University of Arkansas, Fayetteville

ScholarWorks@UARK

Graduate Theses and Dissertations

12-2017

A Molecular Diagnostic Survey of Pathogens and Parasites of Honey Bees, Apis mellifera L., From Arkansas and Oklahoma

Dylan Cleary
University of Arkansas, Fayetteville

Follow this and additional works at: https://scholarworks.uark.edu/etd

Part of the Entomology Commons, and the Parasitology Commons

Citation

Cleary, D. (2017). A Molecular Diagnostic Survey of Pathogens and Parasites of Honey Bees, Apis mellifera L., From Arkansas and Oklahoma. *Graduate Theses and Dissertations* Retrieved from https://scholarworks.uark.edu/etd/2554

This Thesis is brought to you for free and open access by ScholarWorks@UARK. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of ScholarWorks@UARK. For more information, please contact scholar@uark.edu, uarepos@uark.edu.

A Molecular Diagnostic Survey of Pathogens and Parasites of Honey Bees, *Apis mellifera* L., From Arkansas and Oklahoma

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

by

Dylan Alexandra Cleary
Oklahoma State University
Bachelor of Science in Natural Resources, Ecology and Management, 2013

December 2017
University of Arkansas

This thesis is approved for recommendation to the Graduate Council

____
Allen L. Szalanski, Ph.D
Thesis Director

____
Donald Steinkraus, Ph.D
Co-Advisor

____
Jackie Lee, Ph.D
Committee Member

Abstract

The health and viability of colonies of the honey bee, *Apis mellifera,* in the United States have fluctuated dramatically over the past decade. This poses a substantial threat to agricultural production in this country. Currently, no single factor has been identified for this decline. Rather, it has been suggested that the interaction between multiple biotic and abiotic stressors may be responsible. Among these factors are pesticides, habitat loss, climate and weather, parasites and pathogens, and colony management techniques. For this reason, it is important to examine the prevalence of honey bee parasite and pathogen infection at the state level in comparison to national survey data.

In the research described herein, molecular diagnostics were performed on worker honey bee samples from Arkansas hobbyist beekeepers and Oklahoma migratory beekeepers to detect the presence of the following *A. mellifera* pathogens and parasites: protozoans *Nosema apis and N. ceranae;* bacterial pathogens *Spiroplasma apis* and *S. melliferum;* Trypanosomatid parasites *Crithidia mellificae and Lotmaria passim* and the parasitic phorid fly *Apocephalus borealis.* A study including both migratory honey bee colonies and hobbyist managed colonies provides a more comprehensive distribution of where these parasite and pathogen species are occurring and potentially why they are occurring.

The study determined that *N. ceranae* (H=11.6%, M=27.6)*, L. passim* (H=11.3%, M=1.1%)*,* and *V. destructor* (H=45.5%, M=17.2%)*,* occur in both hobbyist and migratory managed colonies. *Nosema ceranae* was more prevalent in the migratory colonies than the hobbyist colonies. *Spiroplasma* was also detected in the Oklahoma migratory colony samples (8.05%), but not in the Arkansas hobbyist colonies. Both *V. destructor* and *L. passim* were more prevalent in the hobbyist managed colonies. This research resulted in the first detection of

Lotmaria passim in Arkansas honey bees, as well as the first documented detection of *L. passim* and *S. melliferum* in Oklahoma. *Apocephalus borealis, C. mellificae, N. apis,* and *S. apis* were not detected in either the migratory nor the hobbyist colonies. This study compares honey bee management practices at the hobbyist and migratory level to better understand how management influences parasite and pathogen spread and abundance. The use of state-level surveys, when examining parasite and pathogen occurrence, allows for a better understanding of how these pests are spreading, as well as how quickly and by what means.

Acknowledgements

I would first like to thank my advisor Dr. Allen Szalanski for his constant support and guidance. His knowledge, advice, and encouragement made this project possible. I would like to acknowledge my lab mates Clinton Trammel and Mary-Kate Williams for their assistance throughout this process, as well as well as my co-advisor Dr. Donald Steinkraus, and committee member Dr. Jackie Lee. Thank you to the University of Arkansas Entomology department's students and faculty. Thank you for sharing your passion and knowledge of science with me.

A sincere thank you to all of the beekeepers from both Arkansas and Oklahoma for their cooperation and participation in this study, without them this project truly would not have been possible.

Lastly, I would like to thank my family and friends. Without their love and support I would not have been able to endure this degree. To Julie and Paul Cleary, thank you for you being the best examples of humans. Your unwavering support and love for me is more than valued. Thank you for encouraging me always to pursue the things I am passionate about. And most importantly, thank you for editing a thesis on insects. To my siblings, Conor and Caitlin Cleary, thank you for cheering me on throughout this process, you are so very important in the completion of this thesis. To Charles Cleary and Brian Threlkeld, thank you for your encouragement and emotional and mental support. Thank you for being my best friends from the beginning and continuing this journey with me to its conclusion. I could not have done this without all of you.

Dedication

This thesis is dedicated to my family: Conor, Caitlin, Julie, Paul, and Charles Cleary.

Table of Contents

Chapter 1: Introduction 1

Background 1
Eusociality linked to increased transmission 3
State-level survey 4
Beekeepers 4
Honey bee parasites and pathogens 7
Microsporidia 8
Bacteria 10
Trypanosomes 12
Apocephalus borealis 13
Varroa destructor 14
Molecular Diagnostic Methods 17
Objectives 19
Literature Cited 20

Chapter 2: Molecular detection of parasites and pathogens in hobbyist honey bee, *Apis mellifera* L., colonies 27

Abstract 27
Introduction 29
Materials and Methods 32
Results 36
Discussion 38
Acknowledgement 43
Literature Cited 44

Chapter 3: Molecular detection of parasites and pathogens in migratory honey bee, *Apis mellifera* L., colonies 64

Abstract 64
Introduction 65
Materials and Methods 68
Results 72
Discussion 73
Acknowledgement 76
Literature cited 77

Chapter 4: Comparison of parasite and pathogen occurrence in hobbyist and migratory honey bee, *Apis mellifera* L., colonies 87

Abstract 88
Introduction 88
Materials and Methods 94
Results and Discussion 96
Acknowledgements 112

Literature cited **101**
Chapter 5: Conclusion **114**

Chapter 1: Introduction

Background

Pollination is vital to the continuation of hundreds of fruit, vegetable, nut, legume and seed crop species (Klein et al. 2007, Potts et al. 2010, Bond et al. 2014). Both wild and commercially managed pollinators are responsible for providing pollination services (Kluser and Neumann 2010, Potts et al. 2010). Valued at an estimated $15 billion (USD) in pollination services alone, the European honey bee, *Apis mellifera* L. is considered both the most widely used commercial pollinator and the most economically significant pollinator species worldwide (Kluser and Neumann 2010, Runckel et al. 2011). Furthermore, *A. mellifera* is responsible for pollinating crops dependent upon pollinators such as almonds, apples, and blueberries. Large-scale monocultures of these pollinator-dependent crops rely upon commercial beekeeping to continue production (vanEngelsdorp et al. 2008). Beyond agricultural crop pollination, honey bees contribute to the pollination of wild and ornamental plants. While the extrinsic value of the honey bee's pollination service for wild plants is not well defined, a level of contribution to biodiversity is achieved (Potts et al. 2010).

Beginning in 2004, U.S. honey bee populations have fluctuated, resulting in all-time low populations in 2008 (Cavigli et al. 2016). These instances of major colony loss have prompted major concern as these losses pose a sizable threat to agricultural production. The decline of pollinator species threatens the world's ability to produce food efficiently (Meeus et al. 2012). In more recent years, honey bee populations have increased, however, not at the same rate as crop species dependent upon insect pollinators have increased (Aizen and Harder 2009, Calderone 2012).

With the decline in honey bee colony health occurring, both biotic and abiotic stressors have been suggested as the cause (Potts et al. 2010). However, no single definitive cause has been conclusively found to be responsible (Cavigli et al. 2016). Multiple stressors acting simultaneously may, in fact, be the root of honey bee decline (Potts et al. 2010). Chief among these stressors are various honey bee parasites and pathogens (vanEngelsdorp et al. 2008, 2009, Kluser and Neumann 2010). The parasites and pathogens studied in my research are the parasitic mite *Varroa destructor* Anderson and Trueman, microsporidian parasites *Nosema apis* Zander and *N. ceranae* Fries; bacterial pathogens *Spiroplasma apis* Mouches and *S. melliferum* Clark; Trypanosomatid parasite species *Crithidia mellificae* Langridge and McGhee and *Lotmaria passim* Schwarz and the parasitic phorid fly *Apocephalus borealis* Brues.

The increasing threat that parasites and pathogens pose to honey bee pollination services has caused alarm among beekeepers specifically and agriculturalists generally (Youngsteadt et al. 2015). Parasites such as the *Varroa* mite or the microsporidian pathogen *Nosema* are among the greatest known threats to honey bee health; while research has identified an increasing number of other threats to *A. mellifera* colonies, there has been scant research on the distribution, prevalence, and effect of these newly identified risks.

Although national surveys in the U.S. have been performed for honey bee pathogens and parasites, there is a lack of statewide surveys examining parasites and pathogens in honey bee colonies. Furthermore, there is a deficiency of information discussing the level of pest occurrence in hobbyist versus commercially managed hives. This information could potentially allow for a better understanding of how parasites and pathogens of honey bees spread.

Efficient pollinators

Apis mellifera is the most common commercially utilized pollinator species worldwide, as well as the most economically significant (Klein et al. 2007, vanEngelsdorp et al. 2008). This is in large part because honey bees are both efficient and reliable pollinators of agricultural crops. Large-scale production of agriculture often utilizes monocultures, many of which are dependent upon pollinators to reproduce (vanEngelsdorp et al. 2008). While many pollinator species are not compatible with specific crops in monoculture or the lack of nutritional diversity, honey bees are capable of ensuring pollinations services (McGregor 1976, Klein et al. 2007, vanEngelsdorp et al. 2008). Honey bees exhibit flower fidelity, facilitating cross pollination within monocultures (Menzel and Muller 1996). Furthermore, because honey bees overwinter as a colony they can pollinate flowering plant species early in the spring, when other bee species consist of overwintered queens (Döke et al. 2015).

A primary reason that honey bees are such efficient pollinators is largely due to their level of sociality. With the high number of acres utilized for monocrops, it is difficult for enough insect pollinators to naturally occur and provide pollination services (McGregor 1976). Honey bee colonies contain thousands of individual bees with division of labor; this high number of individuals allows for successful pollination of large areas (Chapman and Bourke 2001, Simone-Finstrom et al. 2016). Furthermore, because honey bees do reside in colonies, transportation of pollinators is simplified for migratory beekeepers (Simone-Finstrom et al. 2016).

Eusociality linked to increased transmission

The eusociality practices of *A. mellifera* allow for the species to be especially vulnerable to parasite and pathogen transmission (Kurze et al. 2016). Honey bees live in highly social colonies in which individuals interact with other members closely. This close interaction

behavior allows for parasites and pathogens to spread successfully and continuously. Horizontal transmission may be favored, thus, causing larger more prolific outbreaks to occur. Furthermore, lack of coevolution has disallowed for heritable traits linked to resistance and tolerance to be passed down (Locke et al. 2012). For example, the *Varroa* mite is native to Asia, where it coevolved with its native host, *Apis cerana*. *Apis cerana* is far more resilient compared to the European honeybee because of this coevolution (Locke et al. 2012).

State-level survey

While multiple national surveys examining honey bee pathogens have been conducted, few studies surveying occurrence at the state level in the United States have been performed (Szalanski et al. 2013, Traynor et al. 2016). Surveying at the state level may allow for tracking the source location of these parasite and pathogen species.

Beekeepers

Beekeeping typically occurs under three different types of management: commercial, sideliner, or hobbyist. The three differ in terms of number of colonies managed as well as the level of management tactics used and whether colonies remain stationary or are transported (Lee et al. 2015, Simone-Finstrom et al. 2016).

Commercial migratory beekeepers include the migratory beekeepers and the bee brokers. Both beekeepers make a profit from managing hundreds to thousands of colonies; these colonies are rented and transported from one foraging site to the next within the same season to take advantage of honey flows from different crops (Tsutsumi and Oishi 2011, Pilati and Prestamburgo 2016). Commercial beekeeping can be a large source of income depending upon the number of rented colonies.

Large-scale migratory beekeepers may transport colonies thousands of kilometers in one season. As these hives move to new areas across the United States, they are exposed to new and unfamiliar parasites and pathogens. This exposure makes these colonies more susceptible to harmful pests (Cestaro et al. 2017). Bee brokers handle contracts and communicate with both the beekeeper and the grower to properly place migratory colonies in the appropriate areas. The broker charges a brokerage fee.

Commercial migratory beekeeping involves the movement of honey bee colonies to new agricultural locations to provide pollination services (Cestaro et al. 2017). Migratory beekeeping is essential in the pollination and production of many agricultural crops (James and Pitts-Singer 2008). Almond production in California is the number one user of pollination services by honey bees. Annually, between 60-80% of managed, honey producing, colonies in the United States are transported to California to pollinate during the almond bloom (Runckel et al. 2011, Bond et al. 2014, Cavigli et al. 2016).

When transporting, colonies are often moved across the country thousands of kilometers in non-ideal conditions. Poor ventilation, stressful transportation, poor nutrition, and exposure to new parasites, pathogens, viruses, and disease are among the concerns (Bacandritsos et al. 2010). Furthermore, once transported, honey bees are often used to pollinate large monocultures which may not be particularly nutritious to the honey bees (Smith et al. 2013, Hendriksma and Shafir 2016). In terms of management tactics, migratory colonies are typically heavily treated and managed. These colonies are also typically kept in close proximity to large volumes of other colonies, allowing for easy pest and disease transmission (Royce and Rossignol 1990, Lee et al. 2015).

Sideliner beekeepers typically manage between 51-300 honey bee colonies and utilize these colonies as supplemental income (vanEngelsdorp et al. 2012, Lee et al. 2015). This income may be made via selling hive products such honey, wax, and propolis as well as through migrating colonies for pollination services (Connor 2007). These colonies are typically transported regionally, apart from the annual almond pollination in California (Simone-Finstrom et al. 2016). Because sideliner managed colonies likely participate in the almond pollination, they are exposed to similar levels of stress identified above within commercial colonies. Sideliners' management techniques and treatment levels typically are less intensive than commercial beekeepers, however, more intensive than hobbyist beekeepers (Lee et al. 2015).

Honeybee colonies managed by hobbyist beekeepers, also known as backyard beekeepers, remain stationary; thus, they are not being placed under the same levels of transportation stress as the commercially and sideliner managed colonies (Lee et al. 2015). Hobbyist beekeepers have fewer than 50 colonies and do not manage their colonies for large-scale income (Tsutsumi and Oishi 2011, Lee et al. 2015). Typically, hobbyist beekeepers do not manage colonies as intensely, often leaving colonies untreated for various parasites, pathogens, and diseases (Lee et al. 2015). The lack of exposure to other honey bees from across the United States may inhibit newly emerging pests from reaching stationary colonies. Nevertheless, there has been limited research conducted on the occurrence of parasites and pathogens in migratory versus hobbyist beekeepers' colonies. Such a study would be important to understand better how these parasites and pathogens are spreading, and determine how responsible the migration of colonies is for honey bee decline.

In a 2017 study located in Brazil, stationary and migratory colonies of Africanized honey bees, *A. mellifera lineage 'A'* were examined for various parasite and pathogen species (Cestaro

et al. 2017). They found no significant difference in rates of infestation of pathogens between stationary and migratory colonies. This study infers that migratory beekeeping alone does not explain the largescale decrease in honey bee health. The study instead implicates seasonality as a determining factor in colony health.

Honey bee parasites and pathogens

Honey bee populations in the United States continue to fluctuate annually with no single determined explanation (Core et al. 2012). Rather, multiple biotic and abiotic stressors have been suggested as being responsible for this decline (Potts et al. 2010, Goulson et al. 2015). Among these stressors are invasive species of parasites and pathogens (Core et al. 2012, Botías et al. 2013).

While a number of parasites and pathogens are familiar topics of research in honey bee decline, other understudied and less well-known parasites and pathogens are important in order to fully understand the decline in honey bee health (Genersch 2010). The limited research on these pests is cause for concern as many of these species' role in causing mortality or decreased hive health is unknown (Jara et al. 2012, Cavigli et al. 2016).

In a 2016 study performed by Cavigli et al., pathogen prevalence and abundance were examined in honey bee colonies involved in the almond pollination in California. *Nosema ceranae* and trypanosomatids were among the most prevalent pathogens detected in the study. Also, a higher percent of the weak colonies suffered from a higher prevalence of pathogens than stronger colonies.

Microsporidia

Nosema infection (Microsporida: Nosematidae) in honey bees occurs when intracellular microsporidian spores are ingested by the bee, allowing for the spores to multiply and be released. The spores quickly spread via oral-fecal pathways and oral-oral pathways (vanEngelsdorp and Meixner 2010, Evans and Schwarz 2011, Core et al. 2012, Smith 2012). Once the spores enter the bee's body, they then invade the epithelial cells of the midgut (Evans and Schwarz 2011, Uroš et al. 2014).The *Nosema* spores develop, germinate and rapidly multiply in the midgut of the bee, and within 48 hours, the mature spores are released via defecation, allowing for the continued spread of the *Nosema* spores (Evans and Schwarz 2011). The two-known species of *Nosema* affecting honey bees include *Nosema apis* (Zander, 1909) and *N. ceranae* (Fries et al. 1996). These *Nosema* pathogens are obligate pathogens responsible for causing nosemosis in adult honey bees (Fries 2010).

Nosema apis has consistently utilized the European honey bee (*Apis mellifera*) as its host and is well documented in its distribution in the United States being widespread (Matheson 1993). During the winter, fall and early spring, *N. apis* levels tend to be highest; however, 30-35°C is the optimal temperature for *N. apis* to develop (Botías et al. 2013). Dysentery is a primary characteristic of *N. apis*, resulting in defecation within and directly outside the hive, also known as fecal staining (Smith 2012). *Nosema apis* rarely causes major colony losses (Bailey and Ball 2013).

Nosema ceranae switched hosts from the Asian honey bee (*Apis cerana*) as detected in 1990 (Chen et al. 2008). Records show occurrences of *N. ceranae* in the United States dating back to 1995 (Paxton 2010, Smith 2012). The lack of coevolution between the European honey bee and *N. ceranae* may explain why *N. ceranae* has a more detrimental effect on the European

honey bee than *N. apis* (Mayack and Naug 2009, vanEngelsdorp and Meixner 2010). *Nosema ceranae* is also considered to be of higher virulence than *N. apis* (Huang et al. 2015).

The distribution of *N. ceranae* in the United States at the State level is less known than *N. apis*. However, recent research indicates that *N. ceranae* is the more dominant *Nosema* species today and has even displaced *N. apis* as the most common *Nosema* pathogen of honey bees (Matheson 1993, Chen et al. 2008, Fries 2010, Paxton 2010, Smith 2012). Unlike *N. apis, N. ceranae* is highly pathogenic and has been linked to issues in digestion, shortened life span, decreased population, overwintering mortality and reduced honey production (Chen et al. 2008, Pacini et al. 2016).

Both *N. apis* and *N. ceranae* peak in population from January to April in the Northern Hemisphere (Meixner and Conte 2016). The increase of *Nosema* during autumn and winter months is likely due to confinement of individuals and brood levels decreasing (Michalczyk and Rajmund 2014). Also, bees are unable to exit the hive to defecate.

Bicyclohexylammonium fumagillin (fumagillin) is the lone approved treatment for *Nosema* disease in the United States. It has been used in the U.S. for the last 50 years to treat for *N. apis* and more recently has been used to suppress *N. ceranae.* Typically used as a preventative method of controlling *Nosema,* fumagillin is only applied during the late fall and early spring so as to not contaminate honey (Huang et al. 2013). While fumagillin was effective at controlling *N. apis,* recent studies have shown that it is not as effective at preventing *N. ceranae.* In fact, studies have demonstrated that *N. ceranae* is actually capable of developing resistance to fumagillin. Efficacy and degradation are also of concern (Higes et al. 2011, Huang et al. 2013).

The National Honey Bee Pests and Diseases Survey is a collaborative research survey between Bee Informed Partnership (BIP) and the USDA Animal Plant Health Inspection Service

(APHIS). The survey has been conducted annually, beginning in 2009, and aims to monitor for various threats to honey bees, including disease, pests and pathogens on nationwide scale.

The 2013-2014 survey was conducted from June 2013 through September of 2014. The survey collected 648 honey bee samples from across the United States. A total of 32 states were represented in this survey, Arkansas included. *Nosema* spore counts were performed for each honey bee and of those sampled, 47% had detectable spore loads. The 2014-2015 survey included samples from July 2014 to June 2015. The survey examined 551 samples from 26 states, and found detectable spore loads in 50.3% of the samples. This survey did not use molecular diagnostics to identify *Nosema* spores to species.

State level honey bee surveys examining *Nosema* have been conducted, which used molecular diagnostics for species identification. Surveys occurred in Virginia (Traver and Fell 2011), New York and South Dakota (Szalanski et al. 2013). These studies show that 29-44% of the apiaries tested were positive for *Nosema sp.* (Traver and Fell 2011, Szalanski et al. 2013). In Szalanski et al. (2013), *N. ceranae* was detected in 42% of samples collected from South Dakota, and 54% of samples from New York tested positive for *Nosema* sp. Of the samples testing positive for *Nosema*, 97% were identified as *N. ceranae*, with the remainder being *N. apis*.

Bacteria

Spiroplasmas are small Eubacteria which lack a cell wall and descend from Gram-positive bacteria (Regassa and Gasparich 2006). In certain instances, *Spiroplasmas* are particularly destructive pathogens of plants, vertebrates, and insects (Regassa and Gasparich 2006, Zheng and Chen 2014, Tozkar et al. 2015). In an aquaculture study, *Spiroplasma* species were found to be acting as the causative agent in diseases negatively impacting crustaceans (Regassa and Gasparich 2006). *Spiroplasma apis* (Mouches et al. 1983) and *Spiroplasma*

melliferum (Clark et al. 1985) are two *Spiroplasma* bacterium pathogen species (Entomoplasmatales: Spiroplasmataceae) detected in the European honeybee.

The *Spiroplasma* bacteria breaches the barrier of the gut and enters the hemolymph where parthenogenesis occurs (Evans and Schwarz 2011). *Spiroplasma apis* and *S. melliferum* are found in the hemolymph and gut of adult honey bees, the pathogens spread during the spring and summer via fecal contamination from infected individuals on the surface of visited flowers (Evans and Schwarz 2011, Meeus et al. 2012, Hubert et al. 2015). Once the pathogens reach the hemolymph, they can rapidly multiply and ultimately cause mortality in the bee (Regassa and Gasparich 2006).

Furthermore, *Spiroplasma* has been implicated in causing a seasonally occurring neurological disease known as “spiroplasmosis” or “May disease” and increased mortality. Spiroplasmosis results in the bee having difficulty flying, as well as, hive abandonment (Evans and Schwarz 2011, Schwarz et al. 2014). The first reported instance of *Spiroplasma melliferum* was in Beltsville, MD in 1976 (Clark 1977, 1982, Clark et al. 1985). This study revealed plant surfaces act as reservoirs for *S. melliferum*. By 1980, colonies displaying symptoms of spiroplasmosis were observed in France, and *S. apis* was detected in large quantities (Mouches et al. 1982, 1983, 1984, Zheng and Chen 2014). Higher mortality rates have been observed in honey bees which carry spiroplasmosis. As part of a 2013 study, honey bees were infected with *Spiroplasma* via injection into the hemolymph. These bees died within five days unless given tetracycline (Bailey and Ball 2013).

The distribution of *Spiroplasma* is poorly understood. In a 2014 study, 33% of the honey bee colonies surveyed in Maryland, U.S. were positive for *S. apis* or *S. melliferum*; 16.5% of

these colonies were positive for both species of *Spiroplasma* (Schwarz et al. 2014). The occurrence of these bacterial pathogens in other States is unknown.

Currently, a lack of overall research and monitoring of *Spiroplasma* species in honey bees disallows the understanding of its distribution in the U.S. and overall effect on the bee industry (Evans and Schwarz 2011). However, identification of *S. apis* and *S. melliferum* is facilitated due to the sensitivity of multiplex PCR (Meeus et al. 2012).

Trypanosomes

Trypanosomes are obligate protozoan parasites of multiple invertebrate species (Trypanosomatida: Trypanosomatidae) (Evans and Schwarz 2011). Despite the related *Crithidia bombi* being a known parasite of bumbles bees, trypanosome species *Crithidia mellificae* Langridge and McGhee and *Lotmaria passim* Schwarz are among the neglected parasites of the honey bee regarding research (Evans and Schwarz 2011). Both species are thought to occur in the hindgut and rectum of adult honey bees (Runckel et al. 2011).

While the impact of both *C. mellificae* and *L. passim* on *A. mellifera* is not well known, *Crithidia bombi* is a trypanosomatid which targets bumble bees. *Crithidia bombi* has been researched and is associated with decreased *Bombus* health and an increase in bee mortality. In a 2003 study in Switzerland, it was found that colonies with a queen positive for *C. bombi* had a 40% lower fitness level than colonies without an infected queen (Brown et al. 2003).

Since its description in 1967, little research has been performed related to *C. mellificae* (Schwarz et al. 2015). The lack of knowledge on *C. mellificae* is due in large part to the complexity of isolating it and identifying samples using morphological characteristics. There has been confusion and complications in the morphological taxonomy of trypanosomatids (Schmid-Hempel and Tognazzo 2010). In fact, *L. passim* was not identified as a separate species until

2015 in Maryland (Schwarz et al. 2015). *Lotmaria passim* has since been detected in Belgium, Chile, Japan, and Switzerland (Ravoet et al. 2013).

A study in Belgium examined 363 honey bee samples, revealing 70.5% of those samples were positive for *C. mellificae* (Ravoet et al. 2013). A later study in 2015 revealed that *L. passim* was the dominant trypanosome species in Belgium, Japan, and Switzerland (Ravoet et al. 2015). Furthermore, a molecular diagnostic technique has recently been developed to identify and distinguish *L. passim* and *C. mellificae* (Szalanski et al. 2016). This study also detected *L. passim* in honey bees from Hawaii and American Samoa.

In a one-year Chilean study conducted in 2014-15, honey bees were collected from apiaries in the key beekeeping regions of Chile. A total of 189 colonies were sampled, and using PCR with species specific primers, *L. passim* was detected. The study found a prevalence of *L. passim* between 40-90% (Arismendi et al. 2016).

Apocephalus borealis

Apocephalus borealis Brues is a parasitic phorid fly (Diptera: Phoridae) detected in 2008. *A. borealis* is a known parasite of bumble bees and paper wasps. However, it was only recently determined to attack honey bees (Core et al. 2012, Khattab 2014). The fly was first discovered to be parasitizing honey bees when a study used DNA barcoding to determine it was the same species (Core et al. 2012). Since then it has been detected in Oregon, Washington, Vermont, South Dakota and British Columbia, Canada (Core et al. 2012). The fly falls into the genus *Apocephalus*, also known as the decapitating flies. Members of this genus primarily attack ant species, some of which are used as biological control agents of the imported fire ant. However, *A. borealis* is among the subgenus Mesophora, which includes species which use other hosts including spiders, beetles, wasps and other bee species (Core et al. 2012).

The female fly deposits her eggs into the abdomen of the honey bee using her ovipositor. As the larvae feed on the bee's muscles and nervous system, they can mature and begin attacking the brain of the adult, resulting in abnormal behaviors (Khattab 2014). According to a 2011 study by Core et. al, *A. borealis* alters the typical behavior of worker honey bees. The study found bees infected with *A. borealis* flew at night and were disoriented. The bees infected tended to be attracted to light and were dead by the following day. This colony abandonment and mortality in worker honey bees results in decreased population and productivity (Core et al. 2012). The mature fly emerges on average, within a week and usually exits between the thorax and head of the honey bee (Khattab 2014)

In a California study performed in 2012, a total of 7,417 honey bees were collected from the bay area and molecularly and morphologically tested for *A. borealis (Core et al. 2012)*. The study found a mean parasitism rate of 6% among the worker honey bees tested. Furthermore, this study also screened the parasitized honey bees and adult and immature phorid flies for various pathogens. These tests detected both *Nosema ceranae* as well as the deformed winged virus, implicating that the fly can act as a vector.

Little research has been done on the distribution of *A. borealis* infections in honey bees in the United States; however, it has been detected in California, South Dakota, Washington, Oregon, and Vermont, indicating further research on its distribution is necessary (Core et al. 2012).

Varroa destructor

Varroa destructor Anderson and Trueman (Parasitiformes: Varroidae) is an ectoparasitic mite and is currently considered the number one threat to honeybees worldwide (Uroš et al. 2014, Locke 2015). Endemic to Asia, the *Varroa* mite is now distributed worldwide; occurring in

every continent in which honeybees are distributed except Australia (Hood 2000). The *Varroa* mite's original host is the Asian honeybee (*Apis cerana*), a relative of the European honeybee. Because the Asian honeybee coevolved with the *Varroa* mite, it is more resilient compared to the European honeybee (Locke et al. 2012).

The life cycle of the *Varroa* mite has two stages, both of which involve dependency on the honey bee as a host (Hubert et al. 2016). The phoretic stage (transport stage) and the reproductive stage. The phoretic stage lasts 5-11 days and involves the mite attaching to adult honeybees and feeding on the bee's hemolymph. This is essentially a transportation mechanism and is also how *Varroa* mites are capable of spreading to new locations saturated with honeybees (Huang 2012).

The reproductive stage of *V. destructor* involves the fertilized female entering the honeybee hive and identifying a suitable location for reproduction to occur. According to Huang (2012), approximately 15 hours before a brood cell containing a newly deposited bee larva is capped, the mature fertilized female *Varroa* mite enters the cell. The cell is then sealed and the fertilized *Varroa* mite feeds on the hemolymph of the bee larva, this typically occurs nine days after the egg has been laid. The following day, the mite lays an egg every 30 hours. The first egg is the only male produced, all subsequent offspring are female. Once the female offspring become sexually mature, each mite mates with single male mite in the cell. Approximately 21 days after the honeybee egg was laid, the matured bee leaves the cell, transporting the female mites and beginning the phoretic stage (Huang 2012). *Varroa destructor* relies on honeybees and is unable to reproduce without finding a suitable honeybee host (Locke et al. 2012). Once it has found a host the mite is able to reproduce and mature quickly, making it a dangerous parasite (Locke et al. 2012).

The mite's presence within a honey bee colony has been linked to reduced lifespan of bees, deformities, increased occurrence of disease, and an increase in overall colony mortality (Le Conte et al. 2010, Core et al. 2012). Colony mortality may occur if the *Varroa* mite population in a colony is not controlled (Locke et al. 2012). The mite is easily spread to new geographic areas as honeybees are moved or migrated. *Varroa* mites have also been observed ingesting the pathogenic *Spiroplasma* bacteria, suggesting the mite may aid in the spread of this potentially dangerous bacteria (Bruce et al. 1991, Hubert et al. 2015).

Multiple surveys have been performed on the occurrence of *V. destructor* at the national level in the United States. Specifically, the 2013-2014 National Honey Bee Pests and Diseases Survey Report examined *Varroa* mite loads from managed honey bee colonies. The study sampled beekeepers from 32 states and found 98.2% of the 648 honey bee samples were positive for *V. destructor* (Rennich et al. 2015). The 2014-2015 survey examined 551 samples from 26 states, 86% of which were prevalent for *V. destructor.*

A 2008 study sampled honey bee colonies in Arkansas that were not managed for *Varroa* mite, with 65% of the samples from beekeepers who had five or fewer hives. The study found a mean *Varroa* mite infestation level of 3.12, with 0 to 87.95 mites per 100 honey bees in a colony (Zawislak 2008).

Currently, an infallible chemical treatment for *V. destructor* does not exist (Le Conte et al. 2010). While miticides are sometimes used in *Varroa* mite control, there is a concern over their long-term toxicity to the honey bee colony as well as fear of long-term resistance by the mites (Le Conte et al. 2010).

Other reasons for honey bee population decline

Other parasite and pathogen species have also been known to cause reduced colony health. Tracheal mites, small hive beetle, and bacterial brood diseases such as American foulbrood and European foulbrood have shown to be notable threats to honey bee populations (vanEngelsdorp et al. 2008). Additionally, pesticides, habitat loss and fragmentation, and poor nutrition are also factors which have been examined in pollinator loss (Klein et al. 2007, vanEngelsdorp et al. 2008, Potts et al. 2010).

Colony collapse disorder (CCD) is a phenomenon in which large numbers of managed worker honey bees disappear suddenly for no known reason, leaving behind substantial amounts of brood and food (vanEngelsdorp et al. 2009, Williams et al. 2010). Unfortunately, CCD is often confused with other honey bee colony losses which may be explained. In more recent years, as honey bee populations continue to fluctuate, an interaction of multiple stressors causing decrease in honey bee health and even death among honey bee populations is important to examine.

Molecular Diagnostic Methods

Traditional taxonomic identification of parasites and pathogens can be tedious and often unreliable in species closely related and under-researched species. In fact, among the species examined in the study, some of the species lack a morphological description. Also, the differences between pathogen species in this study are so small, that it would be virtually impossible to identify the correct species of pathogen reliably. Molecular diagnostics occurs a more modern technique of molecular diagnostics may be used to identify species correctly. Molecular diagnostic techniques allow for a robust, reliable method of identifying honey bee pathogens and parasites especially from honey bee samples that have been preserved in ethanol.

Furthermore, molecular diagnostics allows for hundreds of samples to be screened accurately and cost-effectively over a short amount of time (Meeus et al. 2012, Arismendi et al. 2016).

Polymerase chain reaction (PCR) is a molecular diagnostic technique in which paired primers are used to replicate a specific DNA sequence in the genome. PCR is an inexpensive and more sensitive technique than traditional spore counts via microscopy (Webster et al. 2004).

Polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) and Multiplex PCR allow for species level identification. Multiplex PCR involves using multiple species-specific primers at once for detection. By using multiple primers multiple species are able to be detected using one PCR. PCR-RFLP uses restriction enzymes on the PCR product to fragment the DNA at a specific sequence.

Need for statewide surveys

Currently, the continents with the most results concerned with pollinator loss include North America and Europe. Research has indicated that in order to improve future policy agreements concerning pollinators, an increase in pollinator research is required to occur in other regions (Potts et al. 2010). Similarly, in the United States there is an underrepresentation of pollinator research being performed at the statewide level. Knowledge of pollinator pest occurrence at the state level is necessary to understand and integrate current knowledge as well as for future policy decisions. Statewide surveys are especially pertinent and relevant in honey bee research, as migratory beekeeping involves the transportation of colonies from one state to the next. A better understanding of parasite and pathogen infections in honey bees, at the statewide level, is crucial in predicting the long-term health of honey bees and gaining more insight into pollinator decline.

Objectives

The objectives of my thesis are:

1. Survey managed Arkansas and Oklahoma honey bee colonies for prevalence and distribution of invasive parasites and pathogens: *Varroa destructor, Nosema apis, N. ceranae, Apocephalus borealis, Spiroplasma apis, S. melliferum, Lotmaria passim and Crithidia mellificae*

2. Test for co-occurrence of parasites and pathogens

3. Use seasonality to compare the distribution and occurrence of parasites and pathogens

4. Compare parasite and pathogen occurrence between Oklahoma migratory beekeeper's samples and Arkansas hobbyist beekeeper's samples

Literature Cited

Aizen, M. A., and L. D. Harder. **2009**. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Curr. Biol. 19: 915–918.

Arismendi, N., A. Bruna, N. Zapata, and M. Vargas. **2016**. PCR-specific detection of recently described *Lotmaria passim* (Trypanosomatidae) in Chilean apiaries. J. Invertebr. Pathol. 134: 1–5.

Bacandritsos, N., A. Granato, G. Budge, I. Papanastasiou, E. Roinioti, M. Caldon, C. Falcaro, A. Gallina, and F. Mutinelli. **2010**. Sudden deaths and colony population decline in Greek honey bee colonies. J. Invertebr. Pathol. 105: 335–340.

Bailey, L., and B. V. Ball. **2013**. Honey bee pathology. Elsevier.

Bond, J., K. Plattner, and K. Hunt. **2014**. Fruit and tree nuts outlook: economic insight U.S. pollination-services market. USDA economic research service situation and outlook FTS-357SA. United States Department of Agriculture.

Botías, C., R. Martín-Hernández, L. Barrios, A. Meana, and M. Higes. **2013**. *Nosema* spp. infection and its negative effects on honey bees (*Apis mellifera* iberiensis) at the colony level. Vet. Res. 44: 25.

Brown, M. J. F., R. Schmid-Hempel, and P. Schmid-Hempel. **2003**. Strong context-dependent virulence in a host-parasite system: reconciling genetic evidence with theory. J. Anim. Ecol. 72: 994–1002.

Bruce, W. A., R. B. Henegar, and K. J. Hackett. **1991**. An artificial membrane for in vitro feeding of *Varroa jacobsoni* and *Acarapis woodi*, mite parasites of honey bees. Apidologie. 22: 503–507.

Calderone, N. W. 2012. Insect pollinated crops, insect pollinators and US agriculture: trend analysis of aggregate data for the period 1992-2009. PLOS ONE. 7: e37235.

Cavigli, I., K. F. Daughenbaugh, M. Martin, M. Lerch, K. Banner, E. Garcia, L. M. Brutscher, and M. L. Flenniken. **2016**. Pathogen prevalence and abundance in honey bee colonies involved in almond pollination. Apidologie. 47: 251–266.

Cestaro, L. G., M. L. T. M. F. Alves, D. Message, M. V. G. B. Silva, and É. W. Teixeira. **2017**. Honey bee (*Apis mellifera*) health in stationary and migratory apiaries. Sociobiology. 64: 42–49.

Chapman, R. E., and A. F. G. Bourke. **2001**. The influence of sociality on the conservation biology of social insects. Ecol. Lett. 4: 650–662.

Chen, Y., J. D. Evans, I. B. Smith, and J. S. Pettis. **2008**. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. J. Invertebr. Pathol. 97: 186–188.

Clark, T. B. 1977. *Spiroplasma* sp., a new pathogen in honey bees. J. Invertebr. Pathol. 29: 112–113.

Clark, T. B. 1982. *Spiroplasmas*: diversity of arthropod reservoirs and host-parasite relationships. Science. 217: 57–59.

Clark, T. B., R. F. Whitcomb, J. G. Tully, C. Mouches, C. Saillard, J. M. BOVe, H. Wroblewski, P. Carle, D. L. Rose, R. B. Henegar, and D. L. Williamson. **1985**. *Spiroplasma melliferum*, a new species from the honeybee (*Apis mellifera*). Int. J. Syst. Bacteriol. 35: 296–308.

Connor, L. 2007. Sideline Beekeepers. Bee Cult. Medina. 135: 22–24.

Core, A., C. Runckel, J. Ivers, C. Quock, T. Siapno, S. DeNault, B. Brown, J. DeRisi, C. D. Smith, and J. Hafernik. **2012**. A new threat to honey bees, the parasitic phorid fly *Apocephalus borealis*. PLOS ONE. 7: e29639.

Döke, M. A., M. Frazier, and C. M. Grozinger. **2015**. Overwintering honey bees: biology and management. Curr. Opin. Insect Sci. 10: 185–193.

Evans, J. D., and R. S. Schwarz. **2011**. Bees brought to their knees: microbes affecting honey bee health. Trends Microbiol. 19: 614–620.

Fries, I. 2010. *Nosema ceranae* in European honey bees (*Apis mellifera*). J. Invertebr. Pathol. 103 Suppl 1: S73-79.

Genersch, E. 2010. Honey bee pathology: current threats to honey bees and beekeeping. Appl. Microbiol. Biotechnol. 87: 87–97.

Goulson, D., E. Nicholls, C. Botías, and E. L. Rotheray. **2015**. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science. 347: 1255957.

Hendriksma, H. P., and S. Shafir. **2016**. Honey bee foragers balance colony nutritional deficiencies. Behav. Ecol. Sociobiol. 70: 509–517.

Higes, M., M. J. Nozal, A. Alvaro, L. Barrios, A. Meana, R. Martín-Hernández, J. L. Bernal, and J. Bernal. **2011**. The stability and effectiveness of fumagillin in controlling *Nosema ceranae* (Microsporidia) infection in honey bees (Apis mellifera) under laboratory and field conditions. Apidologie. 42: 364–377.

Hood, W. 2000. *Varroa* mite control in South Carolina: extension: Clemson University: South Carolina. Clemson Univ.

(http://www.clemson.edu/extension/beekeepers/factsheets/varroa_mite_control_in_sc.html).

Huang, W.F., L. Solter, K. Aronstein, and Z. Huang. **2015**. Infectivity and virulence of *Nosema ceranae* and *Nosema apis* in commercially available North American honey bees. J. Invertebr. Pathol. 124: 107–113.

Huang, W.F., L. F. Solter, P. M. Yau, and B. S. Imai. **2013**. *Nosema ceranae* escapes fumagillin control in honey bees. PLOS Pathog. 9: e1003185.

Huang, Z. Y. 2012. *Varroa* mite reproductive biology. Am. Bee J. 152: 981–985.

Hubert, J., T. Erban, M. Kamler, J. Kopecky, M. Nesvorna, S. Hejdankova, D. Titera, J. Tyl, and L. Zurek. **2015**. Bacteria detected in the honeybee parasitic mite *Varroa destructor* collected from beehive winter debris. J. Appl. Microbiol. 119: 640–654.

Hubert, J., M. Kamler, M. Nesvorna, O. Ledvinka, J. Kopecky, and T. Erban. **2016**. Comparison of Varroa destructor and Worker Honeybee Microbiota Within Hives Indicates Shared Bacteria. Microb. Ecol. 72: 448–459.

James, R., and T. L. Pitts-Singer. **2008**. Bee pollination in agricultural ecosystems. Oxford University Press.

Jara, L., A. Cepero, E. Garrido-Bailón, R. Martín-Hernández, M. Higes, and P. De la Rúa. **2012**. Linking evolutionary lineage with parasite and pathogen prevalence in the Iberian honey bee. J. Invertebr. Pathol. 110: 8–13.

Khattab, M. 2014. The first records of the parasite zombie fly (*Apocephalus borialis* Brues) on honeybee, *Apis mellifera* in Egypt. Int. J. Agric. Sci. Res. 4: 37–42.

Klein, A.M., B. E. Vaissière, J. H. Cane, I. Steffan-Dewenter, S. A. Cunningham, C. Kremen, and T. Tscharntke. **2007**. Importance of pollinators in changing landscapes for world crops. Proc. R. Soc. Lond. B Biol. Sci. 274: 303–313.

Kluser, S., and P. Neumann. **2010**. Global honey bee colony disorder and other threats to insect pollinators.

Kurze, C., J. Routtu, and R. F. A. Moritz. **2016**. Parasite resistance and tolerance in honeybees at the individual and social level. Zoology, SI: Host-Parasite Coevolution. 119: 290–297.

Le Conte, Y., M. Ellis, and W. Ritter. **2010**. *Varroa* mites and honey bee health: can *Varroa* explain part of the colony losses? Apidologie. 41: 353–363.

Lee, K. V., N. Steinhauer, K. Rennich, M. E. Wilson, D. R. Tarpy, D. M. Caron, R. Rose, K. S. Delaplane, K. Baylis, E. J. Lengerich, J. Pettis, J. A. Skinner, J. T. Wilkes, R.

Sagili, and D. vanEngelsdorp. **2015**. A national survey of managed honey bee 2013–2014 annual colony losses in the USA. Apidologie. 46: 292–305.

Locke, B. 2015. Natural *Varroa* mite surviving *Apis mellifera* honeybee populations. Apidologie. 1–16.

Locke, B., Y. L. Conte, D. Crauser, and I. Fries. **2012**. Host adaptations reduce the reproductive success of *Varroa destructor* in two distinct European honey bee populations: Host Adaptations in *Varroa* -Resistant Honey Bees. Ecol. Evol. 2: 1144–1150.

Matheson, A. 1993. World bee health report. Bee World. 74: 176–212.

Mayack, C., and D. Naug. **2009**. Energetic stress in the honeybee *Apis mellifera* from *Nosema ceranae* infection. J. Invertebr. Pathol. 100: 185–188.

McGregor, S. E. 1976. Insect pollination of cultivated crop plants. US Department of Agriculture, Washington, DC.

Meeus, I., V. Vercruysse, and G. Smagghe. **2012**. Molecular detection of *Spiroplasma apis* and *Spiroplasma melliferum* in bees. J. Invertebr. Pathol. 109: 172–174.

Meixner, M. D., and Y. L. Conte. **2016**. A current perspective on honey bee health. Apidologie. 47: 273–275.

Menzel, R., and U. Muller. **1996**. Learning and memory in honeybees: from behavior to neural substrates. Annu. Rev. Neurosci. 19: 379–404.

Michalczyk, M., and S. Rajmund. **2014**. Nosemosis in honey bees. Pol. J. Nat. Sci. 29: 91–99.

Mouches, C., J. M. Bové, and J. Albisetti. **1984**. Pathogenicity of *Spiroplasma apis* and other spiroplasmas for honey-bees in Southwestern France. Ann. Inst. Pasteur Microbiol. 135: 151–155.

Mouches, C., J. M. Bové, J. Albisetti, T. B. Clark, and J. G. Tully. **1982**. A spiroplasma of serogroup IV causes a May-disease-like disorder of honeybees in Southwestern France. Microb. Ecol. 8: 387–399.

Mouches, C., J. Bové, J. G. Tully, D. L. Rose, R. E. McCoy, P. Carle-Junca, M. Garnier, and C. Saillard. **1983**. *Spiroplasma apis*, a new species from the honey-bee *Apis mellifera*. Ann. Inst. Pasteur Microbiol. 134: 383–397.

Pacini, A., A. Mira, A. Molineri, A. Giacobino, N. Bulacio Cagnolo, A. Aignasse, L. Zago, M. Izaguirre, J. Merke, E. Orellano, E. Bertozzi, H. Pietronave, R. Russo, A. Scannapieco, S. Lanzavecchia, L. Schnittger, and M. Signorini. **2016**. Distribution

and prevalence of *Nosema apis* and *N. ceranae* in temperate and subtropical eco-regions of Argentina. J. Invertebr. Pathol. 141: 34–37.

Paxton, R. J. 2010. Does infection by *Nosema ceranae* cause "Colony Collapse Disorder" in honey bees (*Apis mellifera*)? J. Apic. Res. 49: 80–84.

Pilati, L., and M. Prestamburgo. **2016**. Sequential relationship between profitability and sustainability: the case of migratory beekeeping. Sustainability. 8: 94.

Potts, S. G., J. C. Biesmeijer, C. Kremen, P. Neumann, O. Schweiger, and W. E. Kunin. **2010**. Global pollinator declines: trends, impacts and drivers. Trends Ecol. Evol. 25: 345–353.

Ravoet, J., J. Maharramov, I. Meeus, L. De Smet, T. Wenseleers, G. Smagghe, and D. C. de Graaf. **2013**. Comprehensive bee pathogen screening in Belgium reveals *Crithidia mellificae* as a new contributory factor to winter mortality. PLOS ONE. 8: e72443.

Ravoet, J., R. S. Schwarz, T. Descamps, O. Yañez, C. O. Tozkar, R. Martin-Hernandez, C. Bartolomé, L. De Smet, M. Higes, T. Wenseleers, R. Schmid-Hempel, P. Neumann, T. Kadowaki, J. D. Evans, and D. C. de Graaf. **2015**. Differential diagnosis of the honey bee trypanosomatids *Crithidia mellificae* and *Lotmaria passim*. J. Invertebr. Pathol. 130: 21–27.

Regassa, L. B., and G. E. Gasparich. **2006**. Spiroplasmas: evolutionary relationships and biodiversity. Front. Biosci. J. Virtual Libr. 11: 2983–3002.

Rennich, K., G. Kunkel, S. Abban, R. Fahey, H. Eversole, J. Evans, E. Forsgren, V. Levi, D. Lopez, S. Madella, J. Pettis, R. Rose, and D. vanEngelsdorp. **2015**. 2013-2014 National honey bee pests and diseases survey report. University of Maryland.

Royce, L. A., and P. A. Rossignol. **1990**. Epidemiology of honey bee parasites. Parasitol. Today. 6: 348–353.

Runckel, C., M. L. Flenniken, J. C. Engel, J. G. Ruby, D. Ganem, R. Andino, and J. L. DeRisi. **2011**. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, *Nosema*, and *Crithidia*. PLOS ONE. 6: e20656.

Schmid-Hempel, R., and M. Tognazzo. **2010**. Molecular divergence defines two distinct lineages of *Crithidia bombi* (Trypanosomatidae), parasites of bumblebees. J. Eukaryot. Microbiol. 57: 337–345.

Schwarz, R. S., G. R. Bauchan, C. A. Murphy, J. Ravoet, D. C. de Graaf, and J. D. Evans. **2015**. Characterization of two species of Trypanosomatidae from the honey bee *Apis mellifera*: *Crithidia mellificae* Langridge and McGhee, and *Lotmaria passim* n. gen., n. sp. J. Eukaryot. Microbiol. 62: 567–583.

Schwarz, R. S., É. W. Teixeira, J. P. Tauber, J. M. Birke, M. F. Martins, I. Fonseca, and J. D. Evans. **2014**. Honey bee colonies act as reservoirs for two *Spiroplasma* facultative symbionts and incur complex, multiyear infection dynamics. MicrobiologyOpen. 3: 341–355.

Simone-Finstrom, M., H. Li-Byarlay, M. H. Huang, M. K. Strand, O. Rueppell, and D. R. Tarpy. **2016**. Migratory management and environmental conditions affect lifespan and oxidative stress in honey bees. Sci. Rep. 6.

Smith, K. M., E. H. Loh, M. K. Rostal, C. M. Zambrana-Torrelio, L. Mendiola, and P. Daszak. **2013**. Pathogens, pests, and economics: drivers of honey bee colony declines and losses. EcoHealth. 10: 434–445.

Smith, M. L. 2012. The honey bee parasite *Nosema ceranae*: transmissible via food exchange? PLOS ONE. 7: e43319.

Szalanski, A. L., J. Whitaker, A. D. Tripodi, and P. Cappy. **2013**. Prevalence of *Nosema* from managed honey bee colonies in South Dakota and New York. J. Agric. Urban Entomol. 29: 99–104.

Szalanski, A., C. E. Trammel, A. D. Tripodi, D. Cleary, L. Rusert, and D. Downey. **2016**. Molecular diagnostics of the honey bee parasite *Lotmaria Passim*, and *Crithidia* spp. using multiplex PCR. Fla. Entomol.

Tozkar, C. A., M. Kence, A. Kence, Q. Huang, and J. D. Evans. **2015**. Metatranscriptomic analyses of honey bee colonies. Front. Genet. 6.

Traver, B. E., and R. D. Fell. **2011**. Prevalence and infection intensity of *Nosema* in honey bee (*Apis mellifera* L.) colonies in Virginia. J. Invertebr. Pathol. 107: 43–49.

Traynor, K. S., K. Rennich, E. Forsgren, R. Rose, J. Pettis, G. Kunkel, S. Madella, J. Evans, D. Lopez, and D. vanEngelsdorp. **2016**. Multiyear survey targeting disease incidence in US honey bees. Apidologie. 47: 325–347.

Tsutsumi, L., and D. Oishi. **2011**. Farm and forestry production and marketing profile for honey bees. Permanent Agriculture Resources (PAR), Holualoa, HI.

Uroš, G., S. Jevrosima, G. Bojan, S. Predrag, Đ. Spomenka, V. Branislav, and S. Zoran. **2014**. *Nosema ceranae* DNA in honey bee haemolymph and honey bee mite *Varroa destructor*. Acta Vet. (Beogr.). 64.

vanEngelsdorp, D., D. Caron, J. Hayes, R. Underwood, M. Henson, K. Rennich, A. Spleen, M. Andree, R. Snyder, K. Lee, K. Roccasecca, M. Wilson, J. Wilkes, E. Lengerich, and J. Pettis. **2012**. A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. J. Apic. Res. 51: 115–124.

vanEngelsdorp, D., J. D. Evans, C. Saegerman, C. Mullin, E. Haubruge, B. K. Nguyen, M. Frazier, J. Frazier, D. Cox-Foster, Y. Chen, R. Underwood, D. R. Tarpy, and J. S. Pettis. **2009**. Colony collapse disorder: A descriptive study. PLOS ONE. 4: e6481.

vanEngelsdorp, D., J. H. Jr, R. M. Underwood, and J. Pettis. **2008**. A survey of honey bee colony losses in the U.S., fall 2007 to spring 2008. PLOS ONE. 3: e4071.

vanEngelsdorp, D., and M. D. Meixner. **2010**. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. J. Invertebr. Pathol. 103: S80–S95.

Webster, T. C., K. W. Pomper, G. Hunt, E. M. Thacker, and S. C. Jones. **2004**. *Nosema apis* infection in worker and queen *Apis mellifera*. Apidologie. 35: 49–54.

Williams, G. R., D. R. Tarpy, D. vanEngelsdorp, M.-P. Chauzat, D. L. Cox-Foster, K. S. Delaplane, P. Neumann, J. S. Pettis, R. E. L. Rogers, and D. Shutler. **2010**. Colony collapse disorder in context. BioEssays. 32: 845–846.

Youngsteadt, E., R. H. Appler, M. M. López-Uribe, D. R. Tarpy, and S. D. Frank. **2015**. Urbanization increases pathogen pressure on feral and managed honey bees. PLOS ONE. 10: e0142031.

Zawislak, J. 2008. Alternative control of the *Varroa* mite on honey bees using botanical smokes (M.S.).

Zheng, H.Q., and Y. P. Chen. **2014**. Detection of *Spiroplasma melliferum* in honey bee colonies in the US. J. Invertebr. Pathol. 119: 47–49.

Chapter 2: Arkansas

A molecular diagnostic survey of honey bee, *Apis mellifera* L., pathogens and parasites from Arkansas, USA

Abstract

Declines in honey bee, *Apis mellifera* L., health in the United States continues to occur with no single determined explanation. It has instead been implicated that the interaction between multiple biotic and abiotic stressors may be responsible for this decline. Among these stressors are invasive species of parasites and pathogens of honey bees. As parasites and pathogens become a topic of interest, understudied parasites and pathogens may be an important factor in understanding the decline in honey bee health. Specifically, examining parasite and pathogen infection prevalence at the statewide level may provide insight relative to national surveys. Furthermore, few studies have examined parasite and pathogen occurrence in colonies managed by hobbyist beekeepers. Understanding the distribution of these parasite and pathogen species at the hobbyist beekeeping level may allow insight into how prevalent these species are as well as how they may be spreading.

Molecular diagnostics were performed on worker honey bee samples from Arkansas hobbyist beekeepers using PCR to detect the presence of: *Nosema apis* and *N. ceranae;* bacterial pathogens *Spiroplasma apis* and *S. melliferum;* Trypanosomatid species *Crithidia mellificae and Lotmaria passim* and the parasitic phorid fly *Apocephalus borealis.* This study found 11.6% of the colony samples were positive for *N. ceranae* and on average, 11.3% of samples were positive for *L. passim* in Arkansas. This study did not detect *N. apis,* either species of *Spiroplasma, C, mellificae,* or *A. borealis.* Also, using a mite wash *Varroa destructor* was isolated from the

honey bee samples. A total of 45.5% of the honey bee samples had *V. destructor* present. This research documents the first occurrence of *L. passim* in Arkansas honey bees.

Introduction

Managed honey bee colonies in the United States have suffered declines in the past decade, yet no single factor has been identified as the cause. Instead, research indicates that a number of abiotic and biotic factors, acting together, are to blame (Potts et al. 2010, Williams et al. 2010, Runckel et al. 2011, Core et al. 2012). Specifically, a decline in colonies in Arkansas has occurred. In 1998 there was approximately 53,000 honey producing colonies in Arkansas and by 2016 this had dropped to 24,000 (Zawislak 2008, USDA 2017). In 2015, Arkansas' honey production was valued at $3,560,000 by 2016 that had dropped to $ 3,047,000 (USDA 2017).

Parasites and pathogens are major contributors to honey bee health decline (Evans and Schwarz 2011). Microsporidian *Nosema* pathogens and the parasitic honey bee *Varroa* mite are widespread and documented pests of honey bees (Chen et al. 2008, Evans and Schwarz 2011, Core et al. 2012, Jara et al. 2012). While studies have established that these pathogenic and parasitic species are abundant and widely occurring, there are a number of other species of honey bee pathogens and parasites for which adequate research is lacking (Chen et al. 2008, Evans and Schwarz 2011, Core et al. 2012, Jara et al. 2012). The lack of research is in large part due to difficulty in detection using microscopy due to small size, low levels, and unknown or lacking obvious pathology symptoms (Whitaker et al. 2010, Evans and Schwarz 2011). Among these lesser studied parasites and pathogens are *Spiroplasma* species, *S. apis* and *mellifera;* trypanosomatid species, *Crithidia mellificae* and *Lotmaria passim;* and the parasitic phorid fly, *Apocephalus borealis.* The limited research on these pests is cause for concern as the role of many of these species in causing mortality or decreased hive health is unknown (Jara et al. 2012, Cavigli et al. 2016).

Treatment is limited for many species of parasites and pathogens in honey bees. The lone treatment for *Nosema sp.* in honey bees is fumagillin dicyclohexylammonium (fumagillin) (Huang et al. 2013, van den Heever et al. 2014). While fumagillin has shown to remedy *N. apis,* it has in some instances shown to worsen *N. ceranae* (Huang et al. 2013).

Commercial practices in the migratory bee industry are implicated as a major stressor affecting honey bee health (Runckel et al. 2011, Simone-Finstrom et al. 2016). Migratory beekeeping involves the transportation of honey bee colonies to different locations for pollinator services. Colonies are often transported thousands of kilometers during warm months, in hives with poor ventilation, which can cause stress (Simone-Finstrom et al. 2016). Furthermore, once colonies arrive at their destination, they are intermingled with colonies from across the United States, thus, exposing colonies to new diseases, viruses, parasites and pathogens (Goulson et al. 2015, Simone-Finstrom et al. 2016). While it is known that practices associated with migratory beekeeping can cause a decrease in honey bee lifespan, because of the increased exposure to additional parasites and pathogens, it is unknown whether these newly identified parasites and pathogens also can be found in non-migratory colonies. For this reason, this study will focus on non-migratory, hobbyist bee colonies, which makes up the majority of those currently in Arkansas.

For hobbyist's beekeepers, beekeeping is unlikely to be the primary source of income (Tsutsumi and Oishi 2011). Hobbyist beekeepers typically have fewer than 50 hives and do not transport them for pollination services. Because hobbyist beekeepers' colonies are not being exposed to honey bees from different geographic areas, one would expect that these colonies are not as likely to be exposed to parasites and pathogens from different geographical areas of the United States. However, hobbyist may utilize used equipment as well as purchased packaged

bees and queens, all capable of spreading Nosemosis, Varroosis, and potentially other harmful parasites, pathogens and diseases (Mutinelli 2011). Until now, there has been little research focused on the occurrence of parasites and pathogens in migratory versus non-migratory bee colonies. Such a survey is important to better understand how these parasites and pathogens are spreading, and to determine the significance of migratory bees' exposure to a wider range of pathogens and parasites to honey bee decline.

Taxonomic identification and detection of understudied internal parasites and pathogens can be both tedious and unreliable. Furthermore, since these parasites and pathogens have not been the subjects of many scientific studies, there is little in the way of morphological description extant. In addition, since the differences between pathogen species in the same family are so small, it is extremely difficult to reliably identify the correct pathogen species. Because of this, a more modern technique utilizing molecular diagnostics is more successful in correctly identifying specific pathogen species (Weiss and Vossbrinck 1999, Klee et al. 2006). Molecular diagnostics allow for quick, efficient, and reliable testing of large volumes of samples, which can be preserved for extended periods of time in alcohol (Meeus et al. 2012). Furthermore, molecular detection allows identification of species at different life stages. Using species specific primers, researchers can achieve a high degree of success in detecting and identifying these uncommon parasites and pathogens using Polymerase Chain Reaction (PCR) molecular diagnostics (Weiss and Vossbrinck 1999, Klee et al. 2006).

The objectives of this study were to; detect the presence and distribution of parasites and pathogens in Arkansas hobbyist beekeepers' honey bee colonies; determine if seasonality affects when the parasites and pathogens occur; and determine whether co-occurrence exists between any of the parasites and pathogens.

Materials and Methods

Sample Collection

In 2015, as part of a USDA APHIS Cooperative Agreement Award No. 15-8100-1743-CA., the University of Arkansas Insect Genetics lab contacted via mail 1,000 Arkansas hobbyist beekeepers using apiary registration information from the Arkansas State Plant Board to determine their interest in participating in the parasite and pathogen study. The 1,000 beekeepers were chosen from the 1732 registered beekeepers in Arkansas to provide a sampling of all Arkansas counties that had registered beekeepers. Those interested were mailed collection kits that included protocol information instructing them to collect 30-50 bees from up to five hives in their apiary and place them in 70% ethanol in the provided individual 250 ml plastic containers. Samples were mailed to the Insect Genetics Lab, University of Arkansas, Fayetteville, AR, databased, and stored at room temperature. A survey form (Figure 2.1) was also included in the kit, asking beekeepers to provide treatment history, location, queen source, and further comments. Samples were collected and analyzed for 2015. In 2016, collection kits were mailed to all previously participating beekeepers to obtain a second year of samples.

Varroa mite detection

Varroa destructor presence was detected using a mite wash adapted from Oliver (2013). The mite wash allows for separation of *V. destructor* mites from the honey bees (Figure 2.2). The mite wash included two plastic jars with a modified lid with mesh separating the two jars. Each sample, containing 30-50 honey bees, was deposited in one of the containers with 70% ethanol. The mesh lid and second container were then attached and the entire mite wash was shaken, allowing mites to dislodge from bees and fall to the opposite side of the mesh (Figure 2.2). Mites

were counted, recorded and placed in 1.5 ml Eppendorf tubes with 70% ethanol for future research.

DNA Extraction

Following the mite wash, 6-10 worker honey bees from each sample were placed on a paper towel for 3-4 hours to allow all ethanol to evaporate. Thereafter, mitochondrial DNA extraction was performed using a salting-out protocol with in-house reagents as described in Sambrook and Russell (2001). This consisted of adding 2 mL of cell lysis solution in 5 ml tubes and masticating the honey bees. The cell lysis solution was composed of detergents, salts and ions, and buffer. Samples were stored in a -80°C freezer for at least one hour, followed by 5 min. incubation in an 80°C water bath. Each sample received 670 µL of protein precipitation solution, allowing proteins to be salted-out. Samples were next centrifuged at 13.2 X 1000 rpm for 3 min. Subsequently, 300 µL of the supernatant was dispensed in two 1.5 ml labeled tubes for each sample along with 300 µL chilled 100% isopropanol alcohol; the samples were centrifuged at 13.2 X 1000 rpm for 4 min. The supernatant of each sample was poured off, and the tubes were blotted dry, leaving a small DNA pellet at the bottom of each tube. Following, 300 µL of 100% chilled ethanol was added to each tube and centrifuged at 13.2 X 1000 rpm for 4 min. The supernatant was once again discarded and the tubes were blotted and placed, uncapped, on a 65°C heat block for 30 min. The extraction product was then re-suspended in 50 µL Tris: EDTA solution and left at ambient temperature for at least 12 hours. Samples were stored in a -20°C freezer. PCR was performed as described in Szalanski (2000).

Successful DNA extraction was confirmed using 2 µL of extracted DNA solution using honey bee mtDNA COI-COII PCR primers E2 (5'-GGCAGAATAAGTGCATTG-3') and H2 (5'-CAATATC ATTGATGACC-3') and the following thermocycler conditions: denatured

initially for 5 min at 94°C then 40 cycles at 94°C for 45 seconds, 46°C for 1 min, 72°C for 1 min and a final extension of 72°C for 5 min (Garnery et al. 1993) (Table 2.1). PCR products were run on a 2% agarose check gel and visualized using the UV BioDocit station. Successful DNA extraction was indicated by a 600-1200 bp amplicon, the size variation is due to an intergenic spacer region which varies in size among honey bee lineages (Figure 2.3).

Molecular Diagnostics

Nosema

Samples were tested for *Nosema sp.* using the DNA extraction product and PCR primers NosemaSSU-1F (5'-ACAATATGTATTAGATCTGATATA-3') and NosemaSSU-1R (5'-TAATGATATGCTTAAG TTCAAAG-3'). These PCR primers were developed by Szalanski et. al (2014) and amplify a 222 bp amplicon for *N. apis* and a 237 bp amplicon for *N. ceranae* using the small subunit gene region specific for *Nosema* mitochondrial DNA (Table 2.1). The thermocycler condition are as follows: 2 min. at 94°C, then 40 cycles of 94°C for 45 seconds, 50°C for 1 min. and 72°C for 1 min., followed by a final extension of 72°C for 5 min. (Szalanski et al. 2014). PCR products were run on a 2% agarose check gel and visualized using the UV BioDocit station.

The PCR product of samples testing positive for *Nosema,* those resulting in an amplicon of 222 bp or 237 bp, underwent an RFLP digestion (Taylor and Szalanski 1999) to distinguish the *Nosema* species. The RFLP digestion utilizes restriction enzymes *Dra I*, cutting only *N. ceranae* at 79 bp, and *Rsa I*, only cutting *N. apis* at 130 bp. Samples were incubated overnight at 37°C and products were run on a 2% agarose check gel and visualized using the UV BioDocit station.

Phorid fly

The PCR primers Phorid-rRNA-1F (5'-GTACACCTATACATTGGGTTCGTACATT AC-3') and Phorid-rRNA-1R (5'-GAGRGCCATAAAGTAGCTACACC-3') were used to screen for Phorid rRNA (Table 2.1). The following thermocycler conditions were used: 5 min. at 94°C, then 39 cycles of 94°C for 45 seconds, 60°C for 45 seconds and 72°C for 1 min., followed by a final extension of 72°C for 5 min. (Core et al. 2012). PCR products were run on a 2% agarose check gel and visualized using the UV BioDocit station. Samples positive for *A. borealis* were indicated by the presence of a 486 bp on the agarose gel.

Spiroplasma

Multiplex PCR using primers S. apis ITS-F (5'-AATGCCAGAAGCACGTATCC-3'), S.apis ITS-R (5'-GAACGAGATATACTCATAAGCTGTTACAC-3'), Ms-160 F(5'- TTGCA AAAGCTGTTTTAGATGC-3'), Ms-160-R (5'- TGACCAGAAATGTTTGCTGAA-3') was used to detect *S. apis* and *S. melliferum* (Table 2.1). The S.apis ITS primers produce a 190 bp amplicon from the 3' end of 16S rRNA to the ITS-1 region, Ms-160 primers target a spiralin-like gene of *S. melliferum*. The PCR conditions were: 2 min. at 94°C, then 39 cycles of 94°C for 45 seconds, 59°C for 1 min. and 72°C for 1 min., followed by a final extension of 72°C for 5 min. (Schwarz et al. 2014). PCR products were run on a 2% agarose check gel and visualized using the UV BioDocit station. Samples positive for *S. apis* result in a 190 bp amplicon, while samples positive for *S. melliferum* result in a 160 bp amplicon.

Typanosomes

A multiplex PCR using PCR primers CBSSU rRNA-F2, CBSSU rRNA B4 (Schmid-Hempel and Tognazzo 2010), and L. passim18S-F (5'-AGGGATATTTAAACCCATCGAAAATCT-3') was used to detect Trypanosome pathogen

species (Szalanski et al. 2016) (Table 2.1). The CBSSU rRNA primers amplify a small sub unit gene, the L. passim18S primer amplifies only *L. passim*. PCR was done using the following thermocycler program conditions: first denaturing step of 5 min. at 95°C was followed by 40 cycles of 30 seconds at 95°C, primer annealing for 30 seconds at 57°C (Schmid-Hempel and Tognazzo 2010). This PCR resulted in a 608 bp product for samples positive for all trypanosomatids, as well as a 499 bp product for those positive for only *L. passim* species (Szalanski et al. 2016).

Samples positive for only trypanosomatids, but not specifically *L. passim,* underwent a separate multiplex PCR using primers CBSSU rRNA P2, CBSSU rRNA B4, L. passim18S-F (5'-AGGGATATTTAAACCC ATCGAAAATCT-3'), and C. mel 474-F ('5-TTTACGCA TGTCATGCATGCCA-3') under thermocycler program of: 2 min. at 94°C, then 40 cycles of 94°C for 45 seconds, 55°C for 1 min. and 72°C for 1 min., followed by a final extension of 72°C for 5 min. (Szalanski et al. 2016) (Table 2.1). This will display a 716-724 bp product for *Crithidia* spp. and a 245 bp band for samples positive for samples positive only for *Crithidia* sp. (Szalanski et al. 2016).

Results

Over the course of this study, 541 individual honey bee colony samples, containing 30-50 worker honey bees each, were received. The colony samples were received from 107 Arkansas beekeepers (10.7% of surveyed beekeepers, 6.2% of registered beekeepers), which represented 46 of the 75 (61.3%) Arkansas counties (Figure 2.4). Figure 2.4 represents the counties sampled and the number of samples received from each county. The counties sampled were located within the six Arkansas regions (Figure 2.5). In 2015, 80.41% (n=435 colony samples) of the colony samples were received, while 19.59% (n=106) of the samples were received in 2016.

June 2015 returned the largest number of samples with 240 (44.36%). In 2016, April returned the most samples of 44 (8.13%). The lowest number of samples for both 2015 and 2016 were received in January, with a cumulative total of two colony samples. The most substantial number of samples was received from the Ozark region, with 28.28%. Within the Ozark region, Washington county had the highest number of colony samples at 8.13%. The Ouachita region had the fewest number of samples at 10.91%. Comparatively, of the 1732 registered Arkansas beekeepers, 32.6% (565) are located in Ozark county, however, the fewest number of registered beekeepers are in the Delta region with only 6.2% (108) (Table 2.2).

Of the 541 colony samples tested, *N. ceranae, L. passim,* and *V. destructor* were the three parasites and pathogen that were detected. Conversely, none of the samples were positive for *N. apis, A. borealis, S. apis, S. melliferum,* or *C. mellficae.* Of the 46 counties sampled, Saline, Pulaski, and Pike county were the only three counties in which all three of the parasites and pathogens were detected. Both Saline and Pulaski are within the Central region.

Among the parasites and pathogens tested for, *V. destructor* had the highest occurrence with 45.5% of the colony samples being positive (Appendix 2.1). *Varroa destructor* also had the widest distribution, occurring in 43 of the 46 counties sampled from (Figure 2.6). The county with the highest proportion of *V. destructor* occurrence was Ashley county with 90.9% of the 11 samples having detectable mite levels (Figure 2.6). Seasonally, June had the highest cumulative percentage of *Varroa* mite occurrence in 2015, while April had the highest in 2016 (Table 2.3). Furthermore, 2016 (51%) had a higher overall percent of *V. destructor* occurrence than 2015 (43.9%).

Nosema ceranae was detected in 11.6% of the samples (Appendix 2.2). A total of 27 counties out of the 46 were positive for *N. ceranae* (Figure 2.7). Proportionally, Sharp county

had the highest level of occurrence with 80% of the five samples being positive for *N. ceranae* (Figure 2.7). Sebastian and Washington counties received the most colony samples, however each displayed an occurrence of 13% and 2.3% respectively. Furthermore, of the 26 samples from the colonies treated with fumagillin, only one sample was positive for *N. ceranae.* Regionally, *N. ceranae* was concentrated in the Delta region, with 15.7% of the colony samples from the region being positive. Seasonally, June showed the highest proportion of *N. ceranae* occurrence (Table 2.4)

Lotmaria passim was detected in 11.3% of the colony samples (Appendix 2.3). A total of 20 counties out of the 46 sampled had colonies positive for *L. passim* (Figure 2.8). Greene county had the highest number of positive samples (Appendix 2.3). Regionally, *L. passim* was concentrated in the northern portion of Arkansas, within the Ozark region (Figure 2.8). Seasonally, July of 2015 and May of 2016 had the highest levels of occurrence (Table 2.5).

Using Fisher's Exact test in JMP statistical software, I tested for independence among the parasites and pathogen and found that there is significant evidence that *V. destructor* and *L. passim* do not occur independently of each other (P-value=0.0200). Based on our data, *N. ceranae* and *V. destructor* are independent (P-value=0.5035) as well as *N. ceranae* and *L. passim* (P-value=0.5242).

Discussion

This study provides evidence that *V. destructor, N. ceranae,* and *L. passim* occur in Arkansas hobbyist honey bee colonies. Due to *N. ceranae* and *V. destructor* being common and well documented within honey bee apiaries in the United States, it was unsurprising to detect both of these species. However, this study is the first to report the occurrence of *L. passim* in Arkansas. This indicates that migratory beekeeping practices are not the sole spreader of *L.*

passim. Because *L. passim* is a recently observed pathogen species, it is important to continue monitoring, to document its spread. No speculation can be made as to what level of infection is occurring in these colonies. Future research should focus on quantifying levels of infection.

Varroa mite was first detected in Arkansas in the early 1990's (Wenner and Bushing 1996), and is now widespread across the State. Of the 541 Arkansas samples received during 2015-2016, *V. destructor* was detected in 45.7% of the samples. This number is lower than the 98.2% occurrence found in the 2013-2014 National survey, which included 32 states and 648 colony samples (Rennich et al. 2015). This difference in occurrence may be explained by the differing sampling method and management tactics. A 2008 study sampled untreated honey bee colonies in Arkansas, examining a total of 11 colonies. The study detected *Varroa* mites in seven (63.6%) of the colony sampled, which had a mean infestation level of 21.4 ±11.3 mites per 100 bees (Zawislak 2008). Recently, Bee Informed released a preliminary report of their 2017 national citizen science project, MiteCheck (Bee Informed Partnership 2017). Within the study *Varroa* mites were samples from six Arkansas counties, with three counties having 0-3 mites per 100 honey bees and three counties having 4-11 mites per 100 honey bees (Bee Informed Partnership 2017). Mite levels greater than 10 mites per 100 honey bees may result in the loss of the honey bee colony, if treatment methods are not implemented. Within our survey, multiple counties had higher levels of *V. destructor* occurrence, which is concerning and would be detrimental to beekeepers in Arkansas counties. Within both of the above surveys mite loads were measured. In future research, mite loads should be an added component to the survey in order to provide insight on how threatened Arkansas honey bee colonies are.

Furthermore, *Nosema apis* was not detected in any of the 541 colony samples, while *N ceranae* was found in 11.6% of the samples. While *N. apis* was once commonly occurring,

studies suggest that *N. ceranae* is displacing *N. apis* across its range (Chen et al. 2008, Fries 2010, Martín-Hernández et al. 2012, Smith 2012, Milbrath et al. 2015). Our results support this claim, as only *N. ceranae* was detected in Arkansas. The level of *N. ceranae* occurrence (11.6%) found in this study is lower than previous state level surveys in Virginia, New York, and South Dakota (29-44%) (Traver and Fell 2011, Szalanski et al. 2013). While the numbers within our survey are lower, it is important to note that we sampled solely from hobbyist managed colonies. Research has indicated that *Nosema sp.* is more prevalent in migratory colonies than stationary (Meixner and Conte 2016). Furthermore, of the 26 samples treated with fumagillin, only one sample was positive for *N. ceranae.* This differs from previous studies which point to fumagillan actually increasing infection of *N. ceranae* (Huang et al. 2013). In future studies, a larger sample size including beekeeper's known to use fumagillan may allow for more conclusive data.

This study revealed the first documented case of the trypanosome *L. passim* in Arkansas. Additionally, our survey supports previous claims that *L. passim* is the more predominant trypanosome species in honey bees, compared to *C. mellificae* (Schwarz et al. 2015). *Lotmaria passim* was concentrated in the northern portion of Arkansas, within the Ozark region. Within the Delta region, only Green county showed occurrences of *L. passim* with a relatively high proportion (Figure 2.4).

Nosema ceranae and *L. passim* had similar levels of occurrence with 11.6% and 11.3% respectively. *Nosema ceranae's* distribution was fairly scattered across Arkansas with it occurring most frequently in the Central and River Valley regions. Sharp county had the highest proportion of colony samples positive for *N. ceranae* with 80% of the five samples. *Lotmaria passim* was most prevalent in Fulton county with 80% of the 5 total samples being positive.

The Ozarks was the most well-represented region in our study with 28.3% of the honey bee samples coming from colonies within this region. Unsurprisingly, the majority of the samples positive for *N. ceranae* and *L. passim* were found in Ozark county. The *V. destructor* positives came from a relatively even split between the regions, with the Timberlands region having the highest percent of the positive occurrence at 19.9%. Distribution-wise, Ouachita county was the least represented with only 10.9% of the total colony samples.

Of the 541 Arkansas samples received in 2015-2016, none of the samples tested positive for either species of *Spiroplasma* bacteria. This may be attributed to the fact that *Spiroplasma* is a newly occurring bacterial pathogen in the United States. It is likely that the Arkansas hobbyist colonies have not yet been exposed to either species of S*piroplasma.*

None of the Arkansas honey bee samples tested positive for the parasitic phorid fly, *A. borealis*. Because the phorid fly causes hive abandonment, hive sampling is not the best sampling procedure (Core et al. 2012). Future sampling should target honey bees performing abnormal behavior, such as swarming porch lights at night.

The only two parasite and pathogen species which we found to be associated are *V. destructor* and *L. passim* (P-value=0.0200). While *V. destructor* has shown to be associated with and even vectoring multiple species of viruses and disease, no research has been done examining *V. destructor* as a vector of trypanosomes. *Varroa destructor* has been shown to weaken its host, making it more susceptible to other pests, which would explain this correlation (Shutler et al. 2014, Hubert et al. 2017).

The information discovered on the distribution of these parasites and pathogens in Arkansas may aid hobbyist beekeepers in future management decisions. Continued sampling and

monitoring of the colonies in this study may aid in understanding the movement of these parasites and pathogen species.

Future research should involve continued sampling, as well as including more Arkansas counties; as well as, infestation data for parasites and pathogens. Lastly, to further explore co-occurrence among parasites and pathogens, *V. destructor* mites from colony samples should be tested for parasites and pathogens to determine its role in pathogen transmission. The study should continue to sample annually to discover any trends or spread patterns which may be present. A larger sample would allow for a more comprehensive understanding of where and how these parasites and pathogens are spreading.

Acknowledgement

We would like to thank the Arkansas Plant Board for assisting in distributing collection kits as well as the numerous Arkansas hobbyist beekeepers who participated in this study. This research was made possible with the assistance of the USDA APHIS Cooperative Agreement Award No. 15-8100-1743-CA.

Literature Cited

Bee Informed Partnership. **2017**. Bee Informed Partnership - MiteCheck. Bee Informed Partnership.

Cavigli, I., K. F. Daughenbaugh, M. Martin, M. Lerch, K. Banner, E. Garcia, L. M. Brutscher, and M. L. Flenniken. **2016**. Pathogen prevalence and abundance in honey bee colonies involved in almond pollination. Apidologie. 47: 251–266.

Chen, Y., J. D. Evans, I. B. Smith, and J. S. Pettis. **2008**. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. J. Invertebr. Pathol. 97: 186–188.

Core, A., C. Runckel, J. Ivers, C. Quock, T. Siapno, S. DeNault, B. Brown, J. DeRisi, C. D. Smith, and J. Hafernik. **2012**. A new threat to honey bees, the parasitic phorid fly *Apocephalus borealis*. PLOS ONE. 7: e29639.

Evans, J. D., and R. S. Schwarz. **2011**. Bees brought to their knees: microbes affecting honey bee health. Trends Microbiol. 19: 614–620.

Fries, I. 2010. *Nosema ceranae* in European honey bees (*Apis mellifera*). J. Invertebr. Pathol. 103 Suppl 1: S73-79.

Garnery, L., M. Solignac, G. Celebrano, and J.-M. Cornuet. **1993**. A simple test using restricted PCR-amplified mitochondrial DNA to study the genetic structure of *Apis mellifera* L. Experientia. 49: 1016–1021.

Goulson, D., E. Nicholls, C. Botías, and E. L. Rotheray. **2015**. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science. 347: 1255957.

van den Heever, J. P., T. S. Thompson, J. M. Curtis, A. Ibrahim, and S. F. Pernal. **2014**. Fumagillin: An Overview of Recent Scientific Advances and Their Significance for Apiculture. J. Agric. Food Chem. 62: 2728–2737.

Huang, W.F., L. F. Solter, P. M. Yau, and B. S. Imai. **2013**. *Nosema ceranae* escapes fumagillin control in honey bees. PLOS Pathog. 9: e1003185.

Hubert, J., M. Bicianova, O. Ledvinka, M. Kamler, P. J. Lester, M. Nesvorna, J. Kopecky, and T. Erban. **2017**. Changes in the Bacteriome of Honey Bees Associated with the Parasite Varroa destructor, and Pathogens Nosema and Lotmaria passim. Microb. Ecol. 73: 685–698.

Jara, L., A. Cepero, E. Garrido-Bailón, R. Martín-Hernández, M. Higes, and P. De la Rúa. **2012**. Linking evolutionary lineage with parasite and pathogen prevalence in the Iberian honey bee. J. Invertebr. Pathol. 110: 8–13.

Klee, J., W. Tek Tay, and R. J. Paxton. **2006**. Specific and sensitive detection of *Nosema bombi* (Microsporidia: Nosematidae) in bumble bees (*Bombus* spp.; Hymenoptera: Apidae) by PCR of partial rRNA gene sequences. J. Invertebr. Pathol. 91: 98–104.

Martín-Hernández, R., C. Botías, E. G. Bailón, A. Martínez-Salvador, L. Prieto, A. Meana, and M. Higes. **2012**. Microsporidia infecting Apis mellifera: coexistence or competition. Nosema ceranae replacing Nosema apis? Is N. ceranae replacing N. apis? Environ. Microbiol. 14: 2127–2138.

Meeus, I., V. Vercruysse, and G. Smagghe. **2012**. Molecular detection of *Spiroplasma apis* and *Spiroplasma melliferum* in bees. J. Invertebr. Pathol. 109: 172–174.

Meixner, M. D., and Y. L. Conte. **2016**. A current perspective on honey bee health. Apidologie. 47: 273–275.

Milbrath, M. O., T. van Tran, W.-F. Huang, L. F. Solter, D. R. Tarpy, F. Lawrence, and Z. Y. Huang. **2015**. Comparative virulence and competition between Nosema apis and Nosema ceranae in honey bees (Apis mellifera). J. Invertebr. Pathol. 125: 9–15.

Mutinelli, F. 2011. The spread of pathogens through trade in honey bees and their products (including queen bees and semen): overview and recent developments. Rev. Sci. Tech. Int. Off. Epizoot. 30: 257–271.

Potts, S. G., J. C. Biesmeijer, C. Kremen, P. Neumann, O. Schweiger, and W. E. Kunin. **2010**. Global pollinator declines: trends, impacts and drivers. Trends Ecol. Evol. 25: 345–353.

Rennich, K., G. Kunkel, S. Abban, R. Fahey, H. Eversole, J. Evans, E. Forsgren, V. Levi, D. Lopez, S. Madella, J. Pettis, R. Rose, and D. vanEngelsdorp. **2015**. 2013-2014 National honey bee pests and diseases survey report. University of Maryland.

Runckel, C., M. L. Flenniken, J. C. Engel, J. G. Ruby, D. Ganem, R. Andino, and J. L. DeRisi. **2011**. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, *Nosema*, and *Crithidia*. PLOS ONE. 6: e20656.

Sambrook, J., and D. W. Russell. **2001**. Molecular cloning, 3rd ed. ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Schmid-Hempel, R., and M. Tognazzo. **2010**. Molecular divergence defines two distinct lineages of *Crithidia bombi* (Trypanosomatidae), parasites of bumblebees. J. Eukaryot. Microbiol. 57: 337–345.

Schwarz, R. S., G. R. Bauchan, C. A. Murphy, J. Ravoet, D. C. de Graaf, and J. D. Evans. **2015**. Characterization of two species of Trypanosomatidae from the honey bee *Apis*

mellifera: *Crithidia mellificae* Langridge and McGhee, and *Lotmaria passim* n. gen., n. sp. J. Eukaryot. Microbiol. 62: 567–583.

Schwarz, R. S., É. W. Teixeira, J. P. Tauber, J. M. Birke, M. F. Martins, I. Fonseca, and J. D. Evans. **2014**. Honey bee colonies act as reservoirs for two *Spiroplasma* facultative symbionts and incur complex, multiyear infection dynamics. MicrobiologyOpen. 3: 341–355.

Shutler, D., K. Head, K. L. Burgher-MacLellan, M. J. Colwell, A. L. Levitt, N. Ostiguy, and G. R. Williams. **2014**. Honey bee *Apis mellifera* parasites in the absence of *Nosema ceranae* fungi and *Varroa destructor* mites. PLOS ONE. 9: e98599.

Simone-Finstrom, M., H. Li-Byarlay, M. H. Huang, M. K. Strand, O. Rueppell, and D. R. Tarpy. **2016**. Migratory management and environmental conditions affect lifespan and oxidative stress in honey bees. Sci. Rep. 6.

Smith, M. L. 2012. The honey bee parasite *Nosema ceranae*: transmissible via food exchange? PLOS ONE. 7: e43319.

Szalanski, A. L. 2000. Population genetics and phylogenetics of the endangered American burying beetle, Nicrophorus americanus (Coleoptera: Silphidae). Ann. Entomol. Soc. Am. 93: 589–594.

Szalanski, A. L., A. D. Tripodi, and C. E. Trammel. **2014**. Molecular detection of *Nosema apis* and *N. ceranae* from southwestern and south central USA feral Africanized and European honey bees, *Apis mellifera* (Hymenoptera: Apidae). Fla. Entomol. 97: 585–589.

Szalanski, A. L., J. Whitaker, A. D. Tripodi, and P. Cappy. **2013**. Prevalence of *Nosema* from managed honey bee colonies in South Dakota and New York. J. Agric. Urban Entomol. 29: 99–104.

Szalanski, A., C. E. Trammel, A. D. Tripodi, D. Cleary, L. Rusert, and D. Downey. **2016**. Molecular diagnostics of the honey bee parasite *Lotmaria Passim*, and *Crithidia* spp. using multiplex PCR. Fla. Entomol.

Traver, B. E., and R. D. Fell. **2011**. Prevalence and infection intensity of *Nosema* in honey bee (*Apis mellifera* L.) colonies in Virginia. J. Invertebr. Pathol. 107: 43–49.

Tsutsumi, L., and D. Oishi. **2011**. Farm and forestry production and marketing profile for honey bees. Permanent Agriculture Resources (PAR), Holualoa, HI.

USDA. **2017**. Honey- USDA economics, statistics, and market information. National Agricultural Statistics Service (NASS), Agricultural Statistics Board, United States Department of Agriculture (USDA).

Weiss, L. M., and C. R. Vossbrinck. **1999**. Molecular biology, molecular phylogeny, and molecular diagnostic approaches to the microsporidia. 129–171.

Wenner, A. M., and W. W. Bushing. **1996**. Varroa Mite spread in the United States. Bee Cult. 341–343.

Whitaker, J., A. L. Szalanski, and M. Kence. **2010**. Molecular detection of *Nosema ceranae* and *N. apis* from Turkish honey bees. Apidologie.

Williams, G. R., D. R. Tarpy, D. vanEngelsdorp, M.-P. Chauzat, D. L. Cox-Foster, K. S. Delaplane, P. Neumann, J. S. Pettis, R. E. L. Rogers, and D. Shutler. **2010**. Colony collapse disorder in context. BioEssays. 32: 845–846.

Zawislak, J. 2008. Alternative control of the *Varroa* mite on honey bees using botanical smokes (M.S.).

Number written on label on vial:__________

Date:____________ County: _________________

Nearest City: ____________________________________

Fumigilin-B use if known (Y/N): _______________

Beekeeper: _____________________________________

Queen source, if known: ___________________________

Comments: _____________________________________

____

Figure 2.1. Blank sample information survey. Each sampling kit included five surveys as well as collection instructions.

Figure 2.2. Varroa mite wash separating mites from one colony sample.

49

Table 2.1. List of primers used in molecular detection of parasites and pathogens. A: Garnery et al. 1993; B: Szalanski et al. 2014; C: Core et al. 2012; D: Schwarz et al. 2014; E: Schmid-Hempel and Tognazzo 2010; F: Szalanski et al. 2016.

Primer	**Sequence**	**Reference**
E2	F: 5’-GGCAGAATAAGTGCATTG-3’	A
H2	R: 5’-CAATATC ATTGATGACC-3’	A
NosemaSSU-1F	F: 5’-ACAATATGTATTAGATCTGATATA-3’	B
NosemaSSU-1R	R: 5’-TAATGATATGCTTAAG TTCAAAG-3’	B
Phorid-rRNA-1F	F: 5’-GTACACCTATACATTGGGTTCGTACATT AC-3’	C
Phorid-rRNA-1R	R: 5’-GAGRGCCATAAAAGTAGCTACACC-3’	C
S. apis ITS-F	F: 5’-AATGCCAGAAGCACGTATCC-3’	D
S.apis ITS-R	R: 5’-GAACGAGATATACTCATAAGCTGTTACAC-3’	D
Ms-160 F	F: 5’- TTGCA AAAGCTGTTTTAGATGC-3’	D
Ms-160-R	R: 5’- TGACCAGAAATGTTTGCTGAA-3’	D
CBSSU rRNA F2	F: 5’-CTTTTGACGAACAACTGCCCTATC-3’	E
CBSSU rRNA B4	R: 5’- AACCGAACGCACTAAACCCC-3’	E
L. passim18S-F	F: 5’-AGGGATATTTAAACCC ATCGAAAATCT-3’	F
C. mel 474-F	F: 5’-TTTACGCA TGTCATGCATGCCA-3’	F

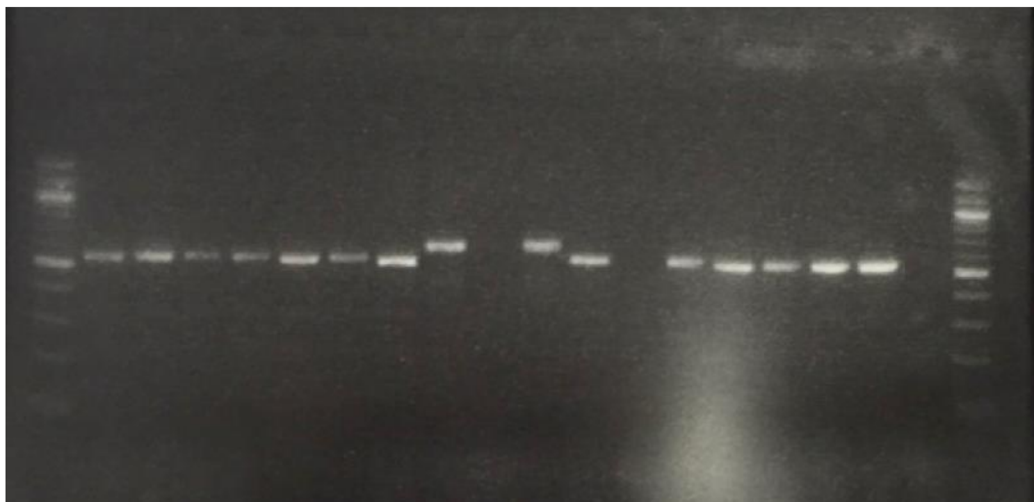

Figure 2.3. Agarose gel visualized using the UV BioDocit station. DNA presence is indicated by a 600-1200 bp amplicon.

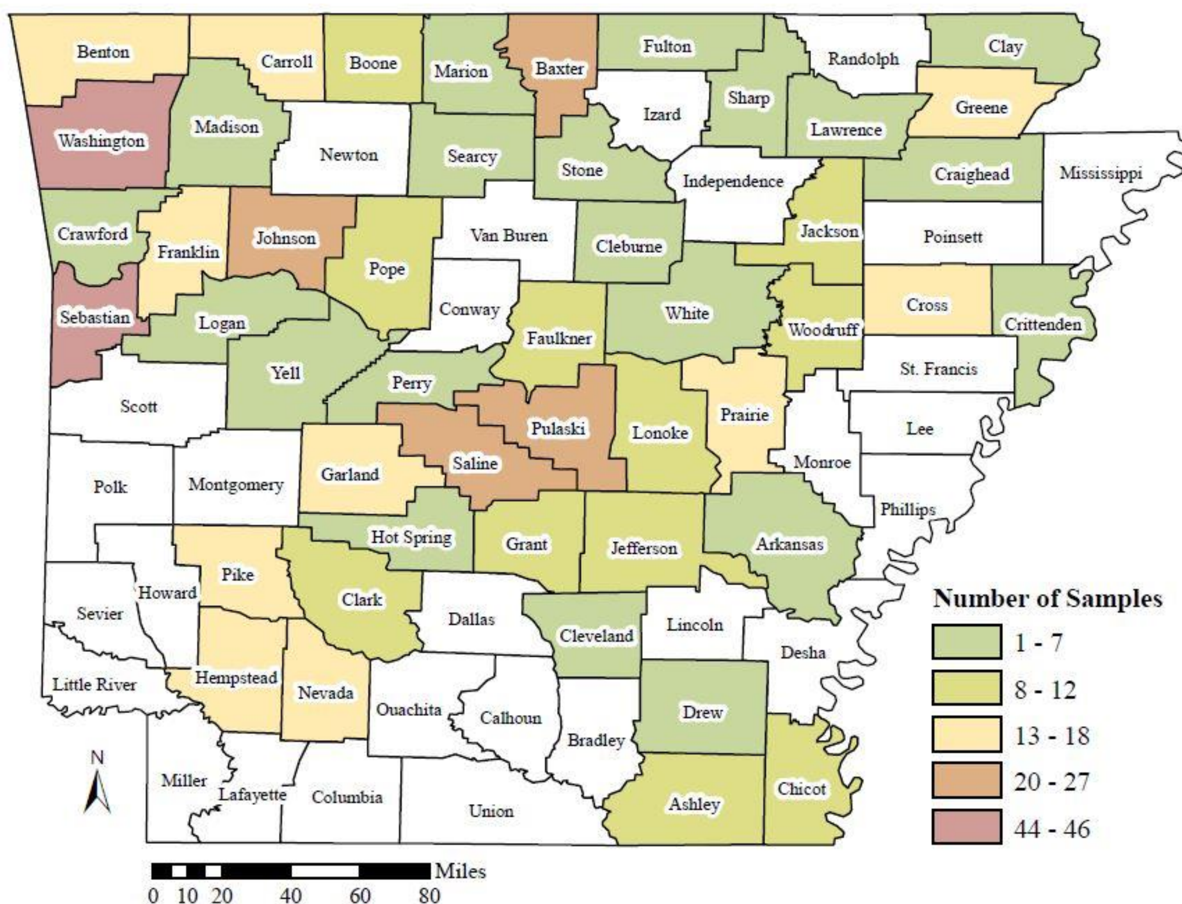

Figure 2.4. Number of honey bee colonies sampled from each county. No samples were received from counties in white.

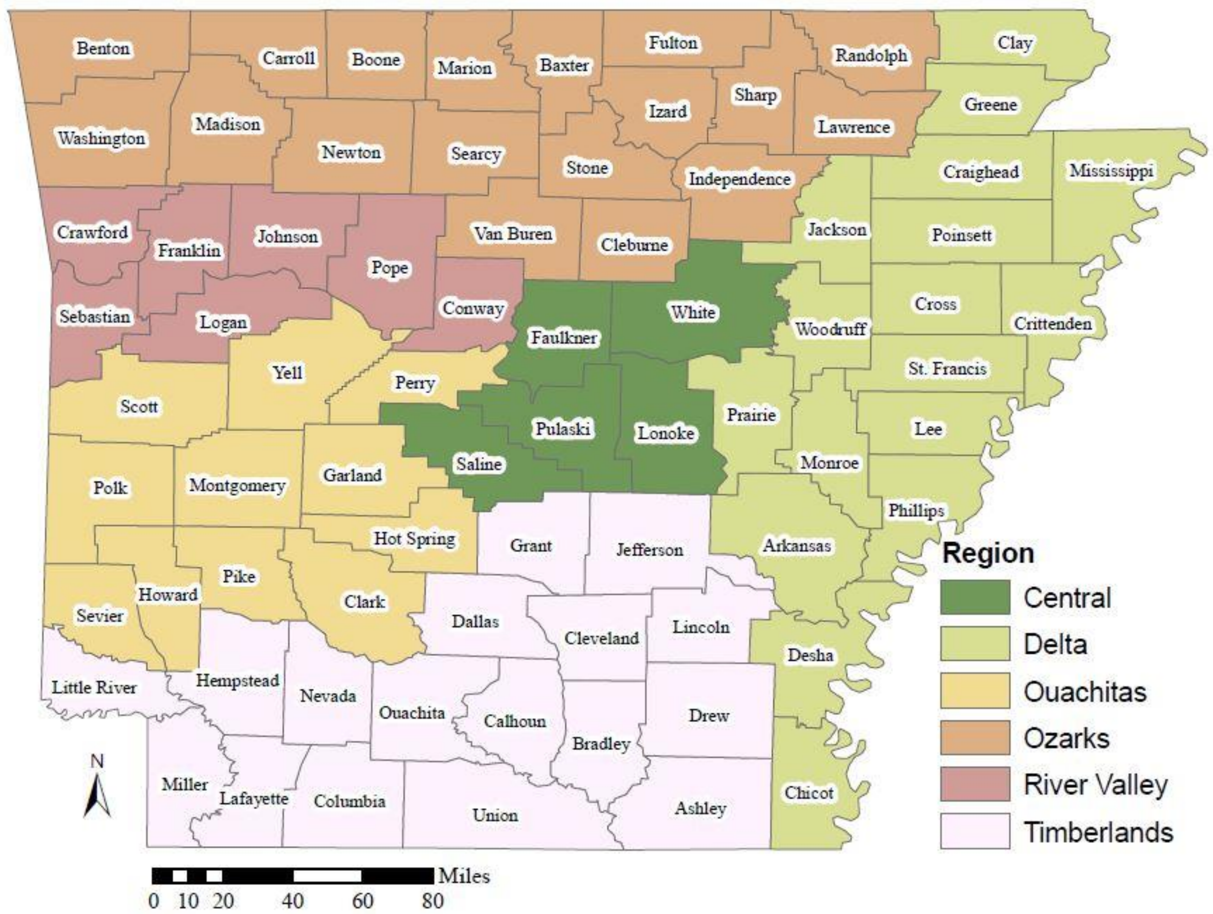

Figure 2.5. Arkansas counties by regions.

Table 2.2. Percent of Arkansas hobbyist beekeepers sampled in each of the six Arkansas regions compared to the percent of total registered Arkansas beekeepers.

Region	Percent of AR Beekeepers Sampled (%) (n=541)	Percent of Total AR Beekeepers (%) (n=1732)
Central	13.1	28.1
Delta	16.5	6.2
Ouachita	10.9	14.2
Ozark	28.3	32.6
River Valley	18.5	10.0
Timberlands	12.8	8.8

Table 2.3. Hobbyist honey bee colony samples from 2015 and 2016 positive for *Varroa destructor* by month.

	2015				2016			
Month	**Positive**	**Total**	**Percent of month (%)**	**Cum. (%)**	**Positive**	**Total**	**Percent of month (%)**	**Cum. (%)**
April	0	0	0	0	18	44	40.9	17.3
May	44	66	66.7	10.1	14	18	77.8	13.5
June	84	240	35.0	19.3	13	17	76.5	12.5
July	36	80	45.0	8.3	3	12	25.0	2.9
Aug	16	27	59.3	3.7	1	2	50.0	1.0
Sept	0	7	0	0.0	3	7	42.9	2.9
Nov	11	15	73.3	2.5	1	4	25.0	1.0
Sum	191	435		**43.9**	53	104		**51.0**

53

Table 2.4. Hobbyist honey bee colony samples from 2015, positive for *Nosema ceranae* by month

Month	Positive	Total	Percent of month (%)	Cum. (%)
May	4	66	6.1	0.9
June	33	240	13.8	7.6
July	15	80	18.8	3.4
Aug	4	27	14.8	0.9
Sept	1	7	14.3	0.2
Oct	2	15	13.3	0.5
Sum	59	435		**13.6**

Table 2.5. Hobbyist honey bee colony samples from 2015 positive for *Lotmaria passim* by month.

	2015				2016			
Month	Positive	Total	Percent of month (%)	Cum. (%)	Positive	Total	Percent of month (%)	Cum. (%)
May	2	66	3	0.5	5	18	27.8	10.6
June	33	240	13.8	8.5	0	17	0.0	0.0
July	15	80	18.8	3.9	5	12	41.7	10.6
Sum	50	386		**12.9**	10	47		**21.3**

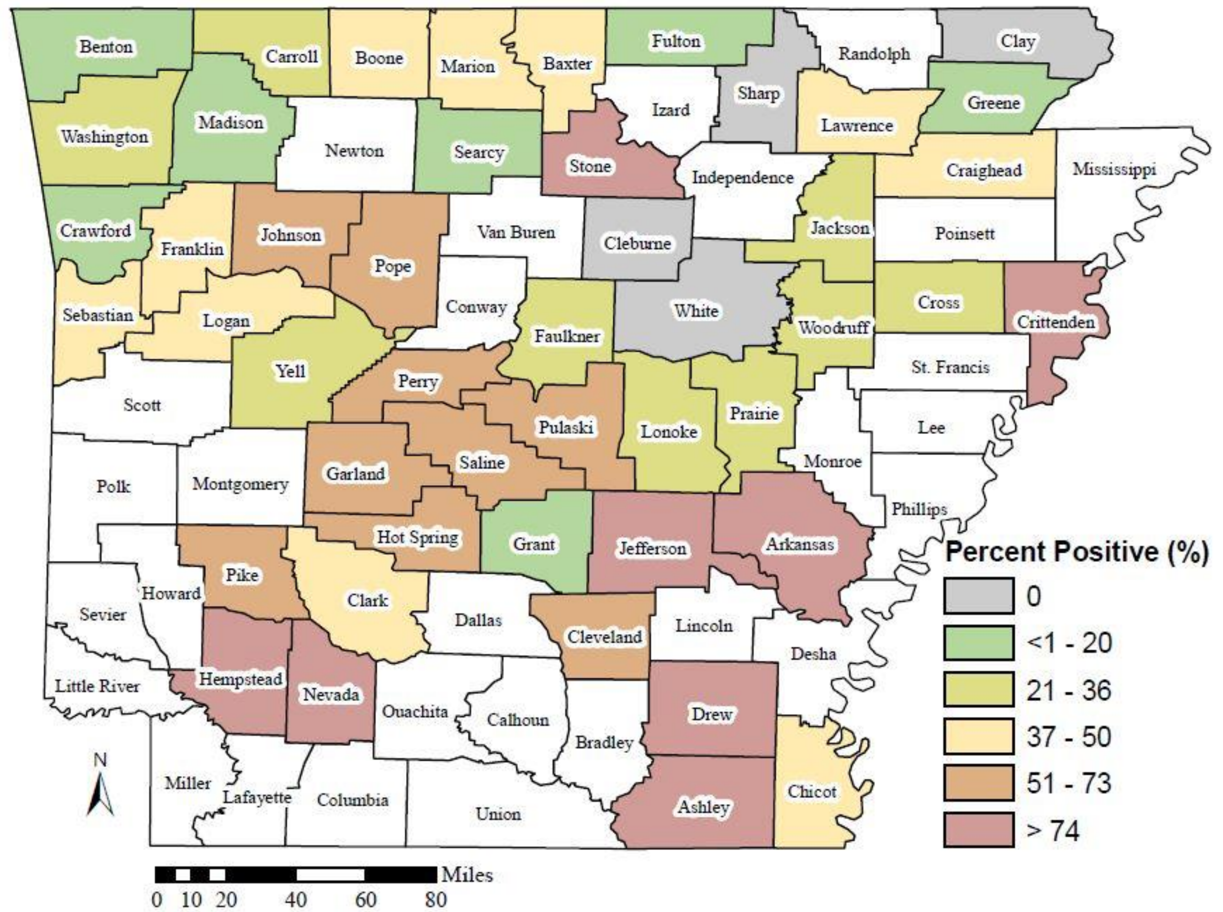

Figure 2.6. Percent of colony samples positive for *Varroa destructor* in each county. No colony samples were received from counties in white.

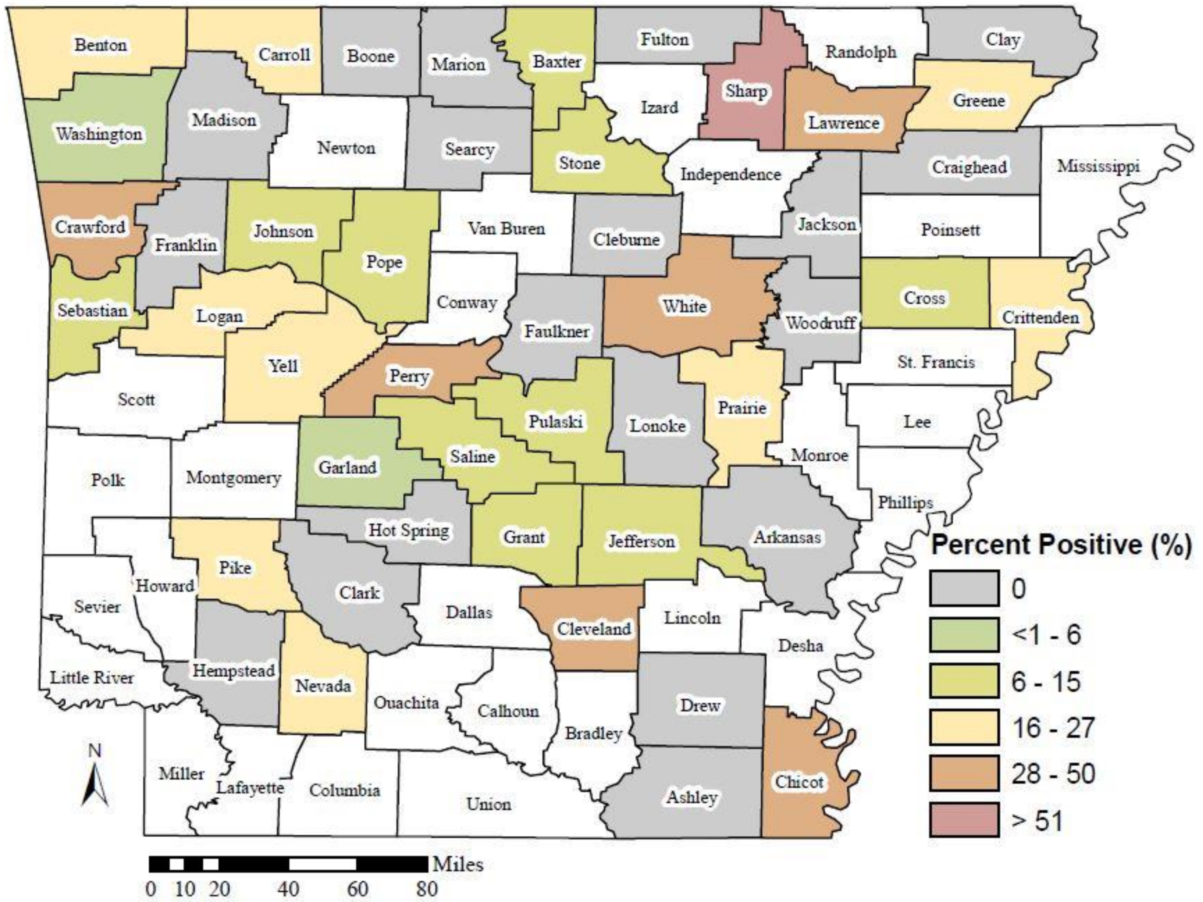

Figure 2.7. Percent of samples positive for *Nosema ceranae* from each Arkansas county. No colony samples were received from counties in white.

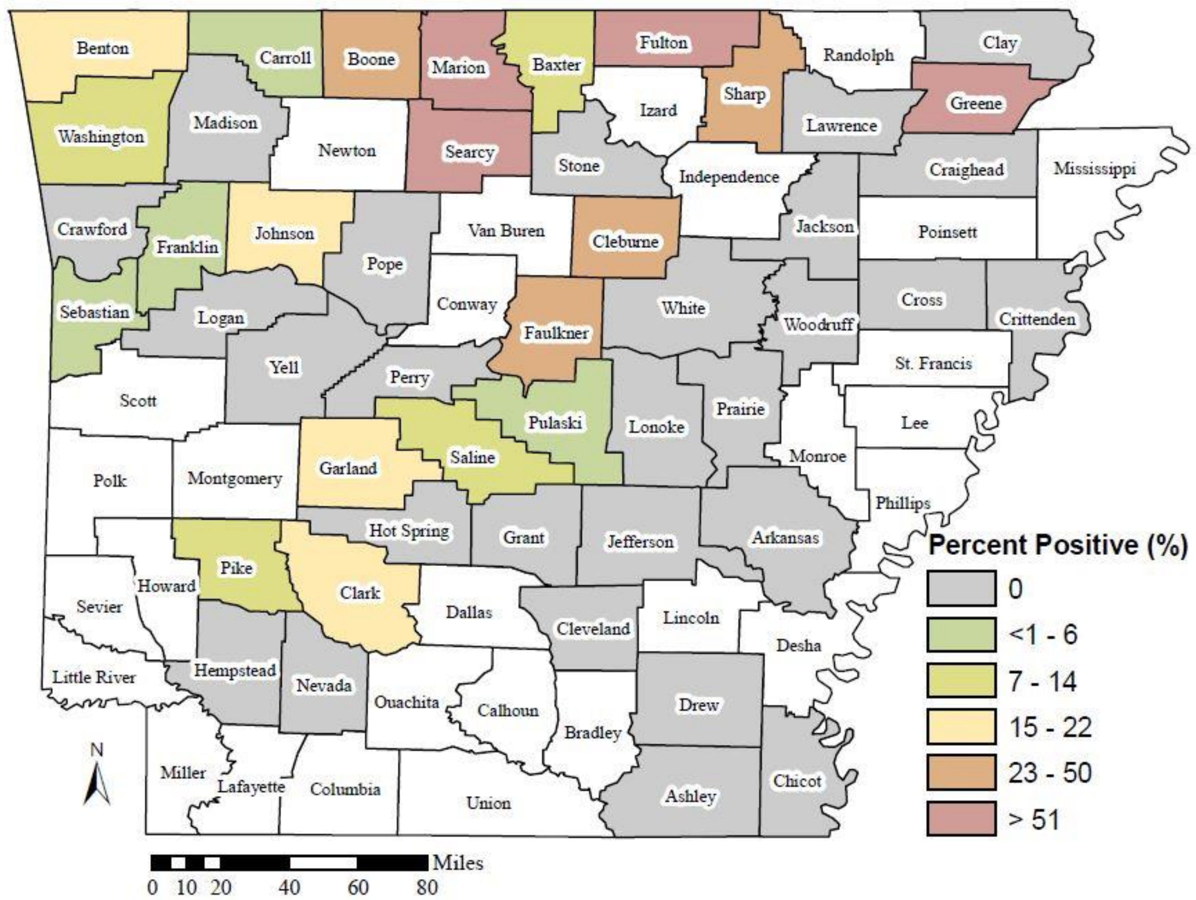

Figure 2.8. Percent of colony samples positive for *Lotmaria passim* in each county. Counties not sampled are indicated in white.

Appendix 2.1. *Nosema ceranae* data by county.

County	Positive	Number of colonies	Positive for *Nosema ceranae* (%)	Beekeepers per county
Arkansas	0	5	0.0	1
Ashley	0	11	0.0	3
Baxter	3	23	13.0	3
Benton	3	18	16.7	3
Boone	0	10	0.0	1
Carroll	3	18	16.7	4
Chicot	4	10	0.0	1
Clark	0	12	0.0	3
Clay	0	2	0.0	1
Cleburne	0	5	0.0	1
Cleveland	2	6	33.3	1
Craighead	0	2	0.0	1
Crawford	2	5	40.0	1
Crittenden	1	5	20.0	1
Cross	2	13	15.4	2
Drew	0	5	0.0	1
Faulkner	0	10	0.0	3
Franklin	0	17	0.0	3
Fulton	0	5	0.0	1
Garland	1	18	5.6	5
Grant	1	8	12.5	2
Greene	3	17	17.6	3
Hempstead	0	13	0.0	4
Hot Springs	0	5	0.0	1
Jackson	0	11	0.0	3
Jefferson	1	9	11.1	2
Johnson	2	20	10.0	2
Lawrence	1	2	50.0	1
Logan	1	4	25.0	1
Lonoke	0	10	0.0	3
Madison	0	6	0.0	2
Marion	0	5	0.0	1
Nevada	3	17	17.6	4
Perry	2	5	40.0	1
Pike	3	15	20.0	3
Pope	1	8	12.5	2
Prairie	4	15	26.7	3
Pulaski	3	21	14.3	5
Saline	3	27	11.1	5
Searcy	0	5	0.0	1
Sebastian	6	46	13.0	8

County	**Positive**	**Number of colonies**	**Positive for *Nosema ceranae* (%)**	**Beekeepers per county**
Sharp	4	5	80.0	1
Stone	1	7	14.3	2
Washington	1	44	2.3	11
White	1	3	33.3	2
Woodruff	0	9	0.0	1
Yell	1	4	25.0	1
Total	63	541	**11.6**	115

Appendix 2.2. *Varroa destructor* data by county.

County	Positive	Number of colonies	Positive for *Varroa destructor* (%)	Beekeepers per county
Arkansas	4	5	80.0	1
Ashley	10	11	90.9	3
Baxter	10	23	43.5	3
Benton	13	18	5.6	3
Boone	17	10	40.0	1
Carroll	6	18	27.8	4
Chicot	13	10	40.0	1
Clark	6	12	41.7	3
Clay	7	2	0	1
Cleburne	2	5	0	1
Cleveland	5	6	66.7	1
Craighead	2	2	50.0	1
Crawford	1	5	20.0	1
Crittenden	4	5	80.0	1
Cross	4	13	30.8	2
Drew	4	5	80.0	1
Faulkner	3	10	30.0	3
Franklin	8	17	47.1	3
Fulton	1	5	20.0	1
Garland	13	18	72.2	5
Grant	1	8	12.5	2
Greene	3	17	17.6	3
Hempstead	10	13	76.9	4
Hot Springs	3	5	60.0	1
Jackson	3	11	27.3	3
Jefferson	7	9	77.8	2
Johnson	12	20	60.0	2
Lawrence	1	2	50.0	1
Logan	2	4	50.0	1
Lonoke	3	10	30.0	3
Madison	1	6	16.7	2
Marion	2	5	40.0	1
Nevada	13	17	76.5	4
Perry	3	5	60.0	1
Pike	11	15	73.3	3
Pope	5	8	62.5	2
Prairie	4	15	26.7	3
Pulaski	13	21	61.9	5
Saline	18	27	66.7	5
Searcy	1	5	20.0	1
Sebastian	18	46	64.3	8
Sharp	0	5	0	1

County	Positive	Number of colonies	Positive for *V. destructor* (%)	Beekeepers per colony
Stone	6	7	85.7	2
Washington	16	44	36.4	11
White	0	3	0	2
Woodruff	3	9	33.3	1
Yell	1	4	25.0	1
Total	295	541	**45.5**	**115**

Appendix 2.3. County data for *Lotmaria passim.*

County	Positive	Number of colonies	Positive for *Lotmaria passim* (%)	Beekeepers per county
Arkansas	0	5	0	1
Ashley	0	11	0	3
Baxter	2	23	8.7	3
Benton	3	18	16.7	3
Boone	5	10	50.0	1
Carroll	1	18	5.6	4
Chicot	0	10	0	1
Clark	2	12	16.7	3
Clay	0	2	0	1
Cleburne	2	5	40.0	1
Cleveland	0	6	0	1
Craighead	0	2	0	1
Crawford	0	5	0	1
Crittenden	0	5	0	1
Cross	0	13	0	2
Drew	0	5	0	1
Faulkner	5	10	50.0	3
Franklin	1	17	5.9	3
Fulton	4	5	80.0	1
Garland	4	18	22.2	5
Grant	0	8	0	2
Greene	9	17	52.9	3
Hempstead	0	13	0	4
Hot Springs	0	5	0	1
Jackson	0	11	0	3
Jefferson	0	9	0	2
Johnson	3	20	15.0	2
Lawrence	0	2	0	1
Logan	0	4	0	1
Lonoke	1	10	0	3
Madison	0	6	0	2
Marion	3	5	60.0	1
Nevada	0	17	0	4
Perry	0	5	0	1
Pike	1	15	6.7	3
Pope	0	8	0	2
Prairie	0	15	0	3
Pulaski	1	21	4.8	5
Saline	3	27	11.1	5
Searcy	3	5	60.0	1
Sebastian	0	46	2.2	8

County	Positive	Number of colonies	Positive for *Lotmaria passim* (%)	Beekeepers per county
Sharp	1	5	40	1
Stone	2	7	0	2
Washington	0	44	13.6	11
White	6	3	0	2
Woodruff	0	9	0	1
Yell	0	4	0	1
Total	61	541	**11.3**	**115**

Chapter 3: Molecular detection of parasites and pathogens in migratory honey bee, *Apis mellifera* L., colonies

Abstract

It is well-documented that bee populations have been fluctuating over the past decade. Among the most significant stressors of honey bees identified to date are parasites and pathogens. Parasites and pathogens threaten honey bee health in multiple ways, both directly and indirectly. While parasites and pathogens have known effects on honey bees, little research has been conducted to survey the occurrence of these pests, especially the lesser known species.

Migratory beekeeping has also become a topic of research interest as being partially responsible for honey bee decline in the U.S. colonies are transported long distances across the country, followed by intermixing colonies from different geographic regions, which potentially expose honey bees to new parasites, pathogens, diseases, and viruses. While migratory beekeeping is speculated as a cause of decline, little research has been conducted to prove such claims.

This study focuses on migratory honey bee colonies sampled from Oklahoma. Molecular diagnostics were used to detect the presence and abundance of various invasive parasite and pathogen species. These species include *Nosema apis, Nosema ceranae, Apocephalus borealis, Spiroplasma apis, Spiroplasma melliferum, Crithidia mellificae, Lotmaria passim,* and *Varroa destructor.* While *N. apis, S. apis, A. borealis,* and *C. mellificae* were not detected in the commercial colonies tested, *N. ceranae* (27.6%), *S. melliferum* (8.05%), *L. passim* (1.1%), and *V. destructor* (17.2%) were detected.

Introduction

The commercially managed honey bee, *Apis mellifera* L., is essential to the production of hundreds of crops in the United States. Honey bees contribute an estimated $15 billion annually in pollination services alone (Kluser and Neumann 2010, Calderone 2012). However, in recent years honey bee populations have fluctuated, with the most dramatic drop occurring in 2008. (Potts et al. 2010, Cavigli et al. 2016). Research suggests that human manipulation and facilitation is partially responsible for the decline, and the migratory bee industry is just one example of this (Dietemann et al. 2006). Migratory beekeeping entails transporting honey bee colonies from one foraging site to the next, often over great distances. As colonies are transported to new foraging sites, the bees' nutritional needs are often compromised (Oldroyd 2007).

Large-scale transportation of honey bees and its effects heighten stress within honey bee colonies, weakening their ability to fight off dangerous parasites, pathogens, diseases and other pests (Cooper 2007, Bacandritsos et al. 2010). Furthermore, as honey bees are transported across the United States to pollinate numerous crops, they are exposed to honey bee colonies from differing areas of the country (Simone-Finstrom et al. 2016). This interaction exposes the bees to new parasites, pathogens, and diseases. The combination of stressed honey bee colonies being exposed to a new range of dangerous parasites and pathogens is likely a major contributor to decline in honey bee health.

While a single factor has not been determined as the cause of honey bee health decline, parasite and pathogen interactions have been identified as key stressors (Neumann et al. 2012, Tritschler et al. 2017). *Varroa destructor* Anderson and Trueman, *Nosema apis* Zander and *Nosema ceranae* Fries are identified threats to honey bee health. Meanwhile, little research has

been conducted on several parasite and pathogen species to determine their presence and possible effect on honey bees in the U.S.

Nosema apis, a microsporidian pathogen has been a recognized pest of honey bees for some time. The spread of *N. apis* has been partially attributed to the movement of honey bees across the United States via migratory beekeeping (Webster et al. 2004). In fact, higher spore loads of *Nosema* have been found in migratory bees (Meixner and Conte 2016). In contrast, the microsporidian species *Nosema ceranae* was first identified as a problem in 1990 when it was discovered that it had switched hosts from *A. ceranae* to *A. mellifera.* This lack of co-occurrence may explain why *N. ceranae* is highly virulent in *A. mellifera* (Mayack and Naug 2009, vanEngelsdorp and Meixner 2010). *Nosema ceranae* has been detected in higher levels than the earlier identified species, *Nosema apis* (Klee et al. 2007, Mayack and Naug 2009, Szalanski et al. 2013). It is thought that *N. ceranae* may actually be replacing *N. apis* in its distribution which is concerning considering it is more pathogenic (Chen et al. 2008, Fries 2010, Smith 2012).

Varroa destructor is the number one threat to honey bees globally (Uroš et al. 2014, Locke 2015). Endemic to Asia, the *Varroa* mite is distributed worldwide, occurring on every continent where honeybees are found except Australia (Hood 2000). An ectoparasite, *V. destructor* attaches externally to both adult and immature honey bees (Rosenkranz et al. 2010). Once attached, *V. destructor* rapidly feeds on the hemolymph of the bee, weakening the bee. The mite's presence within a colony has been linked to reduced lifespan, deformities, and increased occurrence of disease and viruses (Le Conte et al. 2010).

The unicellular, eukaryotic, obligate Trypanosome parasite *Lotmaria passim* Schwarz (Trypanosomatidae) is a newly identified trypanosome species. While research on *L. passim* is limited, it is currently the most prevalent trypanosome species affecting honey bees worldwide

(Schwarz et al. 2015, Tritschler et al. 2017). *Lotmaria passim* has been documented in Europe, North America, South America, and Asia (Ravoet et al. 2013, Schwarz et al. 2015, Arismendi et al. 2016, Szalanski et al. 2016, Tritschler et al. 2017). Another species of trypanosome, *Crithidia mellificae,* has been documented in Belgium as a factor in winter mortality in honey bees (Schwarz et al. 2015).

Spiroplasma apis Mouches and *S. melliferum* Clark are two species of bacterial honey bee pathogens that act as causative agents of neurological disease in bees (Schwarz et al. 2014). The few studies that have examined *Spiroplasma* and its effects on honey bees found the bacterial pathogen detected mostly in spring months (Mouches et al. 1983, 1984). The only current survey of *Spiroplasma* in the United States occurred in a 2014 study in Beltsville, MD. The study found that the prevalence of *S. melliferum* within the colony peaked in the spring, decreasing in the summer, with lowest levels of infection in the winter (Zheng and Chen 2014). At present, these pathogens are considered only occasional pests of honey bees, however, little research has been performed to confirm this or to predict their possible future impact on bee colonies (Schwarz et al. 2014, Hubert et al. 2016).

Apocephalus borealis Brues is a known parasitoid of bumbles bees and vespid wasps and has recently been found to parasitize honey bees. *Apocephalus borealis* can cause abnormal behavior in honey bees, such as flying at night, as well as colony abandonment (Core et al. 2012). The fly has been detected in California, South Dakota, Washington, Oregon and Vermont (Core et al. 2012, Sagili and Marshall 2016).

As new parasite and pathogen species continue to be discovered, it is important to survey for their presence and detect potential sources of their spread. The objectives of this study were

to determine parasite and pathogen occurrence, co-occurrence and distribution among migratory honey bee colonies in Oklahoma.

Materials and Methods

Sample Collection and Preservation

Honey bee samples were collected in March, April, and May in 2015-2016 from four Oklahoma bee broker colonies. The colonies were located adjacent to canola fields and had previously been used in California for almond pollination. Once samples were collected they were mailed to the Arkansas insect genetics lab (University of Arkansas, Fayetteville, AR). Samples were collected within the hive; foraging bees were not included. Samples included approximately 300 worker honey bees per sample. The honey bees were preserved and stored in 70% ethanol.

Varroa mite detection

A mite wash adapted from (Oliver 2013) allowed for *V. destructor* detection (Figure 3.1). Each colony sample was poured into one container, the mesh divider attached to the second container then was attached and the mite wash shaken for approximately 30 seconds, dislodging any mites present. Once the mites dislodged from the honey bees, they were released through the mesh into the second container. Following separation from the honey bees, the mite numbers were recorded, and then placed in 1.5 ml Eppendorf tubes with 70% ethanol and stored at -20°C.

DNA Extraction

DNA mass extraction was modified from Sambrook and Russell (2001), using a salting-out-protocol. This involved allowing 6-10 worker honey bees from each sample to dry on a paper towel for 3-4 hours. This was followed by pulverizing the samples in a 5ml Eppendorf tube combined with 2 mL cell lysis. Samples were left in a -80°C freezer for at least one hour.

Samples were next incubated in an 80°C water bath for five min. followed by pipetting 670 µL of protein precipitate into each sample. The samples were then centrifuged at 13.2 X 1000 rpm for 3 min. Afterwards, 300 µL of the supernatant was dispensed in two 1.5 ml Eppendorf tubes for each sample along with 300 µL 100% chilled isopropanol alcohol. Again, samples were centrifuged, this time at 13.2 X 1000 rpm for 4 min. The supernatant of each sample was discarded, and the tubes were blotted dry. Following, 300 µL of 100% chilled ethanol was added to each tube and again centrifuged at 13.2 X 1000 rpm for 4 min. The supernatant was again discarded and tubes were blotted dry and placed, uncapped, on a 65°C heat block for 30-40 min. The extraction product was then re-suspended in 50 µL Tris: EDTA and left at ambient temperature for at least 12 hours. The DNA was stored in a -20°C freezer.

Once extraction was complete, DNA presence was confirmed using Polymerase Chain Reaction (PCR) with the mtDNA COI-COII honey bee PCR primers E2 and H2 primer set (Table 3.1) (Garnery et al. 1993, Sambrook and Russell 2001). PCR reagents and quantities were per (Szalanski 2000). PCR thermal cycler conditions were as follows: denatured initially for 5 min. at 94°C then 40 cycles at 94°C for 45 seconds, 46°C for 1 min., 72°C for 1 min. and a final extension of 72°C for 5 min. (Garnery et al. 1993). Following PCR, the PCR products were tested on a 2% agarose gel and visualized using a UV BioDocit station. DNA presence was indicated by a 600-1200 bp amplicon.

Parasite and Pathogen detection using molecular diagnostics

PCR

PCR using a species-specific paired primer set Phorid-rRNA-1F (5'-GTACACCTATA CATTGGGTTCGTACATT AC-3') and Phorid-rRNA-1R (5'-GAGRGCCATAAAGTAGCT ACACC-3') was utilized to detect the presence of *Apocephalus borealis* (Core et al. 2012). The

following thermocycler conditions were used: 5 min. at 94°C, then 39 cycles of 94°C for 45 seconds, 60°C for 45 seconds and 72°C for 1 min., followed by a final extension of 72°C for 5 min. (Core et al. 2012). A known positive control and known negative were included to increase reliability. PCR products were run on a 2% agarose check gel and visualized using the UV BioDocit station. Samples positive for *A. borealis* resulted in a 486 bp amplicon.

PCR-RFLP

Samples were tested for *Nosema sp.* using the DNA extraction product and PCR primers NosemaSSU-1F (5'-ACAATATGTATTAGATCTGATATA-3') and NosemaSSU-1R (5'-TAATGATATGCTTAAG TTCAAAG-3') (Szalanski et al. 2014). A known positive control and known negative were included to ensure reliability The thermocycler condition were as follows: 2 min. at 94°C, then 40 cycles of 94°C for 45 seconds, 50°C for 1 min. and 72°C for 1 min., followed by a final extension of 72°C for 5 min. (Szalanski et al. 2014). PCR products were run on a 2% agarose check gel and visualized using the UV BioDocit station. Samples positive for *N. apis* amplified a 222 bp amplicon and samples positive for *N. ceranae* amplified a 237 bp region specific for *Nosema* mitochondrial DNA.

Samples resulting in amplicons between 222 bp and 237 bp, underwent an RFLP digestion to distinguish the *Nosema* species. The RFLP digestion used restriction enzymes *Dra I*, cutting only *N. ceranae* at 79 bp, and *Rsa I*, only cutting *N. apis* at 130 bp. Samples were incubated overnight and products were run on a 2% agarose check gel and visualized using the UV BioDocit station.

Multiplex PCR

Multiplex PCR was used to detect Trypanosome and *Spiroplasma* species using multiple species-specific primers. Primers S.apis ITS-F (5'-AATGCCAGAAGCACGTATCC-3'), S.apis

ITS-R (5'-GAACGAGATATACTCATAAGCTGTTACAC-3'), Ms-160 F(5'- TTGCAAAAGC TGTTTTAGATGC-3'), Ms-160-R (5'- TGACCAGAAATGTTTGCTGAA-3') were used to detect *S. apis* and *S. melliferum*. A known positive control and known negative were included to ensure reliability. The thermocycler conditions were as follows: 5 min. at 95°C followed by 40 cycles of 30 seconds at 95°C, primer annealing for 30 seconds at 57°C (Schmid-Hempel and Tognazzo 2010). Following PCR, the products were run on a 2% agarose check gel and visualized using the UV BioDocit station. Samples positive for *S. apis* resulted in a 190 bp amplicon, while samples positive for *S. melliferum* resulted in a 160 bp amplicon.

Primers CBSSU rRNA-F2, CBSSU rRNA B4, L. passim18S-F (5'-AGGGATATTTAAA CCCATCGAAAATCT-3') were utilized to test for trypanosome and *L. passim* presence (Szalanski et al. 2016). These primers result in a 608 bp product for samples positive for all trypanosomatids, as well as a 499 bp product for those positive for only *L. passim* (Szalanski et al. 2016). A known positive control and known negative were included to ensure reliability. The thermocycler conditions used are those described above. PCR products were run on a 2% agarose check gel and visualized using the UV BioDocit station, and samples positive yielded a 608 bp product if trypanosomatids were present, as well as a 499 bp product for those positive for only *L. passim* (Szalanski et al. 2016).

Samples found positive for *L. passim* or trypanosomatids underwent a separate multiplex PCR using primers CBSSU rRNA P2, CBSSU rRNA B4, L. passim18S-F (5'-AGGGATATTT AAACCCATCGAA AATCT-3'), and C. mel 474-F ('5-TTTACGCATGTCAT GCATGCCA-3') under thermocycler conditions described above (Szalanski et al. 2016). Positive samples displayed a 716-724 bp product for *L. passim* and *Crithidia* spp., a 499 bp band for samples

positive for only *L. passim*, and a 245 bp band for samples positive for *Crithidia* sp. (Szalanski et al. 2016).

Results

Samples were received from 87 different honey bee colonies in four different counties of Oklahoma (Figure 3.2). Seventy-six of the samples (87.4%) were collected in 2015, while 11 (12.6%) were collected in 2016. All of the colony samples were collected during the spring months of March, April, and May in 2015 and 2016. April and May tended to have higher proportions of parasite and pathogen occurrence.

Varroa destructor was detected in 17.2% (n=15) of the colony samples. Kingfisher county had the highest proportion of *V. destructor* compared to the other three counties (Table 3.2). The largest number of *V. destructor* occurrences occurred in May of 2015 (Table 3.3).

Nosema ceranae was detected in 27.6% of the samples. None of the samples were positive for *N. apis.* Logan county had the highest occurrence of *Nosema ceranae* at 76.9% among all of the sampled counties (Table 3.5). April of 2015 had the highest number of *N. ceranae* positive samples at 40% (n=12) (Table 3.6).

The Trypanosome species *Lotmaria passim* was detected in 1.1% of the samples, while *C. mellificae* was not detected in any of the samples. Kingfisher was the only county in which *L. passim* was detected, with only one sample testing positive. The month of April was the only month in which *L. passim* was detected. *Crithidia mellificae* was not detected in any of the samples.

Spiroplasma melliferum was detected in 8.05% of the samples, occurring primarily in Grant county (19.2%) in May (5.3%) (Table 3.7; Table 3.8). *Spiroplasma apis* was not detected in any of the colony samples.

Discussion

Due to the majority of the honey bee colony samples (87.4%) being received in 2015, this would partially explain why 2015 had higher levels of *S. melliferum, N. ceranae,* and *V. destructor.* Our second sampling year (2016) did, however, have the lone instance of *L. passim* from all of the Oklahoma migratory colony samples. While the sample size is not large enough to describe any large-scale trends, it is worth noting for future monitoring.

This survey revealed that 17.7% (n=15) of the honey bee colony samples had *Varroa* mites. This is less than the 2013-14 and 2014-15 National Honey Bee Pests and Diseases Survey Report in which between 86-98% of the colonies sampled were positive for *V. destructor* (Lee et al. 2015, Rennich et al. 2015*).* The colonies from this study were treated for *Varroa* mites with Apistan strips. Frequency and uniformity for treatment is unknown. Because the *Varroa* mite is a known threat honey bees, treatment practices may be more intense than in previous years. Future studies should quantify mite loads to better understand the *Varroa* mites' current threat level in migratory colonies. While the majority of colonies were sampled in April (n=41), it was *V. destructor's* least prevalent month with only 4.87% of samples having detectable mite levels (Figure 3.4). This is likely due to *Varroa* mite populations growing in spring and summer and peaking in the months of September and October.

Nosema ceranae was detected in 27.59% of the colony samples. Again, these infection rates are lower compared to a 2009 study in South Dakota where 42% of samples were positive for *N. ceranae*, and in New York in which 54% samples were PCR-positive for *Nosema* sp., with 96.8% being *N. ceranae* (Szalanski et al. 2013). Similar to this study, none of the South Dakota samples were positive for *N. apis*, and only 0.42% of the New York samples were positive for *N. apis.* In the future, a large sample size should be utilized to gain more comprehensive data.

Nosema treatment history was not available, meaning there is a possibility that the beekeepers treated hives with fumagillan, potentially reducing the levels of *Nosema* sp. *Nosema apis* was not detected in any of the colony samples, supporting the claim that *N. ceranae* is replacing *N. apis* in its distribution.

Spiroplasma melliferum was detected in 8% of the 87 samples. *Spiroplasma* occurred most abundantly in the month of May. These results are fewer than a 2008 study performed in Maryland, in which 33% of colonies sampled were positive for *S. apis* or *S. melliferum.* It is important to note that when the Maryland study began, the infection rate of *Spiroplasma meliferum* was at 5% in the winter, and later increased to 68% in the spring. The prevalence again decreased to 22-25% in the summer (Zheng and Chen 2014). This enforced that temporal studies are needed to determine if *Spiroplasma* infection rates increase or decrease depending upon the season.

Apocephalus borealis was not detected in any of the samples tested. The lack of observance of this species may be due to how the samples were acquired. In previous studies, honey bees displaying abnormal behaviors were collected; including flying at night and crawling in circles on the pavement (Core et al. 2012). Future studies should target these conditions for sampling.

Due to the limited sample size, more research should be performed to collect additional data for a more comprehensive study. These results indicate that as predicted, *Nosema ceranae* and *V. destructor* occur in migratory colonies. Moreover, our data shows detection of two lesser studied pathogen species, *L. passim* and *S. melliferum.* The detection of these species is critical to understanding their prevalence and distribution, but also understanding their future spread. By

conducting state level surveys of parasites and pathogens, occurrence can be monitored and hopefully future spread can be detected and prevented.

Acknowledgement

Thanks to Dr. Jackie Lee and Haley Butler for their assistance in collecting honey bee samples. Thank you to Clinton Trammel and Karen Willard for their technical assistance and help processing the samples. This research was supported in part by the University of Arkansas, Arkansas Agricultural Experiment Station.

Literature cited

Arismendi, N., A. Bruna, N. Zapata, and M. Vargas. **2016**. PCR-specific detection of recently described *Lotmaria passim* (Trypanosomatidae) in Chilean apiaries. J. Invertebr. Pathol. 134: 1–5.

Bacandritsos, N., A. Granato, G. Budge, I. Papanastasiou, E. Roinioti, M. Caldon, C. Falcaro, A. Gallina, and F. Mutinelli. **2010**. Sudden deaths and colony population decline in Greek honey bee colonies. J. Invertebr. Pathol. 105: 335–340.

Calderone, N. W. 2012. Insect pollinated crops, insect pollinators and US agriculture: trend analysis of aggregate data for the period 1992-2009. PLOS ONE. 7: e37235.

Cavigli, I., K. F. Daughenbaugh, M. Martin, M. Lerch, K. Banner, E. Garcia, L. M. Brutscher, and M. L. Flenniken. **2016**. Pathogen prevalence and abundance in honey bee colonies involved in almond pollination. Apidologie. 47: 251–266.

Chen, Y., J. D. Evans, I. B. Smith, and J. S. Pettis. **2008**. *Nosema ceranae* is a long-present and wide-spread microsporidian infection of the European honey bee (*Apis mellifera*) in the United States. J. Invertebr. Pathol. 97: 186–188.

Cooper, E. L. 2007. Colony collapse disorder may affect complementary and alternative medicine. Evid.-Based Complement. Altern. Med. ECAM. 4: 275–277.

Core, A., C. Runckel, J. Ivers, C. Quock, T. Siapno, S. DeNault, B. Brown, J. DeRisi, C. D. Smith, and J. Hafernik. **2012**. A new threat to honey bees, the parasitic phorid fly *Apocephalus borealis*. PLOS ONE. 7: e29639.

Dietemann, V., A. Lubbe, and R. M. Crewe. **2006**. Human factors facilitating the spread of a parasitic honey bee in South Africa. J. Econ. Entomol. 99: 7–13.

Fries, I. 2010. *Nosema ceranae* in European honey bees (*Apis mellifera*). J. Invertebr. Pathol. 103 Suppl 1: S73-79.

Garnery, L., M. Solignac, G. Celebrano, and J.M. Cornuet. **1993**. A simple test using restricted PCR-amplified mitochondrial DNA to study the genetic structure of *Apis mellifera* L. Experientia. 49: 1016–1021.

Hood, W. 2000. *Varroa* mite control in South Carolina: extension: Clemson University: South Carolina. Clemson Univ. (http://www.clemson.edu/extension/beekeepers/factsheets/varroa_mite_control_in_sc.html).

Hubert, J., M. Kamler, M. Nesvorna, O. Ledvinka, J. Kopecky, and T. Erban. **2016**. Comparison of Varroa destructor and Worker Honeybee Microbiota Within Hives Indicates Shared Bacteria. Microb. Ecol. 72: 448–459.

Klee, J., A. M. Besana, E. Genersch, S. Gisder, A. Nanetti, D. Q. Tam, T. X. Chinh, F. Puerta, J. M. Ruz, P. Kryger, D. Message, F. Hatjina, S. Korpela, I. Fries, and R. J. Paxton. **2007**. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. J. Invertebr. Pathol. 96: 1–10.

Kluser, S., and P. Neumann. **2010**. Global honey bee colony disorder and other threats to insect pollinators.

Le Conte, Y., M. Ellis, and W. Ritter. **2010**. *Varroa* mites and honey bee health: can *Varroa* explain part of the colony losses? Apidologie. 41: 353–363.

Lee, K. V., N. Steinhauer, K. Rennich, M. E. Wilson, D. R. Tarpy, D. M. Caron, R. Rose, K. S. Delaplane, K. Baylis, E. J. Lengerich, J. Pettis, J. A. Skinner, J. T. Wilkes, R. Sagili, and D. vanEngelsdorp. **2015**. A national survey of managed honey bee 2013–2014 annual colony losses in the USA. Apidologie. 46: 292–305.

Locke, B. 2015. Natural *Varroa* mite surviving *Apis mellifera* honeybee populations. Apidologie. 1–16.

Mayack, C., and D. Naug. **2009**. Energetic stress in the honeybee *Apis mellifera* from *Nosema ceranae* infection. J. Invertebr. Pathol. 100: 185–188.

Meixner, M. D., and Y. L. Conte. **2016**. A current perspective on honey bee health. Apidologie. 47: 273–275.

Mouches, C., J. M. Bové, and J. Albisetti. **1984**. Pathogenicity of *Spiroplasma apis* and other spiroplasmas for honey-bees in Southwestern France. Ann. Inst. Pasteur Microbiol. 135: 151–155.

Mouches, C., J. Bové, J. G. Tully, D. L. Rose, R. E. McCoy, P. Carle-Junca, M. Garnier, and C. Saillard. **1983**. *Spiroplasma apis*, a new species from the honey-bee *Apis mellifera*. Ann. Inst. Pasteur Microbiol. 134: 383–397.

Neumann, P., O. Yañez, I. Fries, and J. R. de Miranda. **2012**. *Varroa* invasion and virus adaptation. Trends Parasitol. 28: 353–354.

Oldroyd, B. P. 2007. What's killing American honey bees? PLOS Biol. 5: e168.

Oliver, R. 2013. An improved, but not yet perfect, *Varroa* mite washer. Am. Bee Assoc. 153: 1055–1057.

Potts, S. G., J. C. Biesmeijer, C. Kremen, P. Neumann, O. Schweiger, and W. E. Kunin. **2010**. Global pollinator declines: trends, impacts and drivers. Trends Ecol. Evol. 25: 345–353.

Ravoet, J., J. Maharramov, I. Meeus, L. De Smet, T. Wenseleers, G. Smagghe, and D. C. de Graaf. **2013**. Comprehensive bee pathogen screening in Belgium reveals *Crithidia mellificae* as a new contributory factor to winter mortality. PLOS ONE. 8: e72443.

Rennich, K., G. Kunkel, S. Abban, R. Fahey, H. Eversole, J. Evans, E. Forsgren, V. Levi, D. Lopez, S. Madella, J. Pettis, R. Rose, and D. vanEngelsdorp. **2015**. 2013-2014 National honey bee pests and diseases survey report. University of Maryland.

Rosenkranz, P., P. Aumeier, and B. Ziegelmann. **2010**. Biology and control of *Varroa destructor*. J. Invertebr. Pathol. 103: S96–S119.

Sagili, R., and C. Marshall. **2016**. First report of *Apocephalus borealis* Brues, 1924 (Diptera: Phoridae) parasitizing honey bees in Oregon, U.S.A. Pan-Pac. Entomol. 92: 168–169.

Sambrook, J., and D. W. Russell. **2001**. Molecular cloning, 3rd ed. ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Schmid-Hempel, R., and M. Tognazzo. **2010**. Molecular divergence defines two distinct lineages of *Crithidia bombi* (Trypanosomatidae), parasites of bumblebees. J. Eukaryot. Microbiol. 57: 337–345.

Schwarz, R. S., G. R. Bauchan, C. A. Murphy, J. Ravoet, D. C. de Graaf, and J. D. Evans. **2015**. Characterization of two species of Trypanosomatidae from the honey bee *Apis mellifera*: *Crithidia mellificae* Langridge and McGhee, and *Lotmaria passim* n. gen., n. sp. J. Eukaryot. Microbiol. 62: 567–583.

Schwarz, R. S., É. W. Teixeira, J. P. Tauber, J. M. Birke, M. F. Martins, I. Fonseca, and J. D. Evans. **2014**. Honey bee colonies act as reservoirs for two *Spiroplasma* facultative symbionts and incur complex, multiyear infection dynamics. MicrobiologyOpen. 3: 341–355.

Simone-Finstrom, M., H. Li-Byarlay, M. H. Huang, M. K. Strand, O. Rueppell, and D. R. Tarpy. **2016**. Migratory management and environmental conditions affect lifespan and oxidative stress in honey bees. Sci. Rep. 6.

Smith, M. L. 2012. The honey bee parasite *Nosema ceranae*: transmissible via food exchange? PLOS ONE. 7: e43319.

Szalanski, A. L. 2000. Population genetics and phylogenetics of the endangered American burying beetle, Nicrophorus americanus (Coleoptera: Silphidae). Ann. Entomol. Soc. Am. 93: 589–594.

Szalanski, A. L., A. D. Tripodi, and C. E. Trammel. **2014**. Molecular detection of *Nosema apis* and *N. ceranae* from southwestern and south central USA feral Africanized and European honey bees, *Apis mellifera* (Hymenoptera: Apidae). Fla. Entomol. 97: 585–589.

Szalanski, A. L., J. Whitaker, A. D. Tripodi, and P. Cappy. **2013**. Prevalence of *Nosema* from managed honey bee colonies in South Dakota and New York. J. Agric. Urban Entomol. 29: 99–104.

Szalanski, A., C. E. Trammel, A. D. Tripodi, D. Cleary, L. Rusert, and D. Downey. **2016**. Molecular diagnostics of the honey bee parasite *Lotmaria Passim*, and *Crithidia* spp. using multiplex PCR. Fla. Entomol.

Tritschler, M., G. Retschnig, O. Yañez, G. R. Williams, and P. Neumann. **2017**. Host sharing by the honey bee parasites *Lotmaria passim* and *Nosema ceranae*. Ecol. Evol. 7: 1850–1857.

Uroš, G., S. Jevrosima, G. Bojan, S. Predrag, Đ. Spomenka, V. Branislav, and S. Zoran. **2014**. *Nosema ceranae* DNA in honey bee haemolymph and honey bee mite *Varroa destructor*. Acta Vet. (Beogr.). 64.

vanEngelsdorp, D., and M. D. Meixner. **2010**. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. J. Invertebr. Pathol. 103: S80–S95.

Webster, T. C., K. W. Pomper, G. Hunt, E. M. Thacker, and S. C. Jones. **2004**. *Nosema apis* infection in worker and queen *Apis mellifera*. Apidologie. 35: 49–54.

Zheng, H.Q., and Y. P. Chen. **2014**. Detection of *Spiroplasma melliferum* in honey bee colonies in the US. J. Invertebr. Pathol. 119: 47–49.

Table 3.1. List of primers used in molecular detection of parasites and pathogens in honey bee samples. A: Garnery et al. 1993; B: Szalanski et al. 2014; C: Core et al. 2012; D: Schwarz et al. 2014; E: Schmid-Hempel and Tognazzo 2010; F: Szalanski et al. 2016.

Primer	Sequence	Reference
E2	F: 5'-GGCAGAATAAGTGCATTG-3'	A
H2	R: 5'-CAATATC ATTGATGACC-3'	A
NosemaSSU-1F	F: 5'-ACAATATGTATTAGATCTGATATA-3'	B
NosemaSSU-1R	R: 5'-TAATGATATGCTTAAG TTCAAAG-3'	B
Phorid-rRNA-1F	F: 5'-GTACACCTATACATTGGGTTCGTACATT AC-3'	C
Phorid-rRNA-1R	R: 5'-GAGRGCCATAAAAGTAGCTACACC-3'	C
S. apis ITS-F	F: 5'-AATGCCAGAAGCACGTATCC-3'	D
S.apis ITS-R	R: 5'-GAACGAGATATACTCATAAGCTGTTACAC-3'	D
Ms-160 F	F: 5'- TTGCA AAAGCTGTTTTAGATGC-3'	D
Ms-160-R	R: 5'- TGACCAGAAATGTTTGCTGAA-3'	D
CBSSU rRNA F2	F: 5'-CTTTTGACGAACAACTGCCCTATC-3'	E
CBSSU rRNA B4	R: 5'- AACCGAACGCACTAAACCCC-3'	E
L. passim18S-F	F: 5'-AGGGATATTTAAACCC ATCGAAAATCT-3'	F
C. mel 474-F	F: 5'-TTTACGCA TGTCATGCATGCCA-3'	F

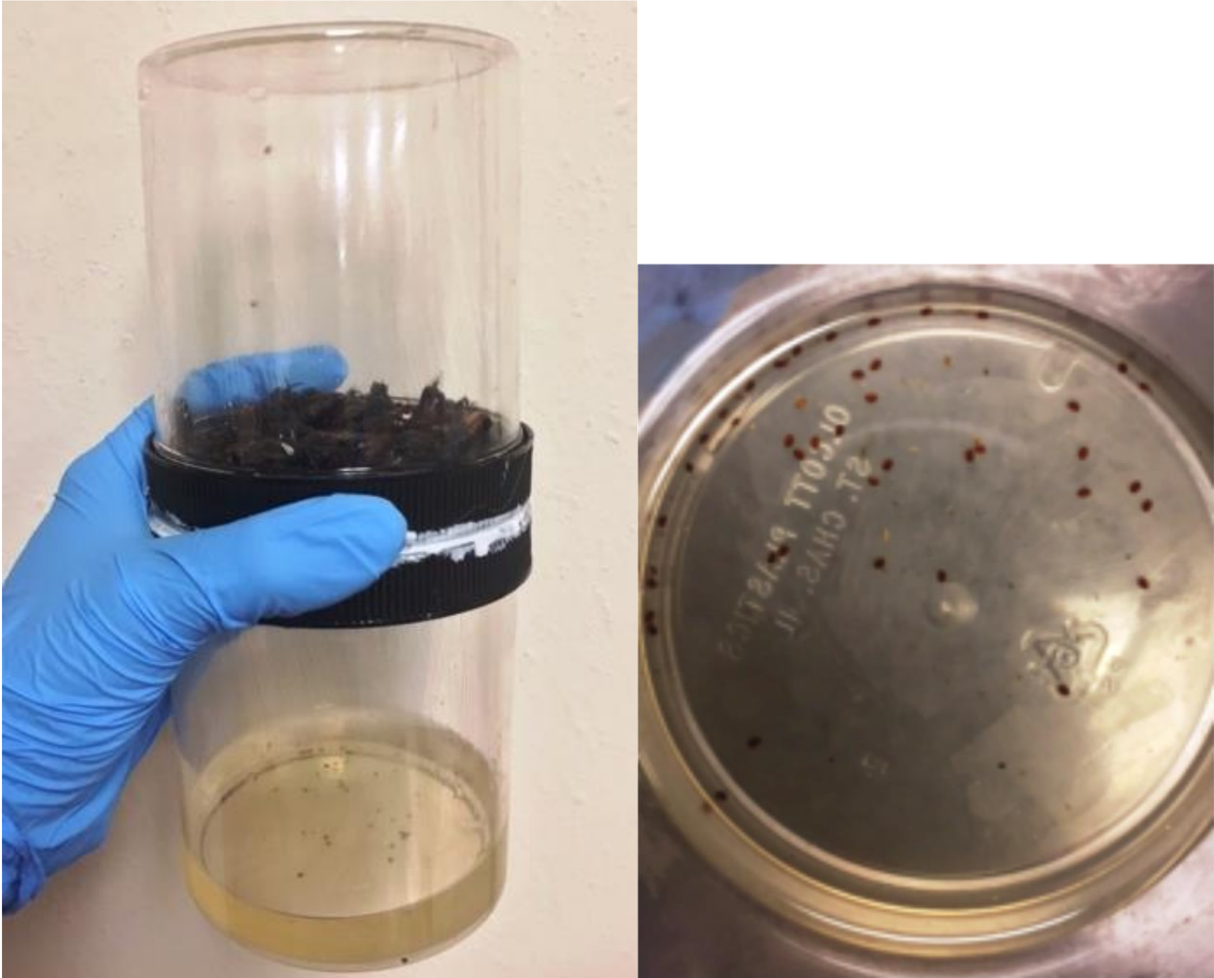

Figure 3.1. Mite wash adapted from Oliver (2013).

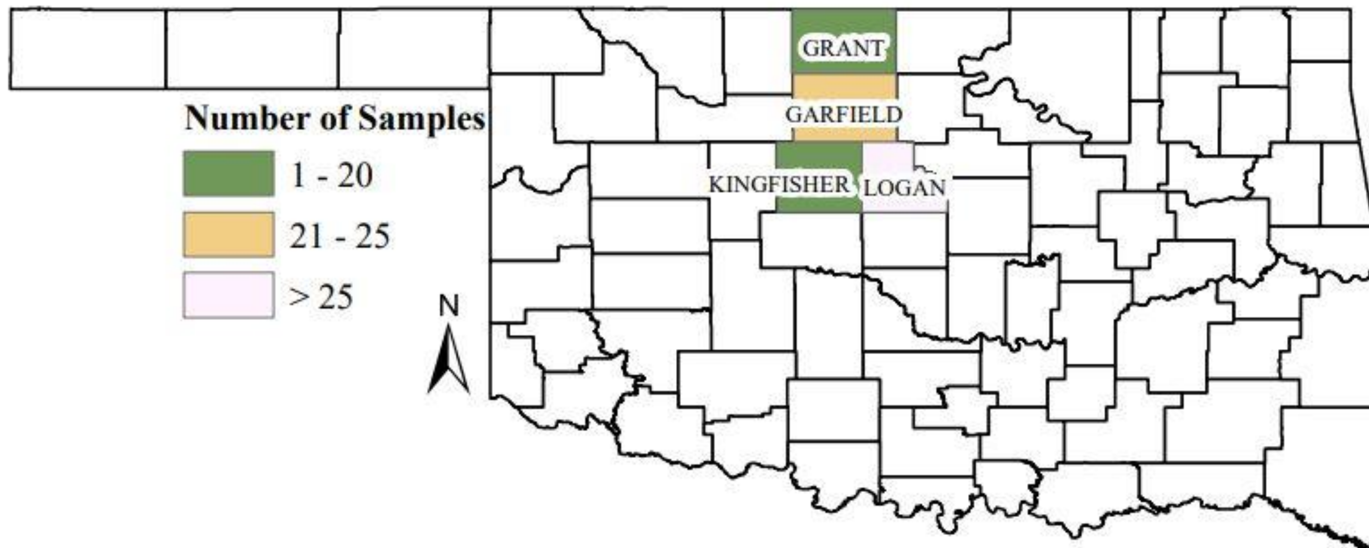

Figure 3.2. County map of Oklahoma indicating the number of samples received from each county. Counties sampled from include: Grant, Garfield, Kingfisher and Logan. Samples were not received from counties in white.

Table 3.2. Percent of honey bee colonies positive for *Varroa destructor* by county.

County	Positive	Number of colonies	Positive for *V. destructor* (%)
Kingfisher	6	20	4.8
Garfield	1	21	15
Logan	5	26	30
Grant	3	20	19.2
Total	15	87	**17.2**

Table 3.3. Honey bee colony samples positive for *Varroa destructor* by month in 2015.

		2015		
Month	**Positive**	**Total**	**Percent (%)**	**Cum. (%)**
March	2	10	20.0	2.6
April	2	30	6.7	2.6
May	11	36	30.6	14.5
Sum	15	76		**19.7**

Table 3.4. Percent of honey bee colony samples positive for *Nosema ceranae* by county.

County	Positive	Number of colonies	Positive for *N. ceranae* (%)
Kingfisher	2	20	10.0
Garfield	7	21	33.3
Logan	10	26	76.9
Grant	5	20	25.0
Total	24	87	**27.6**

Table 3.5. Monthly percent of occurrence of parasites and pathogens for 2015-2016.

	March (n=10)	April (n=41)	May (n=36)
Nosema ceranae	20%	30%	30%
Lotmaria passim	0%	2.5%	0%
Varroa destructor	20%	4.87%	30.5%
Spiroplasma melliferum	20%	2.4%	11.1%

Table 3.6. Colony samples positive for *Nosema ceranae* in 2015.

	2015		
Month	**Positive**	**Total**	**Percent (%)**
March	2	10	20.0
April	12	30	40.0
May	9	36	25.0
Sum	23	76	**30.3**

Table 3.7. Percent of honey bee colony samples positive for *Spiroplasma melliferum* by county.

County	Positive	Number of colonies	Positive for *S. melliferum* (%)
Kingfisher	0	20	0
Garfield	0	21	10.0
Logan	5	26	0
Grant	2	20	19.2
Total	7	87	**8.0**

Table 3.8. Honey bee colony samples positive for *Spiroplasma melliferum* in 2015 by month.

		2015		
Month	**Positive**	**Total**	**Percent (%)**	**Cum. (%)**
March	2	10	20.0	2.6
April	1	30	3.3	1.3
May	4	36	11.1	5.3
Sum	7	76		**9.2**

Chapter 4: Comparison of honey bee, *Apis mellifera* L., parasite and pathogen occurrence between hobbyist beekeepers versus commercial beekeepers

Abstract

With honey bee populations fluctuating over the last decade, colony management is among the possible factors affecting honey bee health. Migratory beekeeping requires movements of millions of honey bee, *Apis mellifera* L., colonies across the United States to provide pollination services. With the large-scale movement of honey bee colonies, criticisms have surfaced, faulting migratory beekeeping as a factor in the health decline of honey bees in the United States. Few studies have compared the occurrence of honey bee parasites and pathogens in commercial migratory operations versus non-migratory hobbyist and sideliner beekeepers' operations to sautinize differing management methods. Using molecular diagnostic techniques, honey bees were screened for parasites and pathogens including, *Nosema apis, Nosema ceranae, Apocephalus borealis, Spiroplasma apis, Spiroplasma melliferum, Crithidia mellificae, Lotmaria passim,* and *Varroa destructor.* When examining all of the colonies sampled, 56.8% were positive for at least one of these parasites or pathogens. *Varroa destructor* and *L. passim* occurrence was more common in the hobbyist managed colonies than the commercially managed colonies. Conversely, *N. ceranae* and *S. melliferum* were more common in the commercially managed colonies. Management techniques may provide insight into how parasite and pathogen species are spreading through commercial and hobby hives.

Introduction

Numerous agricultural crops require animal pollination for productivity. In fact, 70% (87 crops) of the world's primary food crops depend on animal pollination (Klein et al. 2007, Gallai et al. 2009). Among these animal pollinators are birds, bats, and several species of insects (Wardell et al. 1998). While all pollinators are important, managed pollinators allow for consistency, versatility, and help ensure crops receive pollination. The most commonly managed pollinator in North America is the Western honey bee, *Apis mellifera* L. (Klein et al. 2007, Frier et al. 2016, Simone-Finstrom et al. 2016). While honey bee colonies are often managed at the commercial level, hobbyist backyard beekeepers continue to manage several million hives in the Unites States. Moreover, in recent years honey bee populations have displayed high levels of annual loss without a single identified trigger (Glenny et al. 2017). Multiple stressors of honey bees have been implicated, including: parasites, pathogens, diseases, nutrition, habitat loss, pesticides and management practices (Goulson et al. 2015, Simone-Finstrom et al. 2016, Glenny et al. 2017). For the purposes of this study, we will focus on management practice as well as parasite and pathogen occurrence.

Honey bees alone contribute to approximately 90% of managed pollination services (James and Pitts-Singer 2008). Furthermore, the honey bee industry is a $17 billion dollar industry (Calderone 2012, Otto et al. 2016, Traynor et al. 2016). For this reason, the western honey bee, *Apis mellifera* is considered the most economically essential pollinator in North America, and is currently the most commonly managed pollinator (Klein et al. 2007, Frier et al. 2016, Simone-Finstrom et al. 2016). Managed pollinators in easy to transport hives allow for transportation to crops in need of pollination at the specific time of bloom (Simone-Finstrom et

al. 2016). For example, annually, over 60% of the United States honey bee colonies are transported to California for almond pollination.

Over the past decade reductions in honey bee populations have occurred, in some instances resulting in a massive colony die-offs across the United States (Runckel et al. 2011). The number of managed honey bee colonies have shown growth (45% increase) worldwide since measured in 1961. However, while the number of honey bee colonies have increased by 45%, the hectares of crops which depend upon pollination have also increased, by 300% (Aizen and Harder 2009, Smith et al. 2013). Proportionally, there is a higher demand for honey bees than ever (Sumner and Boriss 2006, Runckel et al. 2011).

Multiple factors are suspect for contributing to honey bee colony losses, including parasites, pathogens, disease, habitat loss, pesticides and migratory beekeeping (vanEngelsdorp et al. 2008, Evans and Schwarz 2011). While migratory beekeeping is implicated as a potential cause of honey bee health decline, few long-term studies have been conducted to research such claims (Zhu et al. 2014, Simone-Finstrom et al. 2016, Traynor et al. 2016). In fact, there is little research to indicate how migratory beekeeping affects honey bee physiology at all (Ahn et al. 2012).

Three types of beekeepers exist: commercial, sideliner, or hobbyist. The three differ in the number of colonies that they manage, whether the honey bees are transported, as well as the level of hive management (Lee et al. 2015, Simone-Finstrom et al. 2016). Commercial migratory beekeepers include those that manage over 300 honey bee colonies, and manage honey bees as a primary source of income (Lee et al. 2015). Commercially managed colonies are transported seasonally to new agricultural locations in order to provide pollination services (Cestaro et al. 2017). The Almond pollination in California is the primary user of pollination services by honey

bees in the United States. Between 60-80% of managed colonies in the United States are transported to California annually to pollinate during the almond bloom (Runckel et al. 2011, Bond et al. 2014, Cavigli et al. 2016). Colony transportation often involves moving hives thousands of kilometers in less than ideal conditions. Hives lack ventilation, are threatened by poor nutrition, and are exposed to new parasites, pathogens, viruses, and disease (Bacandritsos et al. 2010, Smith et al. 2013, Hendriksma and Shafir 2016). Regarding management tactics, migratory colonies typically receive heavy treatment and intensive management. Commercially managed colonies are often aggregated with hundreds to thousands of other colonies, allowing for easy pest and disease transmission (Royce and Rossignol 1990, Lee et al. 2015).

Sideliner beekeepers manage between 51-300 honey bee colonies and utilize these colonies as supplemental income; these colonies are moderately managed compared to commercial and hobbyist colonies (vanEngelsdorp et al. 2012, Lee et al. 2015). Sideliners which participate in providing pollinator services, typically only move their colonies regionally, aside from the annual almond pollination in California (Simone-Finstrom et al. 2016). Because sideliner managed colonies likely participate in the almond pollination, they are exposed to similar levels of stress identified above within commercial colonies.

Honeybee colonies managed by hobbyist beekeepers, also known as backyard beekeepers, remain stationary; thus, not being placed under the same levels of transportation stress as the commercially and sideliner managed colonies (Lee et al. 2015). Hobbyist beekeepers have fewer than 50 colonies and do not manage their colonies for large-scale income. Typically, hobbyist beekeepers do not manage colonies as intensely, often leaving colonies untreated for various parasites, pathogens, and diseases (Lee et al. 2015).

As new parasites and pathogens of the honey bee continue to be detected, these organisms require analysis to understand their current distribution and future spread. Among these pests are both external and internal species, affecting various components of the honey bees' physiology (Evans and Schwarz 2011). For our study, we will focus on *Nosema apis* Zander and *N. ceranae* Fries, *Varroa destructor* Anderson and Trueman, *Apocephalus borealis* Brues, *Lotmaria passim* Schwarz and *Crithidia mellificae* Langridge and McGhee, and *Spiroplasma apis* Mouches and *S. melliferum* Clark.

Nosema disease, also known as Nosemosis, is caused by two species of parasitic microsporidia, *Nosema apis* Zander and *Nosema ceranae* Fries (Bailey 1955, Szalanski et al. 2013). The unicellular parasites attack the midgut epithelial cells of the adult honey bee (Evans and Schwarz 2011, Uroš et al. 2014). Populations of *N. apis* and *N. ceranae* have shown to peak from January to April (Forsgren and Fries 2010, Meixner and Conte 2016). While the two species of *Nosema* attack similarly, the effects of *N. apis* are less infectious than *N. ceranae* (Mayack and Naug 2009, vanEngelsdorp and Meixner 2010). *Nosema apis* has been linked to shortening lifespan of worker honey bees and an overall reduction in colony health (Klee et al. 2007). Furthermore, the distribution of *N. apis* is well documented. Records indicate that *N. ceranae* has occurred in the United States since 1995, and is considered to be more virulent than *N. apis* as well as replacing *N. apis* in its distribution (Paxton 2010, Smith et al. 2013). This is problematic because levels of mortality are far higher with *N. ceranae* infections than *N. apis* infections (Forsgren and Fries 2010).

Varroa destructor Anderson and Trueman is a well-documented hemophagous ectoparasitic mite of the honey bee. The mite is considered the number one threat to apiculture worldwide (Rosenkranz et al. 2010, Uroš et al. 2014). Attacking both immature and adult honey

bees, *V. destructor* attaches to the bee, feeding on its hemolymph (Huang 2012). The mite has been linked to deformities, viruses, and reduction in honey bee health (Le Conte et al. 2010). Originally a parasite of the Asian honey bee, *Apis cerana*, *V. destructor* switched hosts to *A. mellifera*, most likely due to transportation of honey bees carrying *V. destructor* (Oldroyd 1999, Rosenkranz et al. 2010). The lack of co-evolution could be to blame for its tremendous impact on honey bee populations (Rath 1999, Rosenkranz et al. 2010).

Protozoan Trypanosomatid species *Lotmaria passim* and *Crithidia mellificae* are obligate parasites of adult honey bees (Schmid-Hempel and Tognazzo 2010, Szalanski et al. 2016). Despite being described in 1967 and the related *Crithidia bombi* Lipa and Triggiani being a known and widespread parasite of bumbles bees, *Crithidia mellificae* Langridge and McGhee is among the neglected parasites of the honey bee regarding research (Schmid-Hempel and Tognazzo 2010, Evans and Schwarz 2011, Schwarz et al. 2015). *Lotmaria passim* has had even less observation considering it was not described as a separate species until 2015 (Schwarz et al. 2015). Both species are thought to occur in the hindgut and rectum of adult honey bees (Runckel et al. 2011). While both species have relatively little research focused on their effect on honey bee health, *L. passim* has been detected in Belgium, Chile, Japan, and Switzerland (Ravoet et al. 2013). While research does not prove that *C. mellificae* has a significant impact on honey bee health, it is important to examine, considering the large effect *Crithidia bombi* has on bumble bee health.

Apocephalus borealis Brues is a parasitic phorid fly originally known to only attack bumble bees and paper wasps (Brown 1993, Otterstatter et al. 2002, Core et al. 2012). A 2012 study documented the first known instance of *A. borealis* using honey bees as a host. The fly lays its eggs inside the host, resulting in abnormal behaviors such as flying at night and hive

abandonment (Core et al. 2012). The fly eventually emerges from the adult honey bee, resulting in honey bee mortality. Studies have also shown that colonies containing *A. borealis* were also positive for Deformed Wing Virus and *N. ceranae,* meaning the fly may act as a vector (Core et al. 2012). While parasitism in bumble bees and papers wasps occurs across the U.S., *A. borealis* has only been documented to parasitize honey bees in Oregon, Washington, Vermont, South Dakota and Vancouver (Runckel et al. 2011, Core et al. 2012).

Spiroplasma apis Mouches and *S. melliferum* Clark are bacterial pathogen species that attack adult honey bees. It has been reported that *Spiroplasma* is transmitted to adult honey bees via plant surfaces (Clark 1982, Evans and Schwarz 2011). Once the bacteria invades the honey bee's gut barriers it enters its hemolymph (Evans and Schwarz 2011, Schwarz et al. 2014). The bacteria has been linked to neurological disease, acting as the causative agent in Spiroplasmosis, a seasonal disease also known as May disease (Schwarz et al. 2014). *Spiroplasma* infection levels have been seasonally abundant, primarily occurring in the spring during the nectar flow (Clark 1982). *Spiroplasma melliferum* has been implicated in high mortality and low colony productivity of honey bee colonies (Schwarz et al. 2014). *Spiroplasma apis* has shown to be lethal when injected and consumed by honey bees (Mouches et al. 1982). The first study targeting *Spiroplasma* detection in honey bees in the Unites Stated was conducted from 2011-2013. This study found that samples positive for either *Spiroplasma* species were also more likely to be susceptible to other *Spiroplasma* species and found that *S. melliferum* is more prevalent than *S. apis* (Schwarz et al. 2014).

Studying both migratory and hobbyist managed colonies may provide insight on the distribution of parasites and pathogens of honey bees. Furthermore, the comparison between the two management practices may provide insight to the impact on honey bee health. Moreover, the

comparison can provide insight on how these honey bee parasites and pathogens are spreading and a better understanding of the distribution may aid in developing better control tactics for honey bee pests.

Materials and Methods

Sample Collection and Preservation

Honey bee samples were collected from 2015-2016 from Arkansas hobbyist managed colonies (Chapter 2) and migratory colonies located in Oklahoma (Chapter 3). Approximately 50-300 worker honey bees were sampled from within the hive. Once samples were collected they were mailed to the Arkansas insect genetics lab (University of Arkansas, Fayetteville, AR). The honey bees were preserved and stored in 70% ethanol at room temperature.

Varroa mite detection

A mite wash adapted from (Oliver 2013) allowed for *V. destructor* detection (Figure 4.1). Colony samples each underwent mite wash, allowing for *Varroa* mites to dislodge from honey bee samples. Following separation from honey bees, mites were recorded, placed in 1.5 ml Eppendorf tubes with 70% ethanol and stored at -80°C.

Molecular Diagnostics

Mass DNA extraction was performed using a salting-out-protocol per Sambrook and Russell (2001). DNA presence was confirmed using the mtDNA COI-COII honey bee PCR primer set E2 (5'-GGCAGAATAAGTGCATTG-3') and H2 (5'-CAATATC ATTGATGACC-3') and the following thermocycler conditions: denatured initially for 5 min. at 94°C then 40 cycles at 94°C for 45 seconds, 46°C for 1 min., 72°C for 1 min. and a final extension of 72°C for 5 min. (Garnery et al. 1993). A total of 2 μL was combined with dye in a 2% agarose gel. Gels were visualized using the UV BioDocit station; DNA was indicated by a 600-1200 BP amplicon.

Following DNA presence verification, samples were screened for parasites and pathogens including: *Nosema apis and N. ceranae;* bacterial pathogens *Spiroplasma apis* and *S. melliferum;* Trypanosomatid parasite species *Crithidia mellificae and Lotmaria passim.* Multiplex PCR and PCR-RFLP were utilized to target these species using molecular diagnostic techniques. A known positive and negative control were included in each PCR to ensure reliability. Primers used to detect each parasite or pathogen species are displayed in (Table 4.1).

Nosema sp. were tested for using the DNA extraction product and PCR primers NosemaSSU-1F (5'-ACAATATGTATTAGATCTGATATA-3') and NosemaSSU-1R (5'-TAATGATATGCTTAAG TTCAAAG-3') (Szalanski et al. 2014). The primer set amplifies a 222 bp amplicon for *N. apis* and a 237 bp amplicon for *N. ceranae* using the small subunit gene region specific for *Nosema* mitochondrial DNA. The thermocycler condition were as follows: 2 min. at 94°C, then 40 cycles of 94°C for 45 seconds, 50°C for 1 min. and 72°C for 1 min., followed by a final extension of 72°C for 5 min. (Szalanski et al. 2014). PCR product was run on a 2% agarose gel and visualized using the UV BioDocit station. Samples positive for *Nosema sp.* underwent PCR-RFLP using restriction enzymes *Dra I* and *Rsa I*. The enzyme *Dra I* cuts *N. ceranae* at 79 bp, while *Rsa I* cuts *N. apis* at 130 bp. Samples were incubated overnight at 37°C followed by visualizing products on a 2% agarose check gel with the UV BioDocit station.

Multiplex PCR was utilized to detect Trypanosome species and *Spiroplasma* species. Primers CBSSU rRNA-F2, CBSSU rRNA B4, and L. passim18S-F (5'-AGGGATATTTAA ACCCATCGAAAATCT-3') were used to detect any trypanosomatid species (608 bp) and only *L. passim* (499 bp) (Szalanski et al. 2016). The thermocycler conditions were as follows denaturing step of 5 min. at 95°C was followed by 40 cycles of 30 seconds at 95°C, primer

annealing for 30 seconds at 57°C (Schmid-Hempel and Tognazzo 2010). PCR products were visualized using a 2% agarose check gel and the UV BioDocit station.

Samples found positive for *L. passim* or trypanosomatids underwent a separate multiplex PCR using primers CBSSU rRNA P2, CBSSU rRNA B4, L. passim18S-F (5'-AGGGATATTT AAACCCATCGAA AATCT-3'), and C. mel 474-F ('5-TTTACGCATGTCAT GCATGCCA-3') under thermocycler conditions described above (Szalanski et al. 2016). Positive samples displayed a 716-724 bp product for *L. passim* and *Crithidia* spp., a 499 bp band for only *L. passim*, and a 245 bp band for *Crithidia* sp. (Szalanski et al. 2016).

Primers S.apis ITS-F (5'-AATGCCAGAAGCACGTATCC-3'), S.apis ITS-R (5'-GAACGAGATATACTCATAAGCTGTTACAC-3'), Ms-160 F(5'- TTGCAAAAGC TGTTTTAGATGC-3'), Ms-160-R (5'- TGACCAGAAATGTTTGCTGAA-3') were used to detect *S. apis* and *S. melliferum*. A known positive and known negative control were included to ensure reliability. The thermocycler conditions were as follows: 5 min. at 95°C followed by 40 cycles of 30 seconds at 95°C, primer annealing for 30 seconds at 57°C (Schmid-Hempel and Tognazzo 2010). Following PCR, the products were run on a 2% agarose check gel and visualized using the UV BioDocit station. Samples positive for *S. apis* resulted in a 190 bp amplicon, while samples positive for *S. melliferum* resulted in a 160 bp amplicon.

Statistical analysis

Statistical analysis was conducted using JMP Pro 13.2. Specifically, a Fisher's Exact Test was utilized to test for independence among the parasite and pathogen species.

Results

A total of 628 honey bee colony samples were received from Arkansas and Oklahoma between 2015 and 2016. All samples received from Arkansas were sampled from hobbyist

beekeepers with fewer than 50 hives. All Oklahoma samples were commercially managed hives, transported most recently from California for the almond pollination to Oklahoma for canola pollination.

Combining hobbyist and migratory managed colonies, 56.8% of the colony samples were positive for at least one of the parasites and pathogens tested for (Figure 4.2). We found that it was most common to encounter one parasite or pathogen species within a honey bee colony than none or more than one parasite or pathogen. Furthermore, a level of association was detected between both *S. melliferum* and *N. ceranae*, as well as between *L. passim* and *V. destructor* (Table 4.2). Concerning individual species occurrence within samples, the most commonly detected species in both 2015 and 2016, were *V. destructor* and *N. ceranae* (Table 4.3). Seasonally, all of the parasites and pathogens were most abundant in June, except for *S. melliferum*, which was most abundant in May (Figure 4.3).

When looking at separate years, 2015 resulted in a total of 511 while 2016 resulted in a total of 117 colony samples. Proportionally, 2015 consistently resulted in more colony samples positive for parasites and pathogens, with the exception of *V. destructor,* which was actually higher, proportionally, in 2016 (Table 4.3).

Between June 2015 and October 2016, a total of 541 hobbyist managed honey bee colonies from 107 different beekeepers and 46 different Arkansas counties were sampled. Samples included worker honey bee samples collected from within the hive. Samples were collected during all four seasons (Summer n=378, Fall n= 33, Winter n=2, Spring n=128).

Between April 2015 and April 2016, a total of 87 commercially managed honey bee colonies from four different Oklahoma counties were sampled. Samples included worker honey

bee samples collected from within the hive. All of the samples were collected during the Spring (n=87).

Nosema ceranae and *S. melliferum* were more abundant, proportionally, in the migratory colonies (Figure 4.4). *Varroa destructor* and *L. passim* were higher in hobbyist managed colonies. *Nosema apis, A. borealis, C. mellificae,* and *S. apis* were not detected in the hobbyist managed colonies nor the migratory managed colonies. Furthermore, none of the hobbyist managed colonies were positive for either species of *Spiroplasma.* The Oklahoma migratory honey bee samples had seven instances of *S. melliferum.*

Discussion

The highest proportion of samples positive for *Nosema* occurred in the month of July with 20% of the samples positive for *N. ceranae.* None of the samples received in January and April were positive for *N. ceranae.* These numbers differ from a multi-year study conducted from 2009-2014 in which *N. ceranae* peaked between the months of January-April (Meixner and Conte 2016, Traynor et al. 2016).

Because the migratory honey bee colony samples were only received in spring we examined monthly occurrence (March n=10, April n=41, May n=36). April had the highest proportion of *N. ceranae* with 31.7% of the samples testing positive. Comparatively, the Hobbyist samples collected in April (n=44) had no occurrence of *N. ceranae.*

Similar to a 2017 Brazilian study, only *Nosema ceranae* was detected in both the migratory and hobbyist colony samples, none of the samples were positive for *N. apis* (Cestaro et al. 2017). *Nosema ceranae* was detected in 11.6% of the hobbyist colony samples compared to 27.59% in the migratory colony samples (Figure 4.4). Similarly, a 2009 study revealed migratory colonies have a higher instance of *Nosema* with it occurring in 59.9% of hives compared to

stationary hives with 46.7% (Traynor et al. 2016). Unfortunately, the treatment history for *Nosema* in the migratory colonies is unavailable. Future studies should include treatment history for both the migratory and hobbyist managed colonies to test if it is a significant factor in pathogen occurrence.

Varroa destructor was detected in 41.1% of the Arkansas hobbyist colony samples tested and 17.24% in the Oklahoma migratory colony samples. This is similar to a survey conducted from 2009 to 2014 in which both prevalence and infestation loads of *V. destructor* were higher in the stationary colonies. The study showed that *V. destructor* was detected in 84.9% of migratory colonies, while stationary colonies showed 97% (Traynor et al. 2016). Commercially managed colonies were treated for *Varroa* mites using Apistan strips. Frequency and uniformity of the strips per colony are unavailable. It is possible that hobbyists' colonies are more likely for *V. destructor* to occur due to less intensive management strategies. Future research should examine infestation levels as well as comprehensive treatment history.

Neither the hobbyist colonies nor the migratory colonies tested positive for *Crithidia mellificae*; however, both groups contained samples positive for *L. passim. Lotmaria* passim was detected in 11.29% of the Arkansas hobbyist colonies, while the Oklahoma migratory colonies had 1.15% of hives positive for *L. passim.* The month of July had the highest proportion of samples positive for *L. passim* at 20%. None of the samples collected in January, April, August, and September were positive for *L. passim.*

Spiroplasma was only detected in two of the migratory. Because *Spiroplasma* is thought to spread via exposure to infected plant surfaces, the migratory samples may have a higher occurrence because these honey bees are exposed to more flowering vegetation in different geographic locations, with more honey bees.

When measuring for independence, a level of association was detected between both *S. melliferum* and *N. ceranae*, as well as between *L. passim* and *V. destructor*. A previous study indicated a similar association between *Nosema ceranae* and *Spiroplasma* infections (Runckel et al. 2011). No previous research has been conducted exploring an association between *V. destructor* and *L. passim.* Further research to explore this association is warranted as means of transmission is currently unknown for *L. passim.* Testing *V. destructor* for *L. passim* may provide insight into whether the mite is acting as a vector of the trypanosomatid.

Furthermore, honey bees serve as a good model for other pollinator species. Many of the parasites and pathogens found in honey bees have comparable species within the same genus which affect native bee species. For example, *Nosema* and *Crithidia* are found in bumble bee species (Fries et al. 2001, Ravoet et al. 2013). Because of accessibility of honey bee colonies, they can be sampled easily. We may be able to apply this research to native bee species in the future.

While this study serves as a foundation for future studies, short comings do exist. Future studies should have a more extensive sample size. When examining migratory colonies, more than one geographic location should be included, which could provide insight as to the source of spread for parasites and pathogens of honey bees. Furthermore, our study only sampled colonies in the spring. Ideally, sampling would occur throughout the year in order to gain information as to when parasite and pathogen species peak seasonally.

Literature cited

Ahn, K., X. Xie, J. Riddle, J. Pettis, and Z. Y. Huang. **2012**. Effects of long distance transportation on honey bee physiology. Psyche J. Entomol. (https://www.hindawi.com/journals/psyche/2012/193029/).

Aizen, M. A., and L. D. Harder. **2009**. The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. Curr. Biol. 19: 915–918.

Bacandritsos, N., A. Granato, G. Budge, I. Papanastasiou, E. Roinioti, M. Caldon, C. Falcaro, A. Gallina, and F. Mutinelli. **2010**. Sudden deaths and colony population decline in Greek honey bee colonies. J. Invertebr. Pathol. 105: 335–340.

Bailey, L. 1955. The epidemiology and control of *Nosema* disease of the honey-bee. Ann. Appl. Biol. 43: 379–389.

Bond, J., K. Plattner, and K. Hunt. **2014**. Fruit and tree nuts outlook: economic insight U.S. pollination-services market. USDA economic research service situation and outlook FTS-357SA. United States Department of Agriculture.

Brown, B. 1993. Taxonomy and preliminary phylogeny of the parasitic genus *Apocephalus*, subgenus Mesophora (Diptera: Phoridae). Syst. Entomol. 18: 191–230.

Calderone, N. W. 2012. Insect pollinated crops, insect pollinators and US agriculture: trend analysis of aggregate data for the period 1992-2009. PLOS ONE. 7: e37235.

Cavigli, I., K. F. Daughenbaugh, M. Martin, M. Lerch, K. Banner, E. Garcia, L. M. Brutscher, and M. L. Flenniken. **2016**. Pathogen prevalence and abundance in honey bee colonies involved in almond pollination. Apidologie. 47: 251–266.

Cestaro, L. G., M. L. T. M. F. Alves, D. Message, M. V. G. B. Silva, and É. W. Teixeira. **2017**. Honey bee (*Apis mellifera*) health in stationary and migratory apiaries. Sociobiology. 64: 42–49.

Clark, T. B. 1982. *Spiroplasmas*: diversity of arthropod reservoirs and host-parasite relationships. Science. 217: 57–59.

Core, A., C. Runckel, J. Ivers, C. Quock, T. Siapno, S. DeNault, B. Brown, J. DeRisi, C. D. Smith, and J. Hafernik. **2012**. A new threat to honey bees, the parasitic phorid fly *Apocephalus borealis*. PLOS ONE. 7: e29639.

Evans, J. D., and R. S. Schwarz. **2011**. Bees brought to their knees: microbes affecting honey bee health. Trends Microbiol. 19: 614–620.

Forsgren, E., and I. Fries. **2010**. Comparative virulence of *Nosema ceranae* and *Nosema apis* in individual European honey bees. Vet. Parasitol. 170: 212–217.

Frier, S. D., C. M. Somers, and C. S. Sheffield. **2016**. Comparing the performance of native and managed pollinators of Haskap (Lonicera caerulea: Caprifoliaceae), an emerging fruit crop. Agric. Ecosyst. Environ. 219: 42–48.

Fries, I., A. de Ruijter, R. J. Paxton, A. J. da Silva, S. B. Slemenda, and N. J. Pieniazek. **2001**. Molecular characterization of Nosema bombi (Microsporidia: Nosematidae) and a note on its sites of infection in Bombus terrestris (Hymenoptera: Apoidea). J. Apic. Res. 40: 91–96.

Gallai, N., J. M. Salles, J. Settele, and B. E. Vaissière. **2009**. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecol. Econ. 68: 810–821.

Garnery, L., M. Solignac, G. Celebrano, and J.-M. Cornuet. **1993**. A simple test using restricted PCR-amplified mitochondrial DNA to study the genetic structure of *Apis mellifera* L. Experientia. 49: 1016–1021.

Glenny, W., I. Cavigli, K. F. Daughenbaugh, R. Radford, S. E. Kegley, and M. L. Flenniken. **2017**. Honey bee (*Apis mellifera*) colony health and pathogen composition in migratory beekeeping operations involved in California almond pollination. PLOS ONE. 12: e0182814.

Goulson, D., E. Nicholls, C. Botías, and E. L. Rotheray. **2015**. Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science. 347: 1255957.

Hendriksma, H. P., and S. Shafir. **2016**. Honey bee foragers balance colony nutritional deficiencies. Behav. Ecol. Sociobiol. 70: 509–517.

Huang, Z. Y. 2012. *Varroa* mite reproductive biology. Am. Bee J. 152: 981–985.

James, R., and T. L. Pitts-Singer. **2008**. Bee pollination in agricultural ecosystems. Oxford University Press.

Klee, J., A. M. Besana, E. Genersch, S. Gisder, A. Nanetti, D. Q. Tam, T. X. Chinh, F. Puerta, J. M. Ruz, P. Kryger, D. Message, F. Hatjina, S. Korpela, I. Fries, and R. J. Paxton. **2007**. Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. J. Invertebr. Pathol. 96: 1–10.

Klein, A.-M., B. E. Vaissière, J. H. Cane, I. Steffan-Dewenter, S. A. Cunningham, C. Kremen, and T. Tscharntke. **2007**. Importance of pollinators in changing landscapes for world crops. Proc. R. Soc. Lond. B Biol. Sci. 274: 303–313.

Le Conte, Y., M. Ellis, and W. Ritter. **2010**. *Varroa* mites and honey bee health: can *Varroa* explain part of the colony losses? Apidologie. 41: 353–363.

Lee, K. V., N. Steinhauer, K. Rennich, M. E. Wilson, D. R. Tarpy, D. M. Caron, R. Rose, K. S. Delaplane, K. Baylis, E. J. Lengerich, J. Pettis, J. A. Skinner, J. T. Wilkes, R. Sagili, and D. vanEngelsdorp. **2015**. A national survey of managed honey bee 2013–2014 annual colony losses in the USA. Apidologie. 46: 292–305.

Mayack, C., and D. Naug. **2009**. Energetic stress in the honeybee *Apis mellifera* from *Nosema ceranae* infection. J. Invertebr. Pathol. 100: 185–188.

Meixner, M. D., and Y. L. Conte. **2016**. A current perspective on honey bee health. Apidologie. 47: 273–275.

Mouches, C., J. M. Bové, J. Albisetti, T. B. Clark, and J. G. Tully. **1982**. A spiroplasma of serogroup IV causes a May-disease-like disorder of honeybees in Southwestern France. Microb. Ecol. 8: 387–399.

Oldroyd, B. P. 1999. Coevolution while you wait: *Varroa jacobsoni*, a new parasite of western honeybees. Trends Ecol. Evol. 14: 312–315.

Oliver, R. 2013. An improved, but not yet perfect, *Varroa* mite washer. Am. Bee Assoc. 153: 1055–1057.

Otterstatter, M. C., T. L. Whidden, and R. E. Owen. **2002**. Contrasting frequencies of parasitism and host mortality among phorid and conopid parasitoids of bumble-bees. Ecol Entomol. 27: 229–237.

Otto, C. R. V., C. L. Roth, B. L. Carlson, and M. D. Smart. **2016**. Land-use change reduces habitat suitability for supporting managed honey bee colonies in the Northern Great Plains. Proc. Natl. Acad. Sci. 113: 10430–10435.

Paxton, R. J. 2010. Does infection by *Nosema ceranae* cause “Colony Collapse Disorder” in honey bees (*Apis mellifera*)? J. Apic. Res. 49: 80–84.

Rath, W. 1999. Co-adaptation of *Apis cerana* Fabr. and *Varroa jacobsoni* Oud. Apidologie. 30: 97–110.

Ravoet, J., J. Maharramov, I. Meeus, L. De Smet, T. Wenseleers, G. Smagghe, and D. C. de Graaf. **2013**. Comprehensive bee pathogen screening in Belgium reveals *Crithidia mellificae* as a new contributory factor to winter mortality. PLOS ONE. 8: e72443.

Rosenkranz, P., P. Aumeier, and B. Ziegelmann. **2010**. Biology and control of *Varroa destructor*. J. Invertebr. Pathol. 103: S96–S119.

Royce, L. A., and P. A. Rossignol. **1990**. Epidemiology of honey bee parasites. Parasitol. Today. 6: 348–353.

Runckel, C., M. L. Flenniken, J. C. Engel, J. G. Ruby, D. Ganem, R. Andino, and J. L. DeRisi. **2011**. Temporal analysis of the honey bee microbiome reveals four novel viruses and seasonal prevalence of known viruses, *Nosema*, and *Crithidia*. PLOS ONE. 6: e20656.

Sambrook, J., and D. W. Russell. **2001**. Molecular cloning, 3rd ed. ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Schmid-Hempel, R., and M. Tognazzo. **2010**. Molecular divergence defines two distinct lineages of *Crithidia bombi* (Trypanosomatidae), parasites of bumblebees. J. Eukaryot. Microbiol. 57: 337–345.

Schwarz, R. S., G. R. Bauchan, C. A. Murphy, J. Ravoet, D. C. de Graaf, and J. D. Evans. **2015**. Characterization of two species of Trypanosomatidae from the honey bee *Apis mellifera*: *Crithidia mellificae* Langridge and McGhee, and *Lotmaria passim* n. gen., n. sp. J. Eukaryot. Microbiol. 62: 567–583.

Schwarz, R. S., É. W. Teixeira, J. P. Tauber, J. M. Birke, M. F. Martins, I. Fonseca, and J. D. Evans. **2014**. Honey bee colonies act as reservoirs for two *Spiroplasma* facultative symbionts and incur complex, multiyear infection dynamics. MicrobiologyOpen. 3: 341–355.

Simone-Finstrom, M., H. Li-Byarlay, M. H. Huang, M. K. Strand, O. Rueppell, and D. R. Tarpy. **2016**. Migratory management and environmental conditions affect lifespan and oxidative stress in honey bees. Sci. Rep. 6.

Smith, K. M., E. H. Loh, M. K. Rostal, C. M. Zambrana-Torrelio, L. Mendiola, and P. Daszak. **2013**. Pathogens, pests, and economics: drivers of honey bee colony declines and losses. EcoHealth. 10: 434–445.

Sumner, D., and H. Boriss. **2006**. Bee-conomics and the leap in pollination fees. Agric. Resour. Econ. Update. 9: 9–11.

Szalanski, A. L., A. D. Tripodi, and C. E. Trammel. **2014**. Molecular detection of *Nosema apis* and *N. ceranae* from southwestern and south central USA feral Africanized and European honey bees, *Apis mellifera* (Hymenoptera: Apidae). Fla. Entomol. 97: 585–589.

Szalanski, A. L., J. Whitaker, A. D. Tripodi, and P. Cappy. **2013**. Prevalence of *Nosema* from managed honey bee colonies in South Dakota and New York. J. Agric. Urban Entomol. 29: 99–104.

Szalanski, A., C. E. Trammel, A. D. Tripodi, D. Cleary, L. Rusert, and D. Downey. **2016**. Molecular diagnostics of the honey bee parasite *Lotmaria Passim*, and *Crithidia* spp. using multiplex PCR. Fla. Entomol.

Traynor, K. S., K. Rennich, E. Forsgren, R. Rose, J. Pettis, G. Kunkel, S. Madella, J. Evans, D. Lopez, and D. vanEngelsdorp. **2016**. Multiyear survey targeting disease incidence in US honey bees. Apidologie. 47: 325–347.

Uroš, G., S. Jevrosima, G. Bojan, S. Predrag, Đ. Spomenka, V. Branislav, and S. Zoran. **2014**. *Nosema ceranae* DNA in honey bee haemolymph and honey bee mite *Varroa destructor*. Acta Vet. (Beogr.). 64.

vanEngelsdorp, D., D. Caron, J. Hayes, R. Underwood, M. Henson, K. Rennich, A. Spleen, M. Andree, R. Snyder, K. Lee, K. Roccasecca, M. Wilson, J. Wilkes, E. Lengerich, and J. Pettis. **2012**. A national survey of managed honey bee 2010–11 winter colony losses in the USA: results from the Bee Informed Partnership. J. Apic. Res. 51: 115–124.

vanEngelsdorp, D., J. H. Jr, R. M. Underwood, and J. Pettis. **2008**. A survey of honey bee colony losses in the U.S., fall 2007 to spring 2008. PLOS ONE. 3: e4071.

vanEngelsdorp, D., and M. D. Meixner. **2010**. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. J. Invertebr. Pathol. 103: S80–S95.

Wardell, A., P. Bernhardt, R. Bitner, A. Burquez, S. Buchmann, J. Cane, P. A. Cox, V. Dalton, P. Feinsinger, M. Ingram, D. Inouye, C. E. Jones, K. Kennedy, P. Kevan, H. Koopowitz, R. Medellin, S. Medellin-Morales, and G. P. Nabhan. **1998**. The potential consequences of pollinator declines on the conservation of biodiversity and stability of food crop yields. Conserv. Biol. 12: 8–17.

Zhu, X., S. Zhou, and Z. Y. Huang. **2014**. Transportation and pollination service increase abundance and prevalence of *Nosema ceranae* in honey bees (*Apis mellifera*). J. Apic. Res. 53: 469–471.

Table 4.1. List of primers used in molecular detection of parasites and pathogens in honey bee samples. A: Garnery et al. 1993; B: Szalanski et al. 2014; C: Core et al. 2012; D: Schwarz et al. 2014; E: Schmid-Hempel and Tognazzo 2010; F: Szalanski et al. 2016.

Primer	Sequence	Reference
E2	F: 5'-GGCAGAATAAGTGCATTG-3'	A
H2	R: 5'-CAATATC ATTGATGACC-3'	A
NosemaSSU-1F	F: 5'-ACAATATGTATTAGATCTGATATA-3'	B
NosemaSSU-1R	R: 5'-TAATGATATGCTTAAG TTCAAAG-3'	B
Phorid-rRNA-1F	F: 5'-GTACACCTATACATTGGGTTCGTACATT AC-3'	C
Phorid-rRNA-1R	R: 5'-GAGRGCCATAAAAGTAGCTACACC-3'	C
S. apis ITS-F	F: 5'-AATGCCAGAAGCACGTATCC-3'	D
S.apis ITS-R	R: 5'-GAACGAGATATACTCATAAGCTGTTACAC-3'	D
Ms-160 F	F: 5'- TTGCA AAAGCTGTTTTAGATGC-3'	D
Ms-160-R	R: 5'- TGACCAGAAATGTTTGCTGAA-3'	D
CBSSU rRNA F2	F: 5'-CTTTTGACGAACAACTGCCCTATC-3'	E
CBSSU rRNA B4	R: 5'- AACCGAACGCACTAAACCCC-3'	E
L. passim18S-F	F: 5'-AGGGATATTTAAACCC ATCGAAAATCT-3'	F
C. mel 474-F	F: 5'-TTTACGCA TGTCATGCATGCCA-3'	F

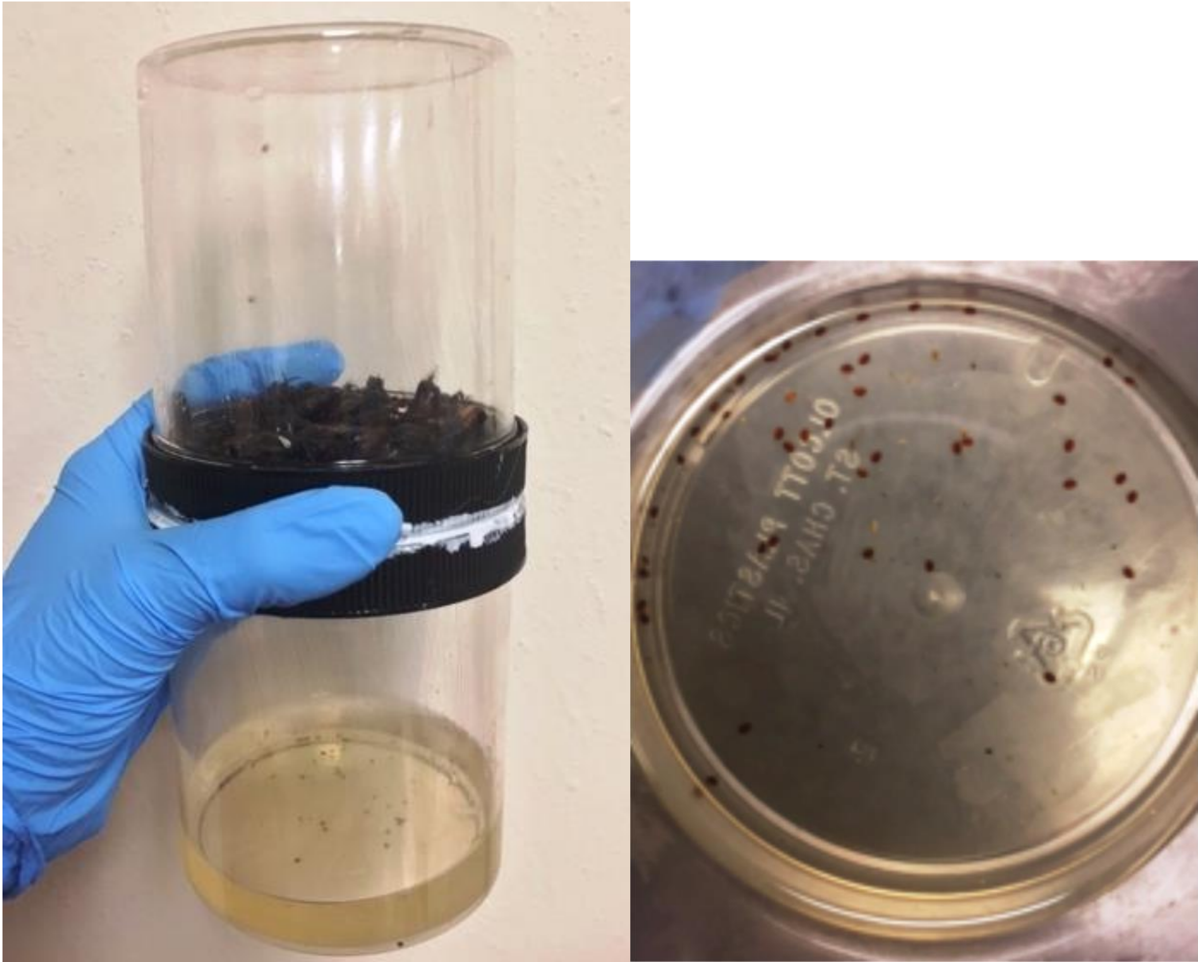

Figure 3.1. Mite wash adapted from Oliver (2013).

Table 4.2. Percent of honey bee samples positive for combinations of parasite and pathogen species occurrence.

	N. ceranae	*L. passim*	*S. melliferum*	*V. destructor*
N. ceranae		1.0%	0.6%*	4.9%
L. passim	1.0%		0.0%	3.0%**
S. melliferum	0.6%*	0.0%		0.5%
V. destructor	4.9%	3.0%**	0.5%	

***P-value=0.0087 **P-value=0.0775**

Table 4.3. Percent of parasite and pathogen species occurrence in colony samples by year.

Parasite/ Pathogen	**2015 Colony Samples (n=511)**	**2016 Colony Samples (n=117)**	**Total Colony Samples (n=628)**
Varroa destructor	40.3	47.0	41.6
Nosema ceranae	16.0	4.3	13.9
Lotmaria passim	10.0	9.4	9.9
Spiroplasma melliferum	1.4	0.0	1.1

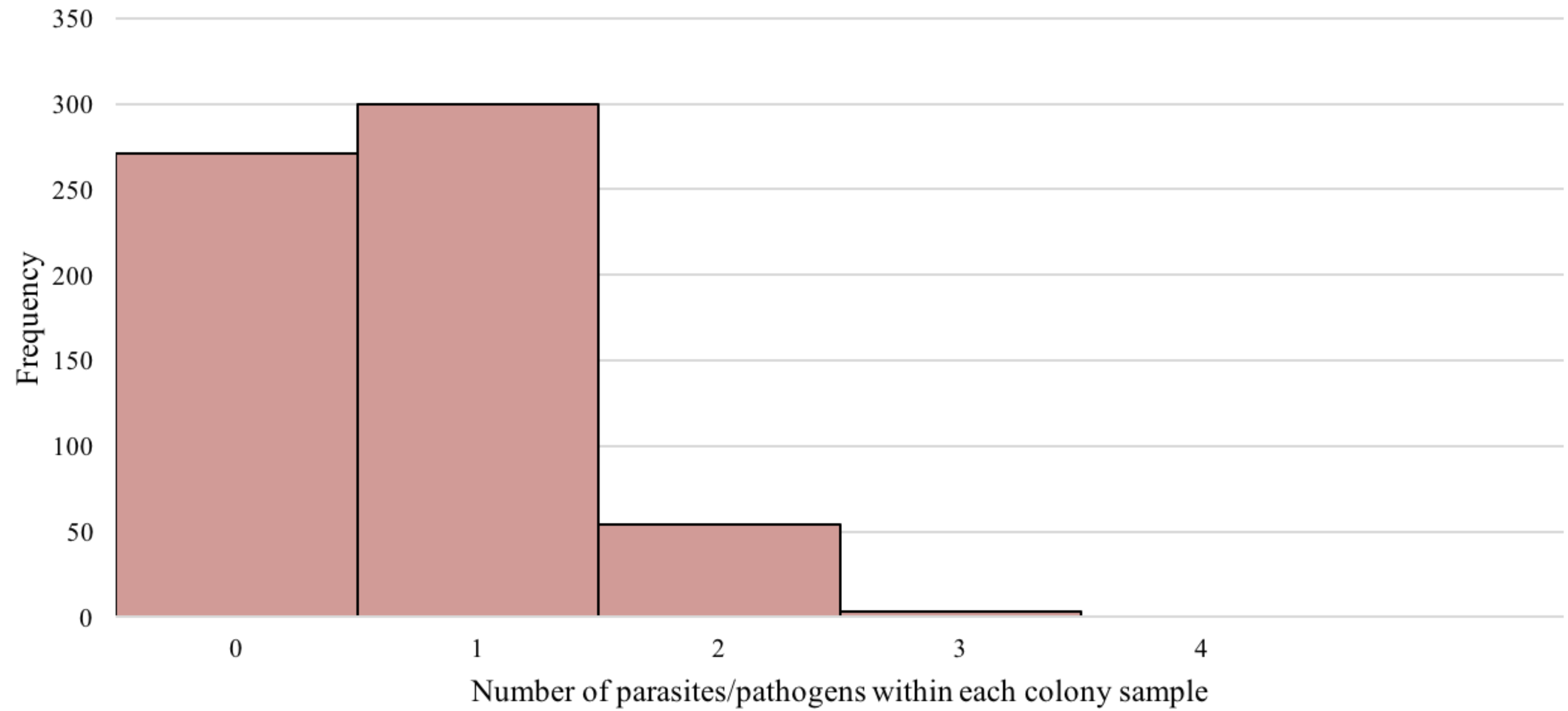

Figure 4.2. Frequency distribution of number of honey bee colony samples positive for 0, 1, 2, 3, or 4 of the tested parasite and pathogen species.

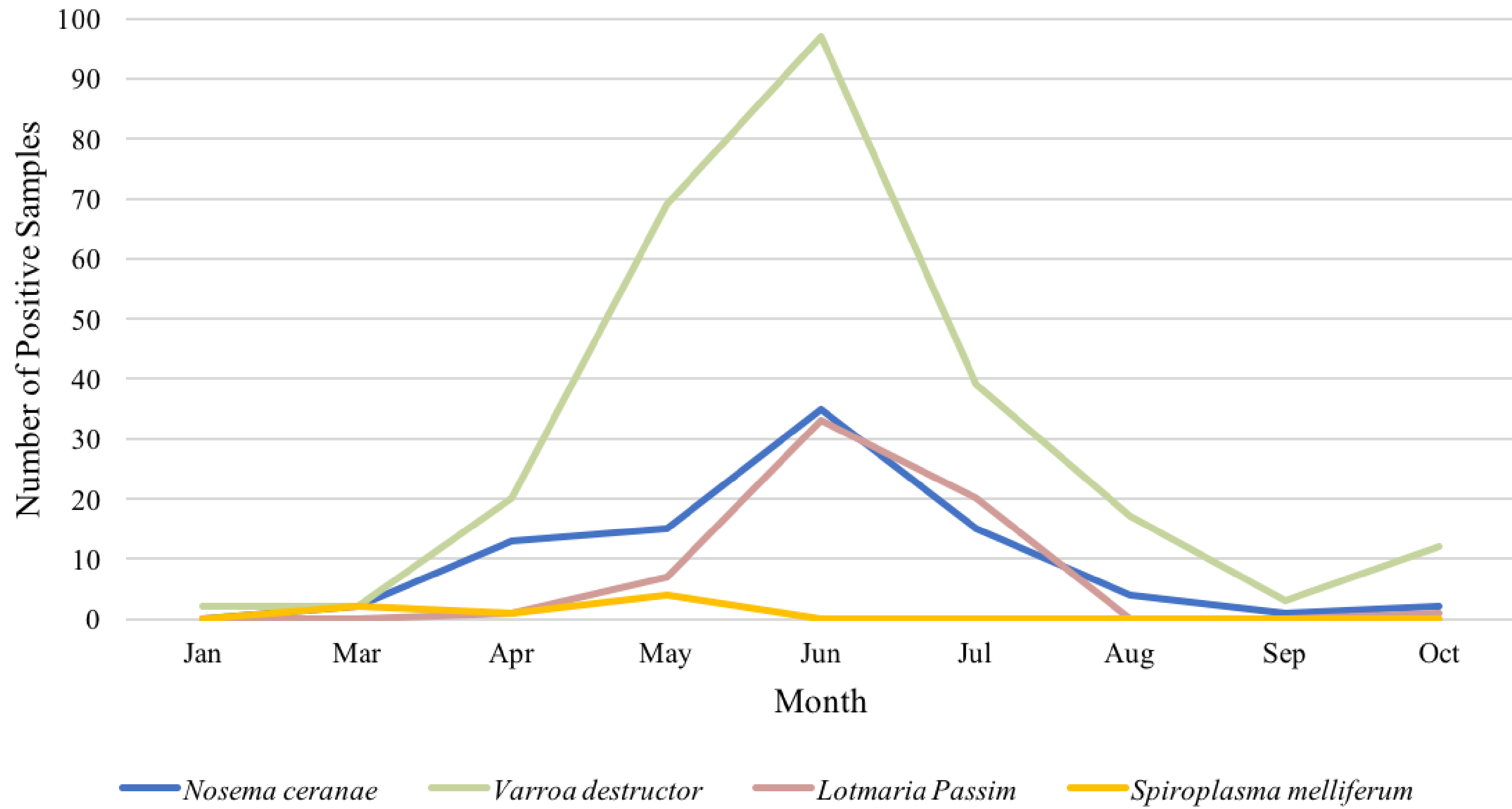

Figure 4.3. Number of colony samples positive for each parasite/pathogen in months sampled.

Table 4.3. Seasonal percentage of occurrence of parasites and pathogens in hobbyist and migratory honey bee colonies. Summer months include June, July, and August. Fall months include September, October, and November. Winter months include December, January, and February. Spring months include March, April, and May. Migratory samples were not collected in the summer, fall, or winter season.

	Summer	Fall	Winter	Spring	
	Hobby (n=378)	Hobby (n=33)	Hobby (n=2)	Hobby (n=128)	Migratory (n=87)
N. ceranae	14.3	9.1	0.0	4.7	27.6
V. destructor	40.5	45.5	100	59.4	17.2
L. passim	14.0	3.0	0.0	5.5	1.1
S. melliferum	0.0	0.0	0.0	0.0	8.0

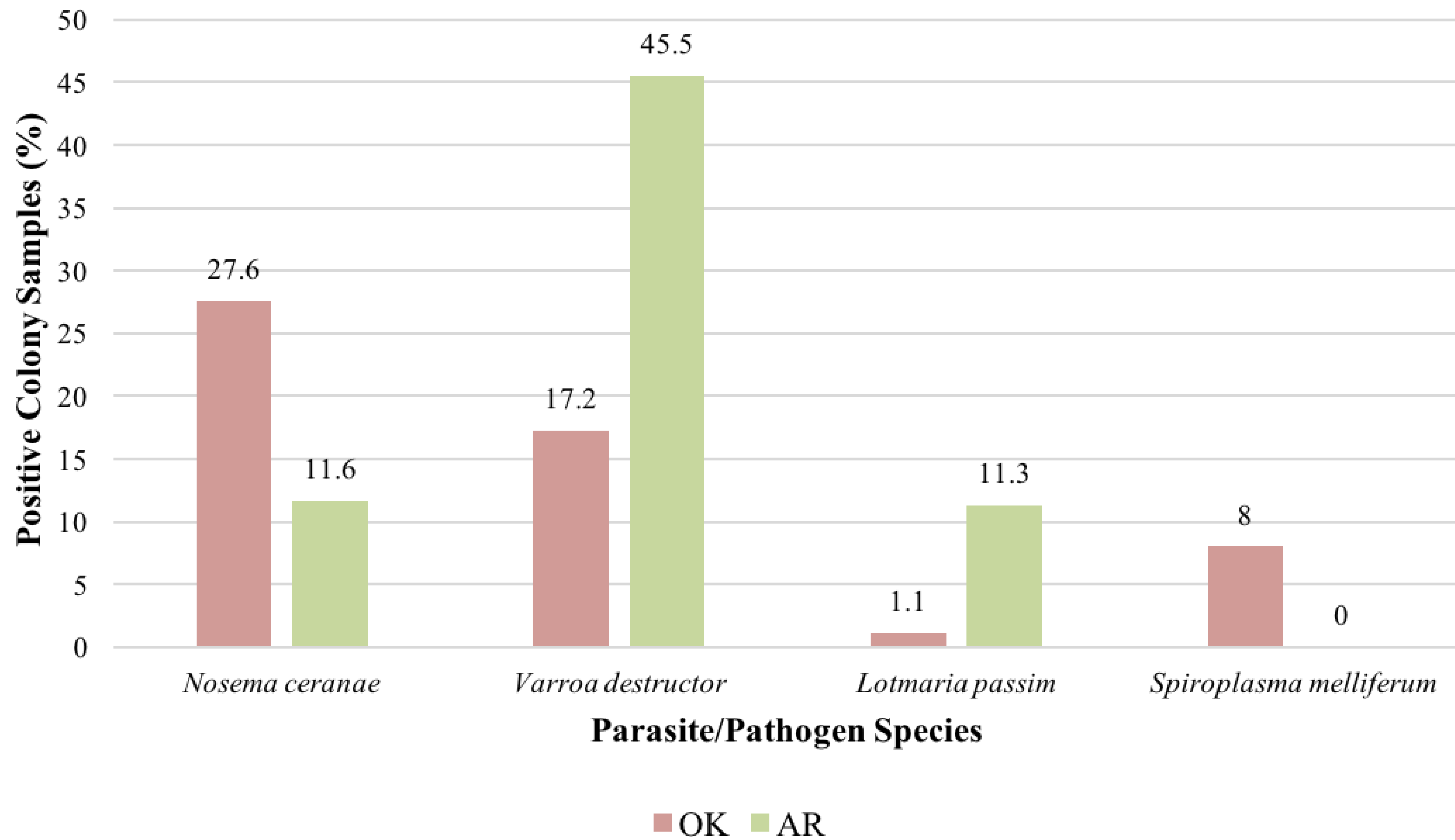

Figure 4.4. Comparison of percent occurrence of parasite and pathogen species in Arkansas hobbyist colonies and Oklahoma migratory colonies.

Acknowledgements

We would like to thank the Arkansas Plant Board for their assistance distributing collection kits as well as the numerous Arkansas and Oklahoma beekeepers for their cooperation and participation in this study. This research was made possible with the assistance of the USDA APHIS Cooperative Agreement Award No. 15-8100-1743-CA.

Chapter 5: Conclusion

Over the last decade the economically significant European honey bee, *Apis mellifera* L., has shown high levels of fluctuation worldwide. Multiple factors are implicated to be responsible for this, including migratory beekeeping practices, spread of parasites, pathogens, viruses, and diseases. In this research, molecular diagnostics were utilized to determine occurrence of widely occurring parasite and pathogen species, as well as, newly-identified species. The research completed in this thesis focus on pathogen and parasite detection among hobbyist and commercially managed honey bee colonies. While further research and monitoring is necessary in order to solidify the implications of this study, this research allows for insight into which parasites and pathogens are occurring in small and medium sized honey bee operations. The species screened for included *Varroa destructor* Anderson and Trueman, *Nosema apis* Zander, *N. ceranae* Fries, *Apocephalus borealis* Brues, *Crithidia mellificae* Langridge and McGhee, *Lotmaria passim* Schwarz, *Spiroplasma apis* Mouches, and *Spiroplasma melliferum* Clark.

This research determined that *N. ceranae, L. passim,* and *V. destructor* occur in both hobbyist and migratory managed colonies, with *S. melliferum* also being detected in the Oklahoma migratory colony samples. This research resulted in the first detection of *Lotmaria passim* in Arkansas honey bees, and the first documented detection of *L. passim* and *S. melliferum* in Oklahoma. The detection of these pest species may provide insight into how they are spreading and from where. Comparatively, while *A. borealis, C. mellificae, N. apis,* and *S. apis* were not detected, it is important to continue surveying and monitoring practices as these species do have the potential to spread. In the future, surveying at night with light traps, for foraging workers bees, may be a more productive sampling method for capturing honey bees infected with *A. borealis.*

Analysis of co-occurrence between the tested parasites and pathogens revealed that an association exists between *S. melliferum* and *N. ceranae,* as well as between *V. destructor* and *L. passim.* Future research should examine *V. destructor* as a vector of parasites and pathogens, specifically of *L. passim* due to this association.

While the honey bee and its importance in modern agriculture is often stressed, few surveys have been conducted targeting risk. This survey detected parasites and pathogens in both stationary and migratory colonies. *Apis mellifera's* significant contribution to pollinations services in agriculture alone make it among the most economically significant organisms worldwide. With continued fluctuations in *A. mellifera* populations, it is essential to continue surveying for these harmful parasites and pathogens in order to understand their spread and abundance. Future studies, could utilize morphological techniques to determine level of infection. Furthermore, laboratory studies could provide an understanding of how the trypanosome and *Spiroplasma* species spread, as this continues to be unknown. Moreover, honey bees serve as a good model for other pollinator species. This survey may provide relevant information to research pertaining to native pollinator pest distribution.

With continued declines in honey bee populations and reductions in honey bee health, it is important to continue monitoring for potential factors disrupting *A. mellifera.* Specifically, examining management practices combined with pest detection may aid in future management decisions.