, tar-like formulation rather than infection from the F52 conidia. Although fewer adults had died from Met52® than nymphs at 14 days PI ( $25.3 \pm 8.3\%$  mortality for adults compared to  $92.3 \pm 4.7\%$  mortality for nymphs), the nymphs that remained after two days PI died at a slower rate than adults. It must be noted that ticks treated directly with the Met52® were initially glued together in the tar-like formulation of this mycoinsecticide—a factor that might have played a role in the mortality of the treated ticks. In order to make a fair treatment comparison against Savoy P10N1, the *M. brunneum* conidia in Met52® would need to be cultured onto synthetic media, and then added to an emulsion of sterile deionized water and Tween 80. Another approach would be to completely sterilize the Met52® EC formulation and use it in bioassays in order to compare its efficacy to pure F52 spores, unsterilized Met52® in formulation, an *M. anisopliae* isolate and a control.

Although there were significant differences in the mortality rates between treatments for adult *A. americanum*, no difference in the mortality rates between treatments was seen for nymphs since the few Met52®-treated nymphs remaining after two days PI died at the same rate as nymphs treated with Savoy P10N1 (Figure 3.5 and 3.6). No significant differences were seen for the infection rates of dead adults between treatments despite the differences in the cumulative sporulation percentages (almost 60% sporulation dead adults treated with Met52® compared to over 72% sporulation for adults treated with Savoy P10N1) observed between treatments at experiment termination. This confirmed that most of the adult mortality was most likely due to *Metarhizium* infection. Conversely, significant differences in the infection rates of dead ticks was observed between treatments likely due to differences in the virulence and fungal sporulation characteristics of the fungal isolates used. However, the cumulative percentages of dead ticks observed to have sporulated by the end of the experiment was roughly the same (roughly 94%) for both treatments showing that most nymphs that died had also most likely died from fungal infection.

Despite being isolated from *A. americanum* ticks, the Savoy P10N1 *M. anisopliae* isolate was not highly virulent to this tick species. Apart from studies conducted in Brazil (da Costa et al. 2002) and in the United States (Benoit et al. 2005; Tuininga et al. 2009), *M. anisopliae* has rarely been isolated from ticks infected in the wild (Samish et al. 2004; 2008). The isolation of a strain of a pathogenic from fungi from ticks, as well as from other arthropods does not always mean that they are the best candidates for the control of a specific arthropod host (2008; Fernandes et al. 2006). However, the virulence of entomopathogenic fungi is known to vary markedly among different species and strains within the same fungal species (Samish et al. 2004; 2008; Ostfeld et al. 2006). Therefore, a good possibility exists that a highly infective strain of *M.*

*anisopliae* towards *A. americanum*—and one that is also effective for the control of this tick species—has still yet to be discovered and tested against this tick species. Nevertheless, the use of entomopathogenic fungi to control ticks in the field is challenged by factors such as temperature, relative humidity, ultraviolet radiation, formulation used and even the microclimate of host skin (Samish et al. 2004; 2008; Polar et al. 2005a; b; 2008; Fernandes and Bittencourt 2008; Fernandes et al. 2012; Nicholson et al 2018). These factors seem to limit entomopathogenic fungi to treating ticks during seasons of heightened tick activity (Maranga et al. 2005; Bharadwaj and Stafford 2010) and at particular times of the day (e.g. during the early morning or early evening hours). With these limitations considered it is likely that the use of entomopathogenic fungi for the control of *A. americanum* will be the most effective when used in an integrated tick management approach (Williams et al. 2018; Nicholson et al. 2018). In a study conducted by Williams et al. (2018), a combination of a broadcast treatment of Met52<sup>®</sup> EC and fipronil impregnated bait boxes to treat ticks on rodents effectively reduced questing *I. scapularis* nymphs from 78-95% each year over the course of a three-year time frame. In addition, the percentage of *I. scapularis* nymphs infected with *Borrelia burgdorferi* Johnson et al. 1984, the causal agent of Lyme disease, was reduced by 66% compared to the untreated control the third year the study was conducted. For such an integrated approach to be implemented against *A. americanum* in Arkansas and in much of the southeastern United States, future research should explore and evaluate additional strains of entomopathogenic fungi that have a high potential for controlling this tick species.

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## Tables and Figures

Table 4.1 Percentage dead *Amblyomma americanum* days post-inoculation (days PI). Ticks were treated with either  $1 \times 10^8$  conidia/ml of Met52® (*Metarhizium brunneum* strain F52) or Savoy P10N1 isolate (*Metarhizium anisopliae*).

Treatment	Life Stage	
	Adults	Nymphs
Met52®		
14 days PI	25.3±8.3	92.3±4.7
28 days PI	65.6±9.8	95.3±2.9
42 days PI	91.5±3.5	97.2±1.8
56 days PI	98.4±0.7	98.3±1.1
70 days PI	100	99.0±0.7
77 days PI	100	100
Savoy P10N1		
14 days PI	0.8±0.4	5.3±3.3
28 days PI	1.9±0.8	8.4±4.9
42 days PI	4.2±1.8	12.9±7.2
56 days PI	9.0±3.7	19.4±10.0
70 days PI	18.4±6.8	28.0±13.0
77 days PI	25.4±8.7	33.1±14.4



Figure 4.1 (A) Sorting *Amblyomma americanum* ticks into glass vials prior to experimental inoculations (B) Pre-sorted *Amblyomma americanum* ticks ready to be treated by immersion. Photo A by Kelly Loftin; photo B by Donald Steinkraus.

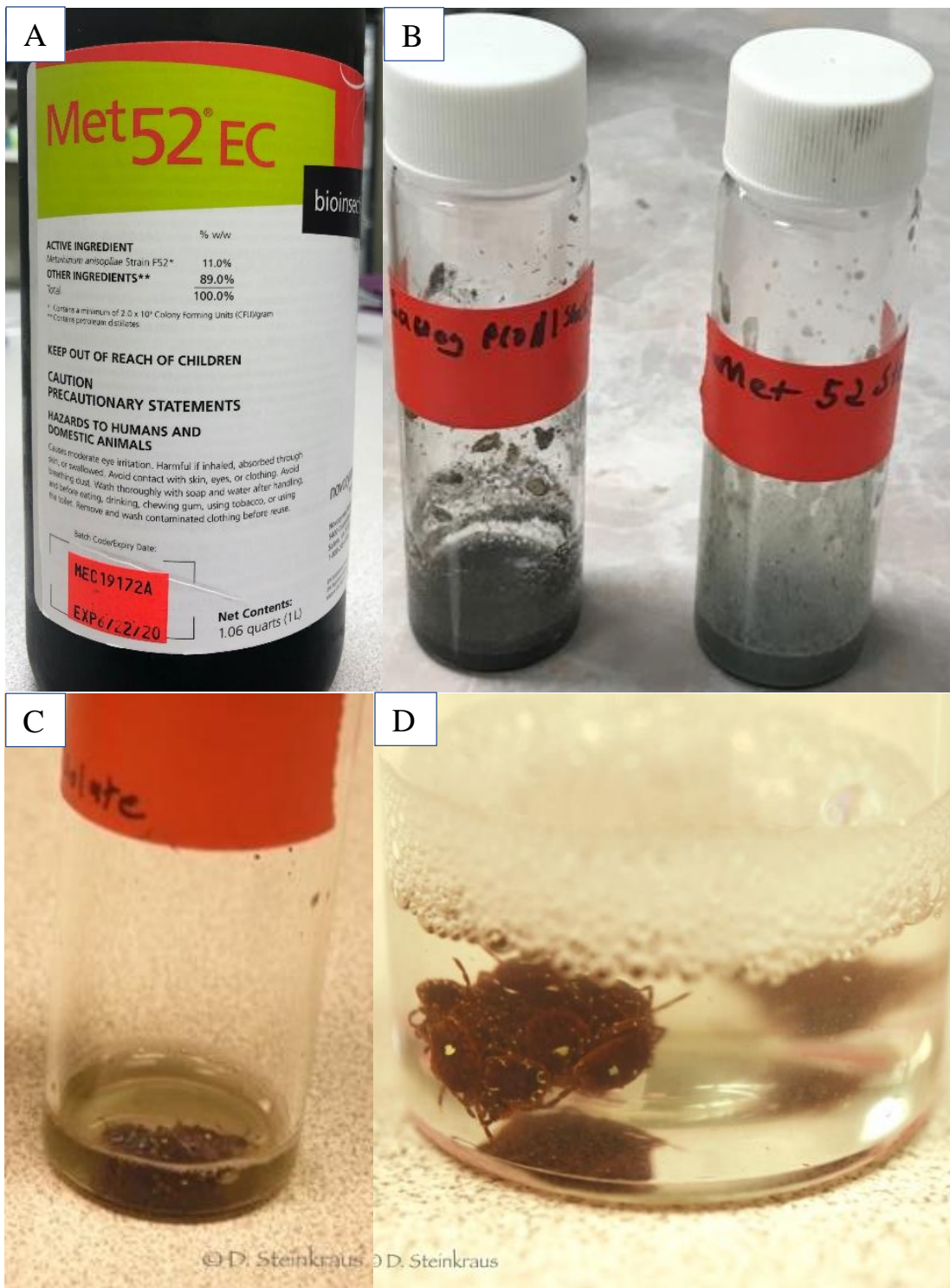


Figure 4.2 Figure 4.3 Treatments used for inoculating *Amblyomma americanum* ticks. (A) Bottle of Met52® EC (B) Treatment suspension of Savoy P10N1 and Met52® EC used for inoculating *Amblyomma americanum*. (C) Ticks immersed in a suspension of Savoy P10N1 (D) Close-up view of ticks immersed in the control emulsion (sterile deionized water and 0.05% Tween 80). Photo (A) by Kelly Loftin; photos C and D by Donald Steinkraus.

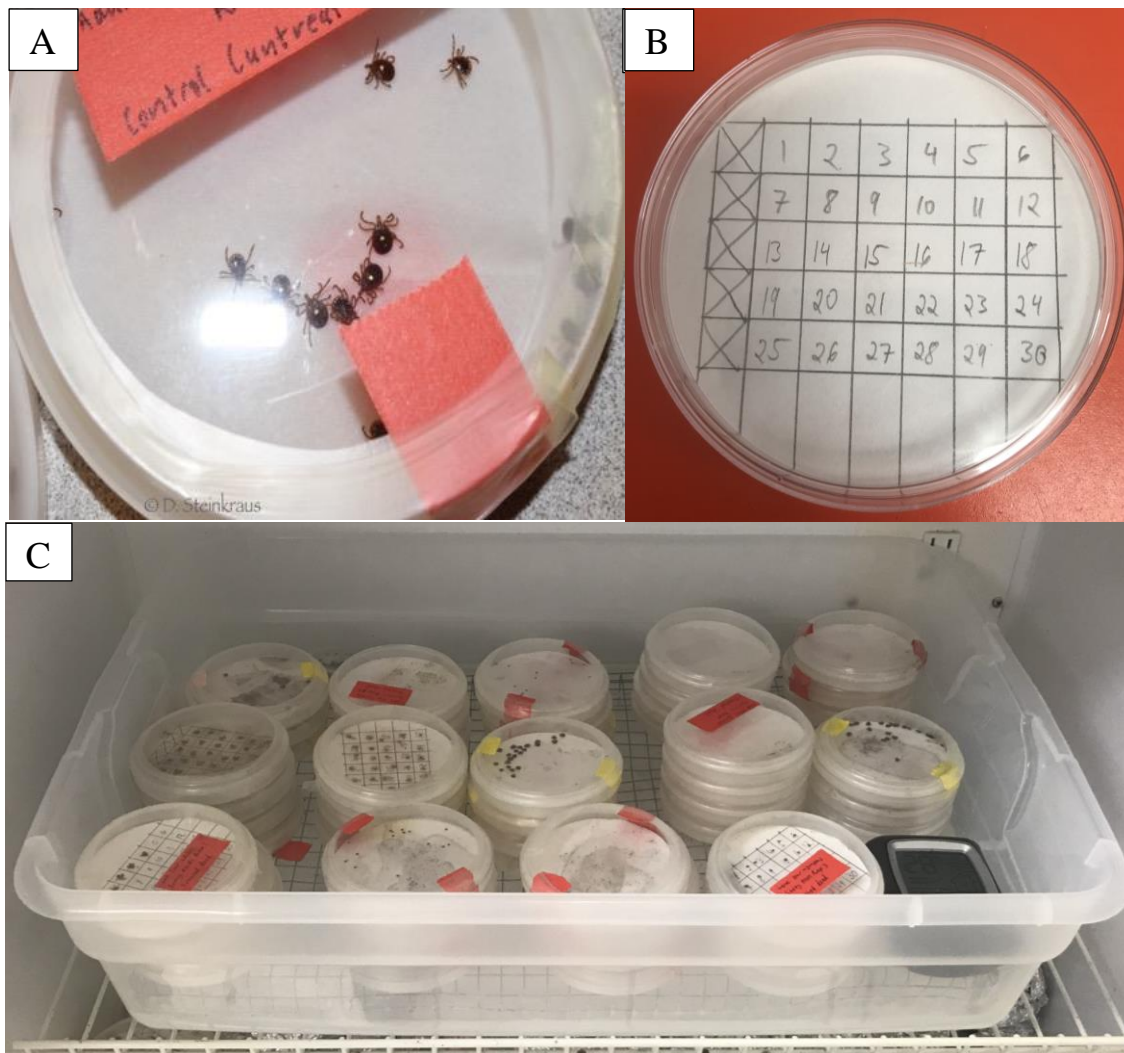


Figure 4.4 (A) Sealed petri dishes with moistened filter paper used for holding treated *Amblyomma americanum* ticks for 77 days. (B) Gridded filter used in petri dishes for holding dead adult and nymphal *Amblyomma americanum* ticks to encourage fungal growth and sporulation. (C) Humidity chamber filled with treated ticks, and a relative humidity/temperature gauge. Photo (A) by Donald Steinkraus.



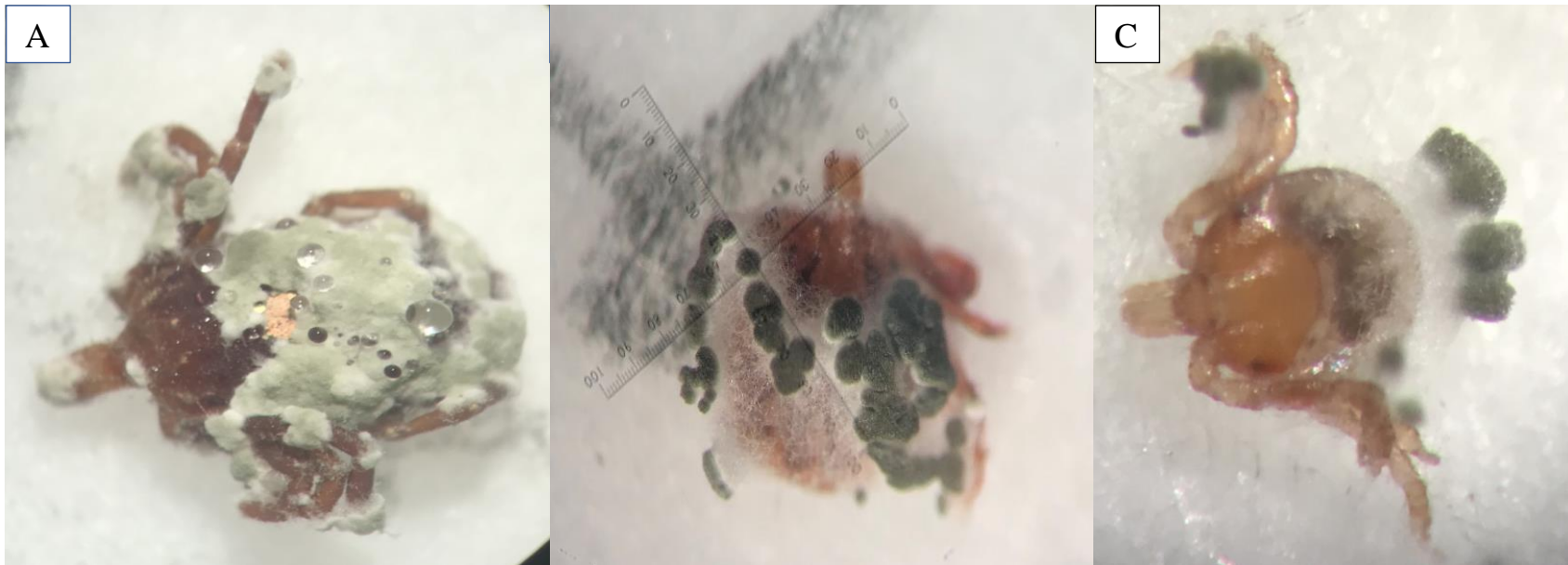


Figure 4.5 Treated *Amblyomma americanum* adult (A), nymph (B) and larva (C) displaying mycoses and green sporulation diagnostic of *Metarhizium* infection. Adult was inoculated with Met52® EC (*M. brunneum* strain F52 conidia) while the nymph and larva were inoculated with the Savoy P10N1 Arkansas isolate (*M. anisopliae*).

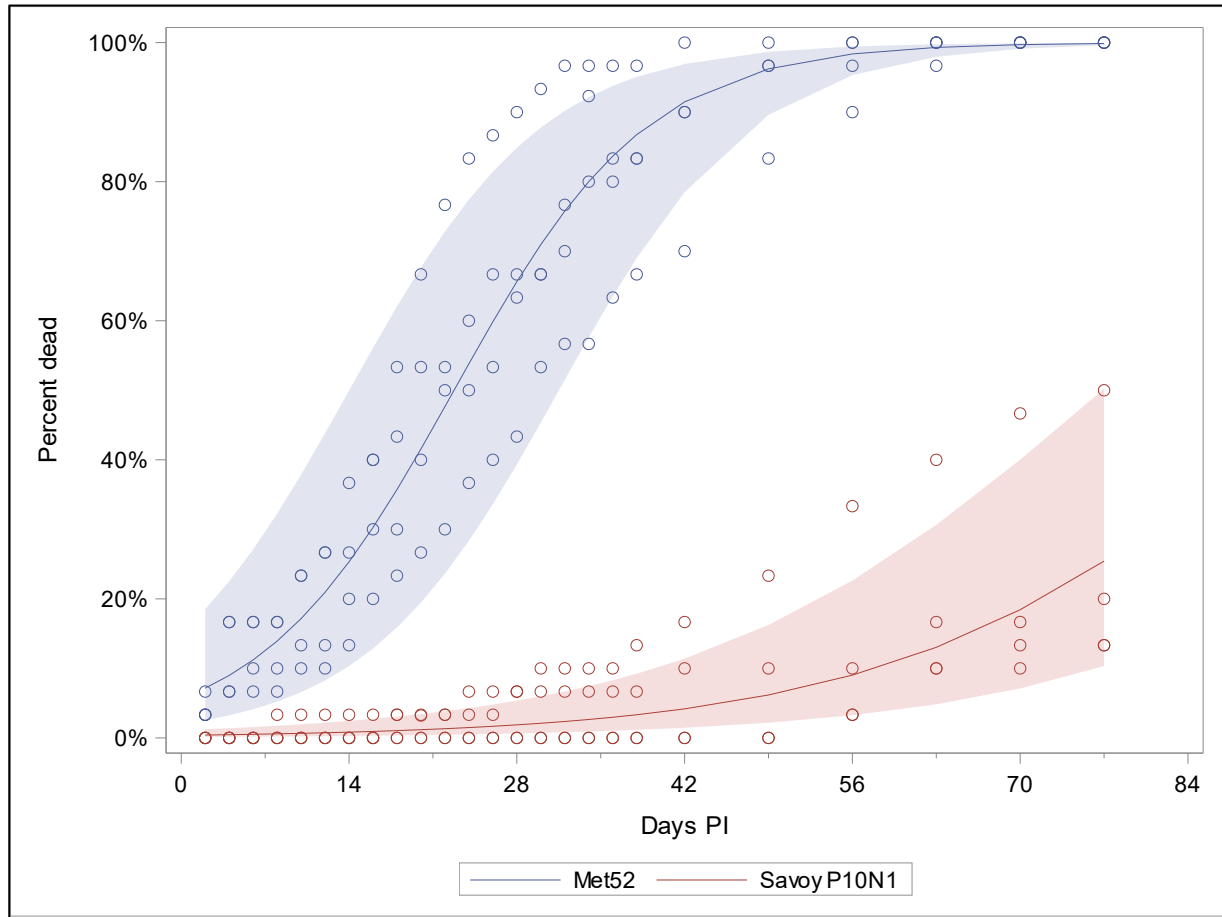


Figure 4.6 Percentage of dead *Amblyomma americanum* adults over the course of 77 days post-inoculation (Days PI). No mortality was seen in the control (not shown). Significant differences were seen in the mortality rates between treatments ( $F = 102.17$ ;  $DF = 1, 196$ ;  $P < 0.0001$ ).

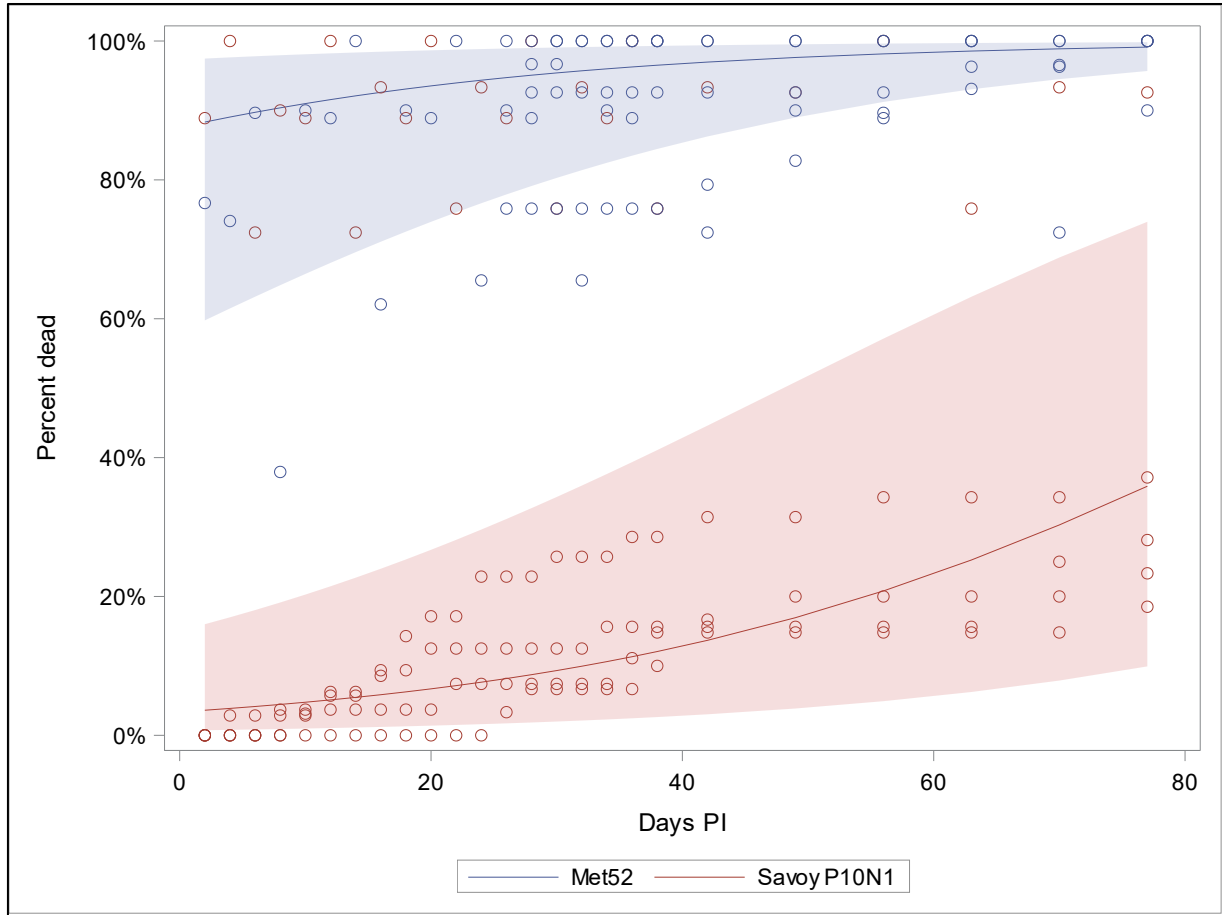


Figure 4.7 Percentage of dead *Amblyomma americanum* nymphs over the course of 77 days post-inoculation (Days PI). No mortality was seen in the control (not shown) until 63 days PI. No significant differences were seen in the mortality rates between treatments ( $F = 0.38$ ;  $DF = 1, 196$ ;  $P = 0.54$ ).

## CHAPTER 5: Conclusions

The lone star tick *Amblyomma americanum* L is the most abundant tick in Arkansas and is also one of the most important tick species in the United States for the transmission of disease-causing pathogens. This tick is a major pest of pets and livestock, is medically significant for its role in the transmission of several bacterial, viral and protozoan pathogens. In addition, components of *A. americanum* saliva can induce a red meat allergy in humans (i.e. alpha-gal syndrome). Despite the extensive research that has been conducted on entomopathogenic fungi in controlling various tick species very little of this work has been utilized for the control of *A. americanum*. The objectives of my research were to: isolate strains of entomopathogenic fungi from wild-caught *A. americanum* ticks that were exposed to soil samples from northwest Arkansas under laboratory conditions; identify isolates of entomopathogenic fungi to species using morphological and molecular techniques; and to assess the effectiveness of a few of these fungal pathogens as potential control agents of *A. americanum* compared to the bio-insecticide Met52® (*Metarhizium brunneum* Petch strain F52). The results of these findings will hopefully allow for further exploration of additional isolates of entomopathogenic fungi to be developed for the control of *A. americanum*.

Adult male, adult female and nymphal *A. americanum* ticks exposed to samples of Arkansan soil displayed signs of infection with entomopathogenic fungi, particularly *Metarhizium*. However, the overall infection percentages of soil-exposed *A. americanum* ticks with *Metarhizium* species from Savoy and West Fork, Arkansas was <2% compared to 19.7-24.2% observed for other fungi. Likewise, the percentages of soil-exposed ticks infected with *Metarhizium* species among the different life stages for *A. americanum* (adult males, adult females, nymphs) were also very low (< 4 %) compared to infection with other fungi that were



observed in this study (11.7%-40%). Natural infection rates of ticks with entomopathogenic can vary substantially < 1% to over 50%. In this study, fungal infection on ticks was induced using a modified arthropod soil-baiting method. Using ticks such as *A. americanum* in a soil exposure assay for isolating entomopathogenic fungi could, if standardized, be an effective way of finding potentially effective strains of pathogenic fungi for the control of ticks such as *A. americanum*. However, isolates of entomopathogenic fungi found from ticks have not always shown to be the most pathogenic isolates to ticks. Isolates of *M. anisopliae* sensu lato (s.l.) (Metchnikoff) Sorokin have shown to be some of the most pathogenic fungi to ticks but few instances of their isolation from ticks have been documented in the literature. This study is the first isolation of *M. anisopliae* from ticks in Arkansas.

Eleven fungal isolates were cultured from wild *A. americanum* ticks exposed to soil from seven sites spread across two different collection locations in northwest Arkansas (Savoy and West Fork, Arkansas). Examination of the morphological characteristics of these fungal isolates showed that eight belonged to the genus *Metarhizium*, while the other three fungal isolates were unidentified. Two of the *Metarhizium* isolates (Savoy P10N1 and West Fork P9N2) were confirmed as *M. anisopliae* through DNA sequencing and a GenBank BLAST analysis. These findings show that *M. anisopliae* can potentially infect *A. americanum* whenever the ticks move to the soil. Further research could take a more in-depth survey of other entomopathogenic fungi, as well as *M. anisopliae*, that infect *A. americanum*. To approach this, fungi could be isolated from naturally infected ticks, ticks exposed to soil samples, and soil baiting methods with *Galleria mellonella* L. larvae/*Tenebrio molitor* L. larvae.

Savoy P10N1, the native Arkansan isolate of *M. anisopliae* assayed against *A. americanum* ticks killed <35% of either adult or nymphal ticks that were immersed in a dose of

$1 \times 10^8$  conidia/ml by the time of experiment termination at 77 days post-inoculation (days PI). On the other hand, mortality seen for nymphal *A. americanum* ticks treated with Met52® was almost 90% after two days post-treatment, and 100% at experiment termination. However, mortality of all nymphs was not achieved until 11 weeks after treatment. By comparison, adult mortality was over > 25% at 14 days post-treatment with 100% mortality reached 10 weeks after treatment.

Despite the shortcomings of the Arkansas isolates of *M. anisopliae* to control *A. americanum*, some control was achieved with Met52®, although it was substantially less than what was reported for studies on the black legged tick, *Ixodes scapularis* Say. Previous lab and field studies with conducted Met52® or with its active ingredient alone (i.e. *M. brunneum* strain F52) have reported effective control against the *I. scapularis*. The implementation of mycoinsecticides, such as Met52®, for the control of *A. americanum* might best be used as part of an integrated tick management program, given the high abundance of this tick throughout its geographic range and its broad host range.

From the small amount of scientific literature that does exist on the biological control of *A. americanum* with entomopathogenic fungi, a few studies have shown that strains of *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin might be an effective control agent for this tick species. Studies on effectiveness of *B. bassiana* as a biological control agent of *A. americanum* would need to be conducted in Arkansas before the implementation of this entomopathogenic fungus as part of an integrated tick control program.