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Molecular Survey for the Honey Bee Trypanosome Parasites Crithidia mellificae and Lotmaria passim

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Molecular Survey for the Honey Bee Trypanosome Parasites
Crithidia mellificae and *Lotmaria passim*

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Abstract

Honey bee populations have been fluctuating within recent years. No one cause has been attributed to colony fluctuations due to the theory that multiple stressors interact with one another to impact colony health. Consequently, microorganisms such as internal parasites of honey bees have been understudied as a contributor to colony health decline.

Molecular diagnostics were utilized to detect the presence of two honey bee trypanosome parasites, *Crithidia mellificae* and *Lotmaria passim*, in managed and feral honey bee populations from eight states in the United States (USA). Because studies on trypanosome infections are lacking in the USA, it is important to know how frequently honey bee colonies are infected and if management techniques are impacting colony susceptibility to trypanosome infections.

This is the first national survey for honey bee trypanosomes in the USA. This study confirmed that *L. passim* is present in the USA, but *C. mellificae* was not observed from the sampled colonies. From the 1,360 honey bee colonies that were screened, 11% were infected with *L. passim*. New York samples had the highest infection rate and Utah samples had the lowest. One state from the survey (Mississippi) did not have any samples positive for *L. passim*. The proportion of samples positive for *L. passim* was significantly different between managed and feral honey bee colonies. Results from this study revealed that *L. passim* has a widespread distribution in the USA and should be monitored as a contributor to honey bee health decline.

Subsequent analyses were performed on the data set to understand trypanosome infections between two honey bee subspecies, co-infection with a fungal pathogen, seasonality in the USA, and if queen breeding facilities are distributing trypanosomes in the USA.

This research demonstrates the importance of learning more about internal parasites because it is unknown to what extent internal parasites impact honey bee health. Therefore, it is

imperative to understand how internal parasites impact honey bees. Further research should be conducted to observe how trypanosomes are spread in the environment and what type of preventative measures should be taken to ensure colonies remain healthy.

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Dedication

To the family. Thank you for the unending support throughout the years and understanding my fascination with insects.

Table of Contents

List of Published Papers

Chapter 2:

Williams, M-K., Tripodi, A. D., and Szalanski, A. L. (2018). Molecular survey for the honey bee (*Apis mellifera* L.) trypanosome parasites *Crithidia mellificae* and *Lotmaria passim* in the United States. J. Api. Res. Submitted.

Chapter 1: Introduction

Background

Honey bees (*Apis mellifera* L.) are one of the most important pollinators in the United States (USA) (vanEngelsdorp et al. 2009, Runckel et al. 2011, Sandrock et al. 2014). Populations have been fluctuating in recent years; however, no one cause has been attributed to population reduction. Recent studies have determined that multiple stressors such as migratory and commercial beekeeping, microorganisms, and pests could influence their decline (vanEngelsdorp et al. 2009, Runckel et al. 2011, Simone-Finstrom et al. 2016). Many microorganisms such as internal parasites have been overlooked as a contributor to their decline. Two trypanosome species, *Crithidia mellificae* Langridge and McGhee, and *Lotmaria passim* Schwarz, have been understudied in honey bee populations and could be attributed to population disease dynamics.

Honey bees in the United States

Honey bees have a natural range stretching from northern Europe to southern Africa, Scandinavia to central Asia, including western Iran and the Arabian Peninsula (Schneider et al. 2004, Le Conte & Navajas 2008, vanEngelsdorp & Meixner 2010). The origin and evolutionary history of honey bees can be described as a specialized divergence from wasps. As described by Winston (1987), "bees are wasps that 'abandoned' predation" to facilitate nests with brood, nectar, and pollen. After bees left a life of predation their mouthparts were modified to lap up nectar and their hind legs developed plumose hairs that allowed them to collect pollen in order to feed brood (Winston 1987).

Honey bees are not native to the Western Hemisphere (Martin et al. 1980). Some of the earliest records state that honey bees were shipped from England to the Colony of Virginia in 1622 and other accounts refer to their in Massachusetts between 1630 to 1633 (Martin et al. 1980). Starting in 1800, honey bees were imported by boats coming in through the Atlantic Ocean and distributed along the Mississippi River (Martin et al. 1980). Russians were reported to have brought bees to Alaska in 1809 and California in 1830 (Martin et al. 1980). Although there are no accounts for how honey bees naturally spread west of the Mississippi River, swarms from California hives or hives transported by settlers may have aided honey bee expansion into Oregon and Washington (Martin et al. 1980).

Once California became the almond orchard hub of the United States (USA), the Almond Board of California was developed in the 1950s to promote relationships between almond orchard owners and beekeepers (Horn 2005). Almond orchards provide commercial hives with nectar and pollen sources that allow commercial beekeepers to build up colonies and increase honey production for successful overwintering colonies (Horn 2005). Once the establishment of commercial pollination took flight, other states followed suit with blueberry orchards in Maine, apples in Washington, cherries in Michigan, cucumbers in Ohio, and Madrid sweet clover in Texas (Horn 2005). Although the pact between almond pollination and commercial beekeepers is important to USA almond production, Sumner and Boriss (2006) state that almond orchards do not provide nutritional nectar for commercial honey bees.

Dependence on a single species for pollination could eventually collapse ecosystems (Potts et al. 2010). It is risky to rely on a single managed species for pollination services since managed North American honey bee stocks have decreased 60% since the 1940s, and today the number of managed colonies are less than half of the original registered number of colonies

(Oldroyd 2007, Aizen et al. 2009, Ellis et al. 2010). However, managed honey bee colonies have increased by 45% worldwide since 1961, and dependence on honey bees for pollination services in agricultural crops has increased by more than 300% (Aizen & Harder 2009). Because bee pollination is responsible for up to 75% of crops used for human food, if pollinators were to disappear crop production would dramatically decrease (Klein et al. 2007, Potts et al. 2010). Pollinator loss in agricultural crops roughly translates to a 12% decrease in fruit production and a 6% decrease for vegetables (Potts et al. 2010). Without the use of honey bees the diversity of food crop production would decrease significantly.

Economic importance of honey bees to the United States

Honey bees are one of the most important contributors to modern agriculture in the USA (vanEngelsdorp & Meixner 2010). The European honey bee (*Apis mellifera ligustica* Spinola, EHB) is responsible for pollinating $215 billion worth of crops worldwide (vanEngelsdorp et al. 2008, Gallai et al. 2009, Smith et al. 2014). Honey bees pollinate numerous crops in the USA that include vegetables, fruits, clover, oilseed, alfalfa seed, nuts, flower seeds, and contribute to seed production for soybeans, hay, and foraging crops (McGregor 1976, Southwick & Southwick 1992). Without the assistance of honey bees, fruit, seed, and nut crop yields would decrease by more than 90% (Southwick & Southwick 1992).

Of the 300 commercially available crops 84% are pollinated by insects and one-third of global food production results from insect pollination (Allsopp et al. 2008). Managed honey bee productions in the USA are valued between $1.6 billion and $14.6 billion (Allsopp et al. 2008). In 2012, Calderone analyzed data gathered over a ten year span (1999 to 2009) to estimate crop production and its relationship with sale prices to evaluate how much pollination was worth in

the USA. By 2009, crops that were directly dependent on honey bees and non-*Apis* pollinators reached a net worth of $11.68 billion and $3.44 billion respectively (Calderone 2012).

Aizen et al. (2009) analyzed 46 years (1961 to 2006) worth of data from the Food and Agriculture Organization of the United Kingdom to quantify the effect of total loss of pollinators on global agricultural and crop production diversity. After their analysis, Aizen et al. (2009) determined that in the absence of pollinators at least 3% to 8% of total agricultural production can be expected (vanEngelsdorp & Meixner 2010). When the demand for agricultural land increases, pollinator shortage will also increase because the pressure on supply of agricultural land contributes to the global environmental change that ultimately affects pollinators (Aizen et al. 2009).

In 1996, insect pollination was valued at $20 billion, but by 2005 the exchange rate of insect pollination was down to $8 billion (Gallai et al. 2009). According to Martin (1975), the value of beef and dairy products is a direct result from seed production (forage legumes, alfalfa) that accounts for 80% of the economic value of insect pollinators (Gallai et al 2009). Honey bees bring in an estimated $40 billion per year valued for pollinating legumes that are in turn fed to cattle (Morse & Calderone 2000, Aizen & Harder 2009).

Probable reasons for honey bee population decline

The most important factors for pollinator declines are urbanization, habitat loss and fragmentation, increasing pesticide application and environmental pollution, climate change, migratory and commercial beekeeping, and spread of pests and pathogens (Sumner & Boriss 2006, Potts et al. 2010, Krupke et al. 2012, Oleksa et al. 2013, Sandrock et al. 2014). Pathogens are known to facilitate transmission by manipulating host behavior in order to increase the

chance of transmission to uninfected colonies (Forfert et al. 2015). Agricultural intensification, increase in monoculture acreage, and overwintering colony losses also contribute to the decline of honey bee colonies in the USA (vanEngelsdorp et al. 2008, vanEngelsdorp & Meixner 2010, Spleen et al. 2013, Smith et al. 2014). Honey bees need high-quality pollen sources in the fall in order to produce long-lived bees that can survive harsh winters (Oldroyd 2007).

One contributing factor to the decreasing amount of honey bee colonies is urbanization. After World War II farmlands once used for agricultural purposes were often turned into housing development areas thus limiting the available resources and ecology for honey bees (Horn 2005). Urbanization is the process of converting natural and agricultural land into suburbs and cities (Appler et al. 2015). Urbanization reduces the area and connectivity of floral resources which in turn forces bees to forage further away from the hive, increases foraging costs, and can reduce energy invested in immune functions therefore reducing honey bee production nationwide (Oldroyd 2007, Appler et al. 2015). Social and economic pressures to produce more food on fewer acres and the need to accommodate a massive number of people is a direct result of urbanization, which attributes to the loss of managed honey bee colonies (Martin et al. 1980). Pathogen pressure has been shown to increase with urbanization and environmental management resulting in a three-fold decline of worker survival (Youngsteadt et al. 2015).

Pesticide exposure has been shown to produce sublethal effects on honey bees (Sandrock et al. 2014, Smith et al. 2014). In recent years the application of pesticides and insecticides has had a direct correlation with honey bee colony deaths (Southwick & Southwick 1992, Sandrock et al. 2014, Smith et al. 2014). Desneux et al. (2007) found that sublethal and low-level pesticide exposure can impair immune system functioning, learning ability, memory, foraging behavior, and odor discrimination of honey bee workers. Wu et al. (2011) reports that pesticide residue

found on contaminated honey bee comb can delay or prolong larval development and adult emergence, shorten adult longevity, have an indirect effect on the colony such as premature shifts in hive roles and foraging activities, and increases reproductive advantages for *Varroa* mites (*Varroa destructor* Anderson and Trueman). Managed honey bee colonies have been documented to be more vulnerable to *Nosema* Nägeli species infection when colonies have residual pesticides lingering within the hive (Wu et al. 2012, Pettis et al. 2013). Because honey bees are being inadvertently hurt by pesticide applications, commercial beekeepers have to keep larger numbers of colonies at different locations in order to make up for pesticide-induced losses (Martin et al. 1980). Application methods for insecticides that are hazardous to bees in decreasing order include dust, wettable powder, flowable, emulsifiable concentrate, soluble powder or liquid solution, and granular formulations (Martin et al. 1980).

Climate change can affect honey bee populations and disease dynamics. It has been previously mentioned that EHB are found almost everywhere in the world and are great at adapting to highly diverse climates (Le Conte & Navajas 2008). Climate change can influence flower development, pollen production, and colony foraging activity (Le Conte & Navajas 2008). Drier climates can reduce nectar production for honey bees to harvest and cause Africanized honey bees (*Apis mellifera scutellata* Lepeletier, AHB) to expand their current distribution range (Le Conte & Navajas 2008).

Change in climate can expand a honey bee's ability to migrate farther and transfer pathogens or pests to uninfected environments (Le Conte & Navajas 2008). Climate change can lead to movement of different honey bee species and races resulting in contact with pathogens that they never co-evolved with (Le Conte & Navajas 2008). Increasing global temperatures can induce warmer winters that may allow honey bees to adapt toward a continual brood cycle and

potentially increase the number of susceptible hosts for tracheal mites (*Acarapis woodi* (Rennie)) (Le Conte & Navajas 2008). Increased temperature and humidity have been documented to increase *Varroa* mite population growth (Harris et al. 2003). Prolonged summer droughts and persistent rainfall are blamed for poor overwintering colonies in the northeastern USA where the fall will provide less than the usual productive amounts of pollen and nectar for honey bees (vanEngelsdorp & Meixner 2010).

Commercial honey bee hives are known to negatively impact honey bee healthy by distributing pathogens to uninfected areas (Klee et al. 2007). Rucker et al. (2001) reports that commercial beekeepers will travel between 59,545 kilometers and 64,373 kilometers per year to pollinate four or more different crops around the USA. In large commercial beekeeping industries tractor-trailer combinations can transport 400 to 500 bee hives at a time during nighttime hours (Rucker et al. 2012). Colla et al. (2006) has also shown that commercially produced bumble bees used in greenhouse pollination tend to have higher levels of pathogens than wild bumble bees, and pathogens can spread to wild bumble bee populations if greenhouse bumble bees forage outside of their intended pollination areas.

A study performed with Montana commercial beekeepers moving their hives to California for almond pollination each year discovered that pathogen prevalence and abundance was higher immediately following almond pollination (Cavigli et al. 2016). This suggests that commercial beekeeping allows migratory hives to acquire new pathogens and viruses that would otherwise be avoided if hives were stationary year-round or only allowed to be used for pollination during certain times of the year. Although it would make sense that managed and commercial colonies have higher pathogen loads than feral colonies, a study performed by Thompson et al. (2014) determined that feral colonies had a higher level of deformed wing virus

than managed colonies, supporting the idea that feral colonies could be potential pathogen reservoirs for uninfected honey bee colonies.

Trypanosomes

Trypanosomes are protozoan parasites that infect several organisms. Trypanosomes that infect insects are found in the family Trypanosomatidae (Podlipaev 2001). Some trypanosome species are obligate parasites that require two hosts to complete their life cycle (dixenous) or one host to complete their life cycle (monoxenous) (Simpson et al. 2006). Those that infect honey bees were first recorded in 1912 and require one host to complete their life cycle (Langridge & McGhee 1967, Ravoet et al. 2015, Schwarz et al. 2015).

Crithidia mellificae was described 51 years ago in 1967 from Australian apiaries. It is an acidophilic trypanosome parasite that infects the rectum of honey bees occurring freely inside the rectum or attached to rectal walls (Langridge & McGhee 1967, Schwarz et al. 2015). Langridge and McGhee (1967) described *C. mellificae* as having an ovoid, truncated anterior, a posterior end that gradually tapers to a slender point, a single kinetoplast located close to the anterior end lateral to the sub-central nucleus, lack of an undulating membrane, and a flagellum that emerges from the posterior end. Molecular identification for *C. mellificae* is determined by using two PCR protocols that target cytochrome b (Cytb) and the sequencing of the small subunit ribosomal RNA (SSU rRNA) (Cersini et al. 2015). Little is known about *C. mellificae*, even though the congener *Crithidia bombi* Lipa and Triggiani, negatively affects bumble bee (*Bombus* spp.) health (Brown et al. 2003, Otterstatter et al. 2005).

A recently described internal trypanosome parasite, *Lotmaria passim* Schwarz, has been documented to be present in honey bee colonies worldwide (Schwarz et al. 2015). *Lotmaria*

passim was described in 2015 by Schwarz et al. (2015) in a molecular study to determine the distinct genetic identity of motile *C. mellificae*. The genus is named after Ruth Lotmar, a microbiologist who produced reports of trypanosomatids in Hymenoptera species during the 20th century (Schwarz et al. 2015). The species name is derived from the Latin word "passim" meaning everywhere in reference to being the dominant trypanosome found in honey bee colonies globally (Schwarz et al. 2015). Schwarz describes *L. passim* promastigotes being tear-drop shaped and containing a single flagellum that lacks a membrane attached to a broad, rounded anterior end and much like *C. mellificae, L. passim* is also acidophilic (Schwarz et al. 2015). *Lotmaria passim* is the predominant honey bee trypanosome in Belgium, Switzerland, and Japan (Morimoto et al. 2013, Ravoet et al. 2015), and was found in 60% to 90% of honey bees from Chile and Serbia (Arismendi et al. 2016, Stevanovic et al. 2016).

The rectum of honey bees is a large thin-walled sac at the posterior end of the abdomen (Snodgrass 1956). The rectum holds solid waste consisting primarily of pollen husks, fat globules, and dead midgut cells (Winston 1987). The rectal sac can hold an immense accumulation of fecal matter and excretion from the Malpighian tubules (Snodgrass 1956). Because honey bees never defecate inside the hive the rectal sac must be large enough to contain feces during winter months occupying any available space inside the abdomen (Snodgrass 1956, Winston 1987). This could explain why *C. mellificae* and *L. passim* are present in hives that are lost over the winter season since both trypanosomes are found inside the rectum of honey bees. Tozkar et al. (2015) found that migratory hives had higher trypanosome abundance than stationary hives, and that urban environments may be linked to impaired honey bee health. Prevalence and persistence of *C. mellificae* and *L. passim* in the USA is unknown among feral and managed colonies, although the presence of *C. mellificae* in USA commercial beekeeping

operations was determined to occur between Mississippi, California, and South Dakota, and has been isolated from a honey bee colony in San Francisco (Runckel et al. 2011).

Feral and managed honey bee colonies

Managed colonies are honey bee colonies that reside in manmade hives maintained by beekeepers and feral colonies are unmanaged hives that reside in trees, buildings, or other cavities (Schiff et al. 1994). Managed honey bee colonies have increased about 45% during the last half century suggesting that economic globalization can drive dynamics of global managed honey bee populations and the increasing demand for agricultural pollination services (Aizen & Harder 2009, Smith et al. 2014). The increase in managed honey bee colonies can be explained by the growth of California's almond industry and an increase in the USA population that corresponds with a 10% increase of food production (Morse & Calderone 2000).

Global honey bee populations are increasing, but not enough to keep pace with pollination demands (vanEngelsdorp & Meixner 2010). Although the global honey bee populations are increasing, EHB populations in Europe and North America are declining at an alarming rate (vanEngelsdorp & Meixner 2010). Managed honey bee populations are influenced by factors such as disease, parasites, pesticides, environment, and socio-economic factors (vanEngelsdorp & Meixner 2010). No one factor can account for all managed honey bee losses over a given period of time because factors are known to influence one another and act together to impact honey bee health (vanEngelsdorp & Meixner 2010).

Difference in honey bee colony health has been poorly understood between feral and managed colonies until recently. Feral bees have been shown to express immune genes two times more than managed bees following immune challenges provided by Youngsteadt et al. (2015).

Youngsteadt et al. (2015) was able to demonstrate that feral colonies had a lower disease burden and stronger immune response than managed colonies, which could influence and shape the future of management strategies for beekeepers. López-Uribe et al. (2017) has also shown that feral honey bees have higher levels of immunecompetence when compared to managed honey bee colonies in North Carolina due to small but significant genetic differences between feral and managed colonies. López-Uribe et al.'s (2017) findings suggest that higher genetic diversity is positively associated with immunocompetence in feral honey bee populations and not managed populations.

The high genetic diversity found in feral honey bees could allow breeders a useful source of genetic variation to improve honey bee health in packaged bee programs (López-Uribe et al. 2017). Most beekeepers become interested in feral honey bee colonies for breeding programs in order to create some sort of "survivor stock" (Magnus et al. 2014). Because feral honey bee colonies have not been manipulated by beekeepers, it is assumed that they have adapted to resist pests, pathogens, and diseases better than managed honey bee colonies (Magnus et al. 2014).

Molecular genetics applied to honey bees

Multiplex-polymerase chain reaction (PCR) is used to amplify multiple sequences of DNA with primer sets that can target unique regions of DNA under a single set of reaction conditions (Markoulatos et al. 2002). Two or more target sequences can be amplified at once by using more than one pair of primers in the same reaction (Markoulatos et al. 2002). Successful multiplex-PCR assays include relative concentration of primers, concentration of PCR buffer, a balance between magnesium chloride and deoxynucleotide concentrations, and cycling temperatures (Markoulatos et al. 2002). Factors such as false amplification products, uneven or

no amplification of target sequences, and difficulties in reproducing results can influence multiplex-PCR reactions (Markoulatos et al. 2002). Multiplex-PCR is very helpful in gene deletion analyses, mutation and polymorphism analysis, quantitative analysis, reserve-transcription (RT)-PCR, and in the study of infectious diseases (identification of viruses, bacteria, and parasites) (Markoulatos et al. 2002). Using more than one primer pair can increase the chance of obtaining false-positive amplification products (primer dimers) (Markoulatos et al. 2002). Primer dimers are formed when the ratio of primer-to-template is too high under a very dilute template or excess primer conditions (Markoulatos et al. 2002).

Molecular genetics can also aid in determining the mitochondrial DNA diversity of honey bees in the USA. In a study performed by Magnus et al. (2014) unmanaged colonies and swarms of honey bees were analyzed using PCR techniques to determine the presence or origin of feral honey bee colonies. To do this, Magnus et al. (2014) sequenced honey bee mitochondrial DNA (mtDNA) using the COI-COII region, which allowed them to determine the lineages of imported honey bee populations. Their results suggest that three of the four lineages that were originally imported into the USA were present in unmanaged colonies (Magnus et al. 2014). This suggests that lineage haplotypes have existed outside of managed honey bee populations for quite some time (Magnus et al. 2014). It has been shown that higher genetic diversity within colonies can prevent infections better than colonies with a small amount of genetic diversity (Tarpy 2002). Tarpy's (2002) analysis states that multiple queen matings can increase genetic diversity thus reducing colony inter-relatedness which can in turn reduce disease prevalence within the hive. However, Tarpy's (2002) conclusion states that genetic diversity alone relieves severe infections, but consistent hygienic behavior is associated with greater disease recovery in the hive. Naug and Camazine (2002) believe that social organisms are more vulnerable to pathogens due to the

homogeneity of the colony, which means colonies that are closely related genetically may be unable to fight off pathogen infections adequately compared to colonies that are more genetically diverse.

Research objectives

1. Conduct a molecular diagnostic survey for *Crithidia mellificae* and *Lotmaria passim* from feral and managed honey bee populations collected from Arkansas, Hawaii, Mississippi, New Mexico, New York, Oklahoma, Texas, and Utah to determine the prevalence of honey bee trypanosomes in the United States.

2. Compare Africanized honey bee and European honey bee samples to determine if there is a difference in trypanosome infection rates between subspecies.

3. Analyze the co-occurrence rates of *Nosema* and trypanosomes from honey bee colonies from previous studies done by the Insect Genetics Laboratory.

4. Determine the temporal occurrence and seasonality of honey bee trypanosomes in the United States.

5. Screen queen breeder samples for trypanosomes to determine if packaged bees can spread trypanosomes across the United States.

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Chapter 2: Molecular survey for the honey bee (*Apis mellifera* L.) trypanosome parasites *Crithidia mellificae* and *Lotmaria passim* in the United States

Abstract

Honey bee populations in the United States have been fluctuating in recent years. Although no one cause has been attributed to this, recent studies have shown that multiple interactions among mircoorganisms may contribute to their decline. Several honey bee internal parasites have been overlooked as probable causes to decline; these include two different species of trypanosomes *Crithidia mellificae* and *Lotmaria passim*. Both parasites have been understudied in honey bee populations and could contribute to population disease dynamics. This study explored a molecular diagnostic survey for *C. mellificae* and *L. passim* using multiplex-PCR. Honey bee samples from both feral and managed populations were collected from eight states. This is the first national honey bee trypanosome survey in the United States. A total of 1,360 samples were surveyed during this study. Of the 1,360 samples screened, 11% were positive for *L. passim*; while no cases of *C. mellificae* were detected using multiplex-PCR analysis. Infection rates of states positive for *L. passim* ranged from 17% (New York) to 0.70% (Utah). Only one state (Mississippi) was not positive for *L. passim*. The proportion of positive *L. passim* samples was significantly different between managed and feral honey bee colonies. Results revealed that the honey bee parasitic trypanosome *L. passim* has a widespread distribution in the United States and should be considered as a potential contributor to honey bee health decline.

Introduction

Trypanosomes have been documented to negatively affect Hymenoptera species by impacting behavior, physiology, fitness, and the immune system making honey bee trypanosomatids imperative to study (Arismendi et al. 2016). Trypanosomes are protozoan parasites that infect numerous organisms, with trypanosomes that infect insects belonging to the family Trypanosomatidae (Podlipaev 2001). Some trypanosome species are obligate parasites that require two hosts to complete their life cycle (dixenous) or one host to complete their life cycle (monoxenous) (Simpson et al. 2006). Those that infect Apidae were first recorded in 1912 and are monoxenous (Langridge & McGhee 1967, Ravoet et al. 2015, Schwarz et al. 2015). Although honey bees are important and well-studied organisms, surprisingly little is known about trypanosomatid diseases in honey bees.

Crithidia bombi Lipa and Triggiani is an intestinal protozoan that occurs in bumble bee populations (Otterstatter et al. 2005). It has been determined that worker bumble bees infected with *C. bombi* have impaired foraging rates and bumble bee queen fitness can be reduced up to 40% (Brown et al. 2003, Otterstatter et al. 2005). Bumble bees can contract *C. bombi* vertically from natal nests or horizontally, acquiring infections while foraging on contaminated flowers (Durrer and Schmid-Hempel 1994). According to Popp et al. (2012), the greatest infection of *C. bombi* in bumble bees occurs in the middle of the foraging season. Tripodi et al. (2018) designed species specific primers to distinguish between *C. bombi* and the recently described *C. expoeki* Schmid-Hempel and Tognazzo due to the morphological similarities of each species' life cycle. Without molecular diagnostics, *Crithidia* species identification would be unreliable using only morphological characteristics, and it is likely that studies prior to the description of *C. expoeki* have conflated the two organisms (Schmid-Hempel & Tognazzo 2010).

Crithidia mellificae has been shown to negatively impact honey bee health (Ravoet et al. 2013). Along with infecting honey bees, *C. mellificae* has been detected in mason bees (*Osmia bicornis* (Linnaeus) and *O. cornuta* Latreille) and yellowjackets (*Vespula squamosa* (Drury)) (Ravoet et al. 2015, Schwarz et al. 2015). Bumble bees have also been documented to carry *C. mellificae* as demonstrated by a molecular survey conducted by Tripodi et al. (2018) and Bartolomé et al. (2018), although other experiments suggest that *C. mellificae* cannot infect bumble bees (Ruiz-Gonzalez & Brown 2006). As determined by Runckel et al. (2011), seasonal occurrence of *C. mellificae* is lighter in the spring and peaks in January. Winter mortality for honey bees in Belgium was associated with the presence of *C. mellificae*, *Nosema ceranae* (Fries), and *Varroa* mites (*Varroa destructor* Anderson and Trueman) (Ravoet et al. 2013). Infection of trypanosomes also seems to increase when the number of individuals in a population is low (Cepero et al. 2016). However, studies prior to 2015 may have mistaken the trypanosomatid species with a close relative, *Lotmaria passim*.

Lotmaria passim Schwarz was described in 2015 by Schwarz et al. (2015) in a molecular study to determine the distinct genetic identity of motile *C. mellificae*. Schwarz describes *L. passim* promastigotes being tear-drop shaped and containing a single flagellum that lacks a membrane attached to a broad, rounded anterior end (Schwarz et al. 2015). *Lotmaria passim* is the predominant honey bee trypanosome in Belgium, Switzerland, Japan, Chile, and Serbia (Morimoto et al. 2013, Ravoet et al. 2015, Arismendi et al. 2016, Stevanovic et al. 2016), and was found in 40% to 90% of honey bee colonies from a regional Chilean study (Arismendi et al. 2016). Seasonal variation of *L. passim* shows increased infection rates in the spring, and like *C. mellificae*, *L. passim* is known to have a positive correlation with *N. ceranae* infection rates (Tozkar et al. 2015, Tritschler et al. 2016). *Lotmaria passim* has also been documented to be

found in bumble bee populations in the United States (Tripodi et al. 2018) and Spain (Bartolomé et al. 2018).

However, it is often problematic to identify trypanosomatid species with morphological characteristics alone. Before molecular techniques were developed, taxonomic diversity was determined by microbial characteristics such as when Langridge and McGhee (1967) described *C. mellificae*. This species was first described by the morphology of flagellates that included characteristics such as kinetoplast placement, lack of an undulating membrane, and the shape of both anterior and posterior ends. Molecular diagnostic methods are now the most reliable way to identify trypanosomatid species infecting honey bee populations (Ravoet et al. 2015, Schwarz et al. 2015). Although Langridge and McGhee's description of *C. mellificae* has been the foundation for identifying honey bee trypanosomatid species, their taxonomic systematics only consisted of using morphological features to describe the species (Morimoto et al. 2013). With the development of molecular diagnostics it has been determined that previous cases positive with *C. mellificae* have been potentially taxonomically misidentified due to the multiple polymorphic identities of trypanasomatid species (Schwarz et al. 2015, Szalanski et al. 2016a). According to Arismendi et al. (2016), the main difference between *C. mellificae* and *L. passim* is a fragment length polymorphism in the ribosomal DNA first internal transcribed spacer (ITS1) region that is amplified using primers that identify trypanosome species in mammals. However, Ravoet et al. (2015) determined that amplification success of ITS1 and ITS1-2 markers is dependent upon the level of trypanosome infection. Additionally, the ITS1 fragment lengths of *L. passim* and *C. bombi* overlap and cannot be distinguished without sequencing (Tripodi et al. 2018).

Prevalence and persistence of *C. mellificae* and *L. passim* in the USA is largely unknown among feral and managed honey bee colonies. Youngsteadt et al. (2015) was able to show that feral colonies had a lower overall disease burden and stronger immune response than managed colonies, and express immune genes two times more than managed honey bees. López-Uribe et al. (2017) found that feral honey bees have higher levels of immunecompetence when compared to managed honey bee colonies in North Carolina due to small but significant genetic differences between feral and managed colonies. Because feral honey bee colonies have not been manipulated by beekeepers, it is assumed that they have adapted to resist pests, pathogens, and diseases better than managed honey bee colonies (Magnus et al. 2014, Appler et al. 2015).

The objective of this study was to conduct a molecular diagnostic survey for the occurrence of *Crithidia mellificae* and *Lotmaria passim* from feral and managed honey bee populations from eight states in the United States: Arkansas; Hawaii; Mississippi; New Mexico; New York; Oklahoma; Texas; and Utah to determine the presence of honey bee trypanosomes in the United States and if management practices influence the presence of parasites in honey bee populations.

Materials and methods

Honey bee samples were collected from both managed and feral honey bee colonies. Managed colonies were defined as honey bee colonies that reside in manmade hives maintained by beekeepers and feral colonies are unmanaged hives that reside in trees, buildings, or other cavities (Schiff et al. 1994). These were obtained from various beekeepers, state agencies and our own collection efforts from 2004 to 2015, preserved in 70 to 90% ethanol, and vouchers are maintained at the Insect Genetics Laboratory, University of Arkansas, Fayetteville, AR, USA. A

total of 1,360 samples were used from eight states (Arkansas: n = 124; Hawaii: n = 346; Mississippi: n = 29; New Mexico: n = 58; New York: n = 350; Oklahoma: n = 173; Texas: n = 131; and Utah: n = 149).

DNA was extracted from worker honey bees collected from individual honey bee colonies using a salting-out procedure with in-house ingredients (Sambrook & Russell 2001). Extracted DNA was resuspended in 50µL Tris and maintained at -20°C until PCR. To confirm that DNA was successfully isolated from the sample, PCR was first done with a primer set to confirm that honey bee DNA is present. This was done using 2µL of extracted DNA with PCR conditions following Szalanski et al. (2016b) and PCR primers E2 (5'-GGCAGAATAAGTGCATTG-3') and H2 (5'-CAATATCATTGATGACC-3') (Garnery et al. 1992). The thermocycler conditions (C1000 Touch Thermocycler, BioRad Labs Inc., Hercules, CA) used are as followed: denature for 2 minutes at 94°C then 39 cycles at 94°C for 45 seconds, 46°C for 1 minute, 72°C for 1 minute, and final extension of 72°C for 5 minutes. PCR products were subjected to electrophoresis on a 2% agarose gel (Owl Separation Systems LLC), stained with ethidium bromide and visualized with UV light (BioDocit™ System, UVP, LLC, Upland, CA) to confirm successful amplification. Positive samples and a negative control were included. Primers E2 and H2 amplify the COI-COII region of honey bee mitochondrial DNA between 530bp to 1230bp (Garnery et al. 1992, Szalanski et al. 2016b).

Multiplex-PCR was performed using a universal primer set CBSSU rRNA F2 (5'-CTTTTGACGAACAACTGCCCTATC-3') and CBSSU rRNA B4 (5'-AACCGAACGCACTAAACCCC-3') to identify any trypanosome species, and L. passim 18S-F forward primer (5'-AGGGATATTTAAACCCATCGAAAATCT-3') was used to identify *L. passim* (Schmid-Hempel & Tognazzo 2010, Szalanski et al. 2016a). Positive PCR reactions yield

a 716-724bp amplicon for *L. passim* and a wide range of trypanosomatid species and a single 499bp product for *L. passim* (Szalanski et al. 2016b). PCR reactions were conducted per Szalanski et al. (2016a), and each batch included a negative control consisting of double-distilled water to ensure cross-contamination did not occur for PCR preparations and positive controls from type strains for *C. mellificae* (30254) and *L. passim* (PRA-422) obtained from the American Type Culture Collection (ATCC, Manassas, VA) to ensure reactions were successful. These were extracted with the same methods as for the honey bee samples. The thermocycler conditions for trypanosome DNA amplification are as followed: denature for 2 minutes at 94°C then 40 cycles at 94°C for 45 seconds, 59°C for 1 minute, 72°C for 1 minute, and final extension of 72°C for 5 minutes. PCR products were run on a 2% agarose check gel and species diagnoses made according to amplicon size.

Select positive samples were purified and sequenced via Eurofins Genomics (Diatherix, Huntsville, AL) for species confirmation using reference sequences available from GenBank (National Center for Biotechnology Information). Additionally, these sequences were visually aligned and a BLAST (National Center for Biotechnology Information) search was conducted with Geneious v.6.1.8 (Auckland, New Zealand) for species confirmation.

Statistical analyses were performed using R v.3.3.1 with the package *fifer* (Fife 2017, R Core Team 2016). States were compared using a Pearson's χ^2 test of independence with pairwise post hoc comparisons and Bonferroni-adjusted p-values. Source of colonies (managed or feral) were compared using a Pearson's χ^2 test of independence.

Results

From this study, we found that 11% (n = 144) of the 1,360 screened honey bee samples were positive for *L. passim*, but no samples were positive for *C. mellificae* or any other species of *Crithidia*. This is the first time *L. passim* has been recorded in Arkansas (n = 17), New Mexico (n = 1), New York (n = 61), Oklahoma (n = 5), and Texas (n = 2) (**Table 2.1**). *Lotmaria passim* was previously recorded in Hawaii by a study performed by Szalanski et al. (2016a) and Utah by Tripodi et al. (2018). The proportion of positive *L. passim* samples was significantly different between the states surveyed ($\chi^2 = 77.153$, df = 7, p = 0.0005). New York had the highest infection rate of 17%, whereas Utah had the lowest with 0.70% (**Table 2.1**). There were no cases of trypanosome infection from Mississippi, which may be due to the small sample size from this state or that all of the samples were from feral colonies (**Table 2.2**). There were significant differences between certain states concerning infection rates when performing the post hoc comparisons. The infection rate in Arkansas was significantly different compared to Oklahoma, Texas, and Utah (p = 0.016, p = 0.0051, p = 0.0002 respectively). The infection rate in Hawaii was significantly different compared to New Mexico, Oklahoma, Texas, and Utah (p = 0.029, p < 0.0001, p < 0.0001, p < 0.0001 respectively). The infection rate in New York was significantly different compared to New Mexico, Oklahoma, Texas, and Utah (p = 0.018, p < 0.0001, p < 0.0001, p < 0.0001 respectively). With this pattern in significant difference, states with higher infection rates and proportionally more managed colonies are significantly different than states with lower infection rates and proportionally more feral colonies. Managed colonies (n = 765) had a higher infection rate of *L. passim* (16%) than feral colonies (n = 595, 4% positive) ($\chi^2 = 49.242$, df = 1, p < 0.0001) (**Table 2.3**). This may be due to climatic or geographical differences between samples states or different beekeeping practices between

commercial and hobbyist beekeepers. Two states (Hawaii and New York) had the most positive samples and also had the highest proportions of managed colonies (**Table 2.2**). *Lotmaria passim* was found from samples dating back to 2005. These were from samples obtained in Oklahoma. Eight samples positive for *L. passim* sent off for sequencing were genetically confirmed as *L. passim* using the program Geneious and comparing the sequencing data through a BLAST search. Sequenced samples were matched with 100% identity match to ten deposited samples on GenBank (accession numbers: KT252547, KT252546-KT252553, KU499927, and KU499926).

Discussion

Infection rates of *L. passim* observed in this study are lower than those observed in Chile (90%) and Serbia (60%) (Arismendi et al. 2016, Stevanovic et al. 2016). Both studies surveyed samples from beekeepers and apiaries rather than conducting analysis on a mix of samples from managed and feral honey bee colonies. Because this study focused on two different sources of hives, where one type was managed by a beekeeper and the other with no aid from a beekeeper, overall infection rates may be different due to management practices among each country. Although the amount of infection rates for managed colonies in this study were still lower (16%) compared to the Chilean and Serbian studies, taking a deeper look into the differences in management practices among countries might be something to consider when comparing infection rates of honey bee trypanosomes and why there is variation between them.

Arismendi et al. (2016) performed a one year study documenting the infection rate of *C. mellificae*, *L. passim*, and *N. ceranae*, focusing on managed honey bee colonies. They discovered that 90% of the honey bee colonies tested was infected with *L. passim*, and 18% of those colonies had a co-infection with *N. ceranae*. A recent study conducted by Xu et al. (2018) found

that 14% of the managed colonies they sampled from Massachusetts, USA were infected with *L. passim* and 5% were infected with *C. mellificae*. Although *C. mellificae* was not detected in our survey, *L. passim* appears to have a widespread and dominant distribution in the USA, and has essentially gone undetected without the use of molecular diagnostics. Real-time PCR analysis could be used in the future to quantify infection of trypanosomes infecting honey bees.

Stevanovic et al. (2016) also studied trypanosomes and microsporidia in Serbia over a span of nine years to develop specific primer sets targeting polymorphic sites in trypanosome mitochondrial DNA. Stevanovic et al. (2016) discovered that *L. passim* has been present in Serbian honey bee colonies since 2007 at moderate to high levels of infection (39-83%) but at the end of their study *L. passim* was detected in at least 60% of the colonies surveyed. *Lotmaria passim* has been present in the United States since 2005 according to this study indicating the importance of using molecular techniques to determine infection of honey bee trypanosomes in USA colonies. Because there are no known vital signs for honey bees infected with trypanosomes the reliance on molecular techniques continues to pave the path for learning more about *L. passim*'s interaction with honey bees.

Tozkar et al. (2015) found that migratory hives had higher trypanosome abundance than stationary hives, and that urban environments may be linked to impaired honey bee health. Pathogen pressure has been shown to increase with urbanization and environmental management resulting in a three-fold decline of worker survival (Youngsteadt et al. 2015). A study performed with Montana commercial beekeepers moving their hives to California for almond pollination each year discovered that pathogen prevalence and abundance was higher immediately following almond pollination (Cavigli et al. 2016). This suggests that commercial beekeeping allows migratory hives to acquire new pathogens and viruses that would otherwise be avoided if hives

were stationary year-round or only allowed to be used for pollination during certain times of the year. Although it would make sense that managed and commercial colonies have higher pathogen loads than feral colonies, a study performed by Thompson et al. (2014) determined that feral colonies had a higher level of deformed wing virus than managed colonies, supporting the idea that feral colonies could be potential pathogen reservoirs for uninfected honey bee colonies.

It is unknown if queen breeders are spreading trypanosomes by infected queens or attendant worker honey bees in the shipped queen cages. It has been documented that *Nosema* has been distributed in packaged queen and packaged honey bee colonies in the United States (Strange et al. 2008). Strange et al. (2008) performed a study to detect the presence of parasites and diseases in honey bee stocks. From their research they found that 15 of 48 screened packages were positive for *Nosema* infection. Queen honey bees can acquire *Nosema* through horizontal transmission from attendant bees confirmed through laboratory studies performed by Higes et al. (2009). Trypanosome acquisition in managed honey bee colonies may have been spread in the same manner as *Nosema* considering that trypanosome infection is widespread in the USA and this should be looked at for future studies.

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Chapter 2 tables

Table 2.1. Samples positive from each state sampled. Mississippi had no infection of *Lotmaria passim*.

State	Sample numbers	Positive for *Lotmaria passim*	% positive samples
Arkansas	124	17	14%
Hawaii	346	57	16%
Mississippi	29	0	0
New Mexico	58	1	2%
New York	350	61	17%
Oklahoma	173	5	3%
Texas	131	2	2%
Utah	149	1	0.70%
Total	1,360	144	11%

Table 2.2. Proportion of total managed and feral honey bee colonies per state, including the proportion of positive samples from each state.

State	Managed colony numbers	Feral colony numbers	Total sample numbers per state	% positive for *Lotmaria passim*
Arkansas	74	50	124	14%
Hawaii	306	40	346	16%
Mississippi	0	29	29	0
New Mexico	0	58	58	2%
New York	350	0	350	17%
Oklahoma	10	163	173	3%
Texas	0	131	131	2%
Utah	25	124	149	0.70%
Total	765	595	1,360	11%

Table 2.3. Samples positive from managed and feral honey bee colonies.

Source of sample	Sample numbers	Positive for *Lotmaria passim*	% positive samples
Managed	765	121	16%
Feral	595	23	4%
Total	1,360	144	11%

Chapter 3: Other analyses concerning honey bee trypanosomes in the United States

Abstract

Data from the previous chapter was further analyzed to study: the presence of *Lotmaria passim* between Africanized honey bees and European honey bees; if there was a significant relationship between bees infected with both *L. passim* and a fungal pathogen (*Nosema*); the temporal occurrence of *L. passim* infections in the United States (USA); and if the spread of *L. passim* in the USA could be due to queen breeds. A total of 532 samples documented to have Africanized or European origins were used to analyze infection prevalence. Of these 532 samples, 3% were positive for *L. passim*; Africanized samples were 2% positive for *L. passim* while 6% of European samples were positive. The proportion of samples positive for *L. passim* were not significantly different between Africanized and European honey bee colonies. A total of 745 samples were analyzed for pathogen and parasite co-occurrence concerning *Nosema* and *L. passim*. Of the 745 samples 4% were co-infected with both *Nosema* and *L. passim*, while 11% were infected with *Nosema* alone and 6% with *L. passim* alone. Statistical analysis revealed that *Nosema* and *L. passim* do not occur independently from the samples screened. From the temporal analysis, year and month were significant indicators for *L. passim* infections in honey bees from the USA. The highest *L. passim* infections occurred in the year of 2009 and lowest in the year of 2006. Samples infected with *L. passim* were also highest in August and lowest in April. Samples infected with *L. passim* were highest in the summer and lowest in the winter. Results from the queen breeder samples were inconclusive and did not provide insight into how *L. passim* is being distributed throughout the USA.

Introduction

Africanized and European honey bees

Africanized honey bees (*Apis mellifera scutellata* Lepeletier, AHB) are hybrids of the European honey bee (*Apis mellifera ligustica* Spinola, EHB) and African honey bees (*A. m. scutellata*). AHB migrated to Texas in 1990 after being imported for scientific research in Brazil to breed a honey bee that could tolerate tropical climates (Schneider et al. 2004). In the United States (USA) AHB are restricted to the southern states because they have a reduced capacity for winter survival (Schneider et al. 2004). Identification of AHB from EHB using only morphological diagnostics is a daunting task. However, the use of molecular diagnostics decreases the amount of time for correct identification and increases the reliability of subspecies identification (Szalanski & McKern 2007).

Migratory beekeeping can also influence the spread of AHB. One million colonies are moved throughout the USA for pollination services (Schneider et al. 2004). Queens can be lost in transport to pollination sites, and upon arrival colonies will try to requeen themselves in regions where AHB drones are present therefore integrating African traits into future brood (Schneider et al. 2004). After pollination season is over commercial beekeepers can potentially transport African populations back to apiaries in other parts of the USA (Schneider et al. 2004).

A study performed by Szalanski et al. (2014) suggests that feral colonies can be separated and analyzed for differences between AHB and EHB. Although no significant difference was observed between AHB and EHB for occurrence or prevalence of *Nosema* Nägeli infection, a comparison can still be completed concerning other honey bee parasites such as trypanosomes. Studies comparing AHB and EHB pathogens and parasite infections are lacking in the USA and

would benefit the scientific community on how molecular genetics plays a role in aiding against infections.

Pathogen co-occurrence

Nosema are obligate microsporidia that exist outside of host cells as inactive spores and attack the midgut wall of adult honey bees (Le Conte & Navajas 2008, Genersch 2010). *Nosema* can develop with no visible symptoms, are more likely found in weak colonies during spring after long wet winters, can reduce colony performance, increase winter mortality, reduce honey bee lifespan, and can cause dysentery within honey bee hives (Le Conte & Navajas 2008, vanEngelsdorp & Meixner 2010, Smith et al. 2014). Nurse bees can pick up spores left inside of the hive and transmit infections horizontally among susceptible bees (vanEngelsdorp & Meixner 2010). Two different species of *Nosema* exist in the world. *Nosema apis* Zander infect *Apis mellifera* L., and *Nosema ceranae* (Fries) infect *Apis cerana* F.. *Nosema ceranae* has been present in the USA since at least 1995 after being introduced from Asia (vanEngelsdorp & Meixner 2010). It was originally thought that both *Nosema* were exclusive to *A. cerana*, but it has since been determined that *N. ceranae* is found in *A. mellifera* and is more virulent compared to *N. apis* (Genersch 2010, vanEngelsdorp & Meixner 2010).

According to Ravoet et al. (2013) the presence of the trypanosomes *Crithidia mellificae* Langridge and McGhee and *N. ceranae* are predictive markers for winter mortality when infested during the summer, and act in a negative synergistic way in terms of effecting colony mortality. Although a study performed by Higes et al. (2016) determined that the highest mortality rates for honey bees in cage studies was due to infections of *N. ceranae* alone rather than co-infected with *C. mellificae*. However, Schwarz and Evans (2013) determined that co-infections of *C. mellificae*

and *N. ceranae* modify the host immune response, alter local and systemic immune gene transcription, and reduce cellular immunity of honey bees suggesting that a co-infection of both parasite and pathogen may lead to undesired death in honey bees. Studies concerning co-infections of *Nosema* and *Lotmaria passim* Schwarz and their impact on honey bee immune response are few and far between. Nonetheless, studies looking at co-infections of *Nosema* and *C. mellificae* are enough to support that there is an interaction between the trypanosome parasite and fungal pathogen.

Pathogen temporal analysis

Although it has been stated that *C. mellificae* and *L. passim* have seasonal occurrences (Runckel et al. 2011, Cersini et al. 2015, Tozkar et al. 2015, Vejnovic et al. 2018), an extensive temporal analysis for the USA is lacking. It has been shown in a commercial beekeeping study performed by Cavigli et al. (2016) that trypanosome infection was higher during the months of March to April after almond pollination than before (Oct.-Dec.) or during (February) almond pollination. A similar exploration could be conducted to observe seasonal variation in the samples for this study although mortality will not be a factor in data analysis. According to Popp et al. (2012), the greatest infection of *C. bombi* in bumble bees occurs in the middle of the foraging season. Transmission rates tend to decrease when more individuals become infected and the number of susceptible hosts decrease (Popp et al. 2012).

Queen breeders and parasite distribution

Packaged queens are used to replace deceased colonies lost over the winter, increase colony numbers, or rejuvenate ongoing colonies (Farrar 1947, Martin et al. 1980, Strange et al.

2008). Although the purpose of raising queens and packaged bees is beneficial to beekeepers and effective when replacing colonies, breeding honey bees and then dispersing them to different areas of the USA can spread disease, parasites, and undesirable stocks (Strange et al. 2008). Strange et al. (2008) performed a study to detect the presence of parasites and diseases in honey bee stocks. From their research Strange et al. (2008) found that 15 of 48 screened packages were positive for *Nosema* infection. Their findings suggest that queen breeding and packaged bees can spread *Nosema* across the USA, and that beekeepers should monitor the quality of their purchased bees for pests and diseases.

It is unknown if queen breeders are spreading disease by providing particular honey bee breed stocks or distributing pathogens across the USA via infected queens or workers. Queen honey bees can acquire *Nosema* through horizontal transmission from attendant bees confirmed through laboratory studies performed by Higes et al. (2009). As of 2011 there were approximately 100 honey bee queen breeders in the USA (Magnus et al. 2011). The initial purpose for breeding queens in the 1980s was to select particular traits that showed resistance against *Varroa* mites and honey bee diseases (Magnus et al. 2011). Once different lines became available, beekeepers could pick and choose what stock they preferred to work with based on productivity and behavior. Magnus et al. (2011) found that of the 14 queen breeders they sampled from the USA, the sampled honey bees represented only five mitochondrial DNA COI-COII lineages. Their results concluded that there are relatively few subspecies produced in the USA from European lineages because beekeepers want stocks that are productive and relaxed in nature.

According to Farrar (1947), *Nosema* has been a leading cause in abnormal supersedure of packaged queens. *Nosema* infections can cause packaged queens to stop laying eggs after two

months, have sluggish behavior, and lay eggs that will shrivel and fail to hatch (Farrar 1947). Following Farrar's (1947) queen supersedure and packaged bee documentation, Moeller (1948) also observed that *Nosema* can cause queen supersedure failure in packaged bee colonies. Moeller's (1948) data suggests that queen bees and attendant bees vary in *Nosema* infection depending on colony conditions before being shipped. Camazine et al. (1998) reported that colonies with queens being superseded are less productive and are at a more significant risk of supersedure failure that leads to queenlessness. According to Mutinelli (2011), *N. ceranae* can be spread through different honey bee products including packaged worker bees, queen bees, wax, and pollen. As of 2011 there were no restrictions or preventative measures for limiting the sale of honey bee packages infected with *Nosema* (Mutinelli 2011).

Alaux et al. (2010) performed a study on *N. ceranae* and its impact on queen physiology. *Nosema*-induced supersedure is not uncommon in honey bee colonies where *Nosema* infection can compromise the renewal and stability of worker populations (Alaux et al. 2010). According to Fyg (1964) and Liu (1992), infected queens have decreased ovary development that leads to infertility and causes frequent supersedure from old or failing queens. Poor queens are a major cause of colony loss because the queen is responsible for reproduction and regulation of her society with pheromones (vanEngelsdorp et al. 2008, Alaux et al. 2010). Although *Nosema* did not have as many negative effects on queen honey bee health, infected queens have a shorter lifespan compared to healthy queens suggesting that infected queens cannot cope with physiological stress for long periods of time (Camazine et al. 1998, Higes et al. 2009, Alaux et al. 2010, vanEngelsdorp & Meixner 2010).

The decline in amount of honey bee breeders could lead to loss of genetic diversity and result in inbreeding and increase susceptibility of colonies to pests and diseases (Bourgeois et al.

2015). Even though queen breeders perform outcrossing to maintain genetic diversity, a limited gene pool of queens can affect genetic diversity in the USA (Bourgeois et al. 2015). A genetic bottleneck can also result from a reduction of queen breeders or disease outbreaks that can lead to a decrease in colony numbers from 5 million to 2.4 million (Bourgeois et al. 2015).

Materials and methods

Africanized and European honey bees

Honey bee samples were collected from feral honey bee colonies to test if the subspecies have variation of trypanosome parasite infection rates. These were obtained from various beekeepers, state agencies and our own collection efforts from 2009 and 2014, preserved in 70 to 90% ethanol, and vouchers maintained at the Insect Genetics Laboratory (University of Arkansas, Fayetteville, AR, USA). A total of 532 samples were previously recorded to be of Africanized or European origins from seven states were used in this analysis (Arkansas: n = 46; Hawaii: n = 22; Mississippi: n = 10; New Mexico: n = 56; Oklahoma: n = 160; Texas: n = 122; and Utah: n = 116). The previously mentioned samples were determined to be of African or European origins by Szalanski and Magnus (2010) and unpublished data from the Insect Genetics Laboratory.

Multiplex-PCR used to detect *C. mellificae* and *L. passim* was previously described in **Chapter 2** of this thesis.

Statistical analyses were performed using R v.3.3.1 with the package *fifer* (Fife 2017, R Core Team 2016). Origin of colonies (Africanized or European) infected with trypanosomes were compared using a Pearson's χ^2 test of independence.

Pathogen co-occurrence

Samples were previously collected and analyzed by the Insect Genetics Laboratory for *Nosema* infection (Szalanski et al. 2013, Szalanski et al. 2014, Szalanski et al. 2016, unpublished data from the Insect Genetics Laboratory) and used to analyze co-occurrence with honey bee trypanosomes. A total of 745 samples were analyzed for co-occurrence of trypanosomes and *Nosema* (Arkansas: n = 97; Hawaii: n = 185; Mississippi: n = 29; New Mexico: n = 53; New York: n = 4; Oklahoma: n = 131; Texas: n = 129; and Utah: n = 117).

Multiplex PCR used to detect *C. mellificae* and *L. passim* was previously described in **Chapter 2** of this thesis. *Nosema* was previously screened during studies performed by Szalanski et al. (2013), Szalanski et al. (2014), Szalanski et al. (2016), and unpublished data provided by the Insect Genetics Laboratory.

Co-occurrence of *Nosema* and *L. passim* were compared using a two-tailed Fisher's exact test for count data in R v.3.3.1 (R Core Team 2016).

Pathogen temporal analysis

Samples tested for honey bee trypanosomes from **Chapter 2** of this thesis recorded with a month and year of collection were further analyzed for year-to-year, month-to-month, and seasonal infection rates. The years 2004 and 2010 to 2012 were excluded from this analysis because no trypanosome infections were detected within those years. A total of 1,286 samples were analyzed (Arkansas: n = 110; Hawaii: n = 336; Mississippi: n = 29; New Mexico: n = 58; New York: n = 347; Oklahoma: n = 154; Texas: n = 128; and Utah: n = 124).

Multiplex-PCR used to detect *C. mellificae* and *L. passim* was previously described in **Chapter 2** of this thesis.

A nominal generalized linear model (GLM) with a binomial distribution and logit link function was used to analyze which parameter (year, month, or season) was significant concerning trypanosome infection among honey bees using JMP Pro v.13.1.0 (SAS Institute Inc. 2016). Seasonality and year-to-year infections were analyzed further using a Pearson's χ^2 test for independence in R v.3.3.1 with the package *fifer* (Fife 2017, R Core Team 2016). Month-to-month infections were compared using a Pearson's χ^2 test of independence with pairwise post hoc comparisons and Bonferroni-adjusted p-values in R v.3.3.1 with the package *fifer* (Fife 2017, R Core Team 2016).

Queen breeders and parasite distribution

Queen breeder samples were previously collected and analyzed by Magnus et al. (2011) and the Insect Genetics Laboratory for studies on haplotype diversity. These queen breeder samples were screened for trypanosomes to investigate if distribution of honey bee trypanosomes could be explained by queen breeder sales. A total of 244 samples from the Insect Genetics Laboratory database were acquired from 14 different queen breeders across the USA (Alabama: n = 1, California: n = 3, Florida: n = 1, Georgia: n = 1, Hawaii: n = 2, Louisiana: n = 2, North Carolina: n = 1, Oregon: n = 1, Tennessee: n = 1, Washington: n = 1).

Multiplex-PCR used to detect *C. mellificae* and *L. passim* was previously described in **Chapter 2** of this thesis.

Statistical analyses were not made for this dataset (see **Results** subsection *Queen breeders and parasite distribution*).

Results

Africanized and European honey bees

European honey bee colonies (n = 286) had a higher infection rate of *L. passim* (6%) than Africanized honey bee colonies (n = 246, 2% positive) but there was no significant difference in infection rates (χ^2 = 2.7611, df = 1, p = 0.09658) (**Table 3.1**). This is the first study to compare trypanosome infections between AHB and EHB. Africanized colonies from Texas (n = 2) had the highest number of samples infected with *L. passim*, but colonies from Oklahoma, Texas, and Utah had the same infection rate (2%) (**Table 3.2**). European colonies from Arkansas (n = 5) had the highest number of samples infected with *L. passim*, but Hawaii had the highest infection rate of *L. passim* (14%) (**Table 3.3**). Although there was no significant difference in infection rates between subspecies, EHB had more infected samples (n = 13) than AHB (n = 4). Because there is no significant difference in infection rates it is unclear if there is any difference in internal parasite infections between honey bee subspecies.

Pathogen co-occurrence

There was a significant association between *Nosema* and *L. passim* from the samples surveyed in this study (p < 0.0001). This means that *Nosema* and *L. passim* do not occur independently from the samples surveyed. Only 4% (n = 32) of the samples analyzed were co-infected with *Nosema* and *L. passim* (**Table 3.4**), while 11% of the samples were infected with *Nosema* alone and 6% of the samples were infected with *L. passim* alone (**Table 3.4**).

Pathogen temporal analysis

The GLM was significant ($p < 0.0001$) and demonstrated that year and month are significant indicators for *L. passim* infections in honey bees (both: $p < 0.0001$) and season was not significant ($p = 0.5681$). However, seasons and months are correlated with each other, therefore subsequent GLMs were made between years and months, and years and seasons. According to the year-month GLM, year and month are still significant indicators for *L. passim* infection ($p < 0.0001$). The year-season model also had a similar result in that both year ($p < 0.0001$) and season ($p = 0.0006$) were significant indicators for *L. passim* infection. Despite which GLM was used, it is apparent that year, month, and season play a role in trypanosome infection of honey bees.

Samples were analyzed to observe year-to-year infections of *L. passim.* The proportion of positive *L. passim* samples were significantly different between years ($\chi^2 = 74.825$, df = 7, $p < 0.0001$). Samples infected with *L. passim* were highest in 2009 (n = 66) and lowest in 2006 (n = 1) (**Table 3.5**). Infection rates were highest in 2013 (26%) and lowest in 2005 and 2006 (both: 2%) (**Table 3.5**).

Months were also analyzed for infections of *L. passim*. The proportion of positive *L. passim* samples was significantly different between months ($\chi^2 = 46.841$, df = 11, $p = 0.0005$). Samples infected with *L. passim* were highest in August (n = 20) and lowest in April (n = 1) (**Table 3.6**). Infection rates were also highest in August and December (both: 21%), and lowest in April (2%) (**Table 3.6**). There were significant differences between particular months from the survey. August was significantly different compared to April, May, and June ($p = 0.003$, $p = 0.0003$, $p = 0.005$ respectively).

Months were pooled into seasons: winter (Dec.-Feb.); spring (Mar.-May); summer (Jun.-Aug.); and fall (Sept.-Nov.). The proportion of positive *L. passim* samples was significantly different between seasons ($\chi^2 = 14.579$, df = 3, p = 0.002). Samples infected with *L. passim* were highest in the summer (n = 76) and lowest in the spring (n = 13) (**Table 3.7**). Infection rates were also highest in summer (14%) and lowest in the spring (5%) (**Table 3.7**).

Queen breeders and parasite distribution

Only two queen breeding facilities were positive for *L. passim*; one from Hawaii and one from California (**Table 3.8**). Out of the 244 samples screened, three were positive for *L. passim* (**Table 3.9**). From the lack of infected samples it appears as though the spread of *L. passim* is not due to queen breeder packages with worker honey bees from the conducted survey.

Discussion

Africanized and European honey bees

Not many studies have been conducted to compare internal parasite prevalence and infection rates between AHB and EHB. Currently, *Varroa* mite infestations have been well studied among each subspecies because AHB have demonstrated greater disease and parasite tolerance than EHB (Teixeira et al. 2013). AHB have lower levels of *Varroa* mite infestation compared to EHB due to AHB having better grooming and hygiene, and difference in genetic diversity (Moretto & de Mello Jr. 1999, Vandame et al. 2002). In recent studies comparing AHB and EHB infections with *N. ceranae,* EHB had the highest infection compared to AHB (Herrera et al. 2017). However, AHB had a higher *N. ceranae* infection compared to Carniolan bees (*Apis mellifera carnica* Pollman), a subspecies of honey bee originally from Slovenia and countries

from Eastern Europe (Garnery et al. 1998, De la Rúa et al. 2009, Herrera et al. 2017). Gregorc et al. (2016) found similar results in which AHB died faster than Carniolan bees while infected with *N. ceranae*. Although Gregorc et al. (2016) determined that AHB died faster while infected with *N. ceranae*, part of their study determined that nutritional source and colony management could cause interference of immunological response before honey bees are infected with *N. ceranae*. Although AHB do not seem to be more resistant to internal parasite infections compared to Carniolan bees, AHB appear to be more resistant to infections compared to EHB (Herrera et al. 2017). This is the first study to compare honey bee trypanosome infections between AHB and EHB. Results reflect that there are less trypanosome infections in AHB than EHB which coincide with the results found in Herrera et al. (2017). Because AHB seem to have less internal parasite infections compared to EHB, AHB traits could be incorporated into EHB populations in order to increase genetic diversity and tolerance to internal parasites and pathogens. However, this study determined that there was no significant difference in infection rates between the two subspecies. It is currently unknown if there is a difference of internal parasite infections between honey bee subspecies, but because all colonies screened were of feral origins and it has been determined that feral colonies have significantly less parasite infection rates compared to managed colonies the true difference in colony infections may lie within management techniques. Further research on trypanosome infection prevalence between Africanized and European honey bee colonies should be conducted to determine the true underlying mechanism of infection rates between subspecies.

Pathogen co-occurrence

This study analyzed natural infections of *Nosema* and *L. passim*. None of the honey bees were administered fungal spores or cultures of trypanosomes in order to stimulate co-occurrence in one individual colony. This study is not saying that a co-infection of *Nosema* and *L. passim* has a negative impact on honey bee health, but rather that the likelihood of co-occurrence of each pathogen is not independent from the samples that were surveyed. Tritschler et al. (2016) found that there is a positive correlation of infection loads between *N. ceranae* and *L. passim*, but that there may not be a significant interaction between the two species. No significant interaction between *N. ceranae* and *L. passim* is possibly due to where each species occurs in the honey bee: *Nosema* occurs in the midgut and *L. passim* infections occur in the hindgut (Tritschler et al. 2016). Although the spatial separation between parasite infection sites may reduce the negative synergistic impact on honey bee health, because there was a positive correlation between infection loads, there is at least some interaction between *Nosema* and *L. passim* that may impact honey bee health to some degree. More studies should be conducted between the two species order to establish the impact a co-infection of parasites and pathogens have on honey bee health.

Pathogen temporal analysis

After performing the GLM to determine the significance of year, month, and season on trypanosome prevalence, month and season may be correlated when compared within the same model. When all parameters were ran together only year and month were significant while season was not, implying that month and year are better indicators of determining the time of parasite occurrence in the USA. However, when month was ran just with year and season ran just with year, both month and season are significant. A more specific model could be used with the

data such as a GLIMMIX model in order to dispel any underlying uncertainty to whether season is a significant factor for trypanosome infections in honey bee colonies across the USA.

Fluctuations in *L. passim* positive samples occurred between 2005 and 2007 but the number of infections ultimately decreased between 2007 and 2009, and there was a decrease in number of infected *L. passim* samples between 2013 and 2015. Despite the fluctuation in positive *L. passim* samples, there were different total number of samples for each year instead of a consistent number of total samples. This is probably why there is such a high positive sample count for the year 2009 (total samples: n = 530, positive for *L. passim*: n = 66) compared to other years where total sample numbers and those positive for *L. passim* were lower. The year 2006 had the lowest number of infected *L. passim* samples compared to the year 2009 where there were more positive *L. passim* samples. According to weather data collected between 2005 and 2015 from the National Centers for Environmental information provided through the National Oceanic and Atmospheric Administration (NOAA), average annual temperatures were higher in 2006 compared to 2009 where they were lower (NOAA 2018). This could be a link of finding lower infections in warmer temperatures and higher infections in cooler temperatures. A follow-up analysis could be conducted where a similar number of total samples can be screened from more recent years to analyze more current yearly infection rates of honey bee trypanosomes.

The sample data for months were not completely biased but still varied in number from month to month. The highest number of samples occurred between the months of June to August which comprised the entire season of summer. The highest infection of *L. passim* occurred in August which occurs in the summer season. The highest season with positive *L. passim* samples was summer as opposed to winter. According to Vejnovic et al. (2018) winter is a favorable time for the development of parasites inside of a host like honey bees, but according to my data winter

had the second lowest count for positive *L. passim* samples. Similar results have been shown in a study performed by Runckel et al. (2011) where *C. mellificae* infections peaked in January during the winter season. However, results coincide with a study conducted by D'Alvise et al. (2018) in which summer had the highest trypanosome infection (81% of summer samples) and the winter season had the lowest (53% of winter samples).

Because seasonality studies are lacking in the USA, a follow-up study would be imperative in order to learn more about the seasonality of honey bee trypanosome occurrence. The follow-up study should be conducted to monitor the same hive for winter, spring, summer, and fall occurrences of honey bee trypanosomes using quantitative PCR techniques. A further study should also look at hives from different states since seasonal changes occur at different paces during the year depending on where one is located in the USA. Seasonal variation in trypanosome infection also varies by what type of beekeeper you are as according to Cavigli et al. (2016) the highest amount of trypanosome infection occurred between March and April (spring months) following almond pollination in California (Cavigli et al. 2016), whereas the data presented in my study showed the lowest infection of *L. passim* occurred during the spring.

Queen breeders and parasite distribution

Although it is inconclusive if queen breeding facilities are spreading trypanosome infections across the USA, infections of *L. passim* were found from two states in the USA (California: n = 1; Hawaii: n = 2) which means that *L. passim* does occur in queen breeding facilities. A follow-up study could be conducted with packaged bees bought from different rearing companies or queen breeding sites and screened for internal parasites and pathogens. Screening the facilities may also open up discussion about incorporating new traits to increase

genetic diversity of the reared honey bees and their queens, and lead to the development of better management tactics to prevent contamination of uninfected colonies.

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Chapter 3 tables

Table 3.1. Samples positive for *Lotmaria passim* from Africanized (AHB) and European honey bee (EHB) feral colonies.

Origin of sample	Sample numbers	Positive for *Lotmaria passim*	% positive samples
AHB	246	4	2%
EHB	286	13	6%
Total	532	17	3%

Table 3.2. Africanized honey bee samples (AHB) infected with *Lotmaria passim* per state.

State	Sample numbers	Positive for *Lotmaria passim*	% positive samples
Arkansas	1	0	0
New Mexico	39	0	0
Oklahoma	52	1	2%
Texas	93	2	2%
Utah	61	1	2%
Total	246	4	2%

Table 3.3. European honey bee samples (EHB) infected with *Lotmaria passim* per state.

State	Sample numbers	Positive for *Lotmaria passim*	% positive samples
Arkansas	45	5	11%
Hawaii	22	3	14%
Mississippi	10	0	0
New Mexico	17	1	6%
Oklahoma	108	4	4%
Texas	29	0	0
Utah	55	0	0
Total	286	13	6%

Table 3.4. Occurrence of *Nosema* and *Lotmaria passim* from screened samples.

Occurrence	Sample numbers	% occurrence
Only *Nosema*	85	11%
Only *L. passim*	41	6%
Both	32	4%
Neither	587	79%
Total	745	

Table 3.5. Year-to-year data on *Lotmaria passim* infections from screened samples.

Year	Sample number per year	Positive for *Lotmaria passim*	% positive samples
2005	174	4	2%
2006	62	1	2%
2007	67	4	6%
2008	110	11	10%
2009	530	66	12%
2013	139	36	26%
2014	69	17	25%
2015	135	4	3%
Total	1,286	143	11%

Table 3.6. Month-to-month data of *Lotmaria passim* infections from screened samples.

Month	Sample number per month	Positive for *Lotmaria passim*	% positive samples
January	51	4	8%
February	95	8	8%
March	85	6	7%
April	64	1	2%
May	117	6	5%
June	172	9	6%
July	148	19	13%
August	230	48	21%
September	154	20	13%
October	87	9	10%
November	35	3	9%
December	48	10	21%
Total	1,286	143	11%

Table 3.7. Seasonal data on *Lotmaria passim* infections from screened samples.

Season	Sample number season	Positive for *Lotmaria passim*	% positive samples
Winter	194	22	11%
Spring	266	13	5%
Summer	550	76	14%
Fall	276	32	12%
Total	1,286	143	11%

Table 3.8. Queen breeder facilities screened for *Lotmaria passim*.

State	Number of queen breeder facilities	Number of queen breeder facilities positive for *Lotmaria passim*	% positive samples
Alabama	1	0	0
California	3	1	33%
Florida	1	0	0
Georgia	1	0	0
Hawaii	2	1	50%
Louisiana	2	0	0
N. Carolina	1	0	0
Oregon	1	0	0
Tennessee	1	0	0
Washington	1	0	0
Total	14	2	14%

Table 3.9. Number of total queen breeder samples screened for *Lotmaria passim*.

State	Total sample numbers	Positive for *Lotmaria passim*	% positive samples
Alabama	15	0	0
California	66	1	2%
Florida	20	0	0
Georgia	24	0	0
Hawaii	20	2	10%
Louisiana	31	0	0
N. Carolina	20	0	0
Oregon	18	0	0
Tennessee	20	0	0
Washington	10	0	0
Total	244	3	1%

Chapter 4: Conclusion

Fluctuations in honey bee (*Apis mellifera* L.) populations have been occurring in recent years. Although no one cause has been attributed to the fluctuation of honey bee populations, internal parasites have been overlooked compared to external parasites such as *Varroa* mites (*Varroa destructor* Anderson and Trueman). Internal parasites such as trypanosomes have been understudied as a contribution to honey bee health decline. For this research project molecular techniques were used to explore five objectives concerning internal parasite presence in the USA, the difference in infection between sources and origin of the colonies, co-occurrence with a fungal pathogen, seasonality, and potential distribution pathways for spreading infection of honey bee trypanosomes. More research is needed to determine how honey bee trypanosomes are inflicting damage on honey bee health, the transmission between infected honey bees to uninfected ones, and to develop a more thorough approach to studying seasonality and occurrence of trypanosomes in the USA.

The research performed determined that *Lotmaria passim* Schwarz occurs in seven states but *Crithidia mellificae* Langridge and McGhee does not occur in the states sampled. *Lotmaria passim* was detected in Arkansas, New Mexico, New York, Oklahoma, and Texas where it was previously undetected. The oldest sample to date was documented to be from a 2005 feral colony in Oklahoma. Essentially, this honey bee trypanosome has been present in the USA without proper detection. *Lotmaria passim* occurred in both managed and feral honey bee populations, but occurs more frequently in managed colonies. Management practices, techniques, and options should be developed to prevent the acquisition of internal parasites such as *L. passim*. Incorporating traits of feral colonies in to managed bee populations should be considered to

potentially increase the honey bee's ability to combat internal parasite infections at the genetic and cellular levels.

Further analysis was made to compare infection presence between Africanized (*A. m. scutellata* Lepeletier, AHB) and European honey bees (*A. m. ligustica* Spinola, EHB). The analysis determined that there is no significant difference in *L. passim* infections between EHB colonies and AHB colonies. More studies should be conducted to solidify if there is a true difference between infection prevalence depending on the genetic origin of the colony.

Samples were also analyzed to reveal if there was any association between *L. passim* and fungal pathogens in the genus *Nosema* Nägeli. After analysis of samples that were recorded to have been tested for *Nosema*, it was determined that there was an association between samples infected with *L. passim* and *Nosema*. Although this research did not determine if both pathogen and parasite work synergistically to impact honey bee health in the USA, the data shows that there is some sort of relationship concerning the presence of internal parasites and pathogens.

Seasonality was observed for *L. passim* in the USA. Overall the amount of samples infected with *L. passim* decreased over the years observed and had a higher rate of infection in summer months as opposed to other times during the year. Although sample size is a potential issue for this research, a future study should be conducted to observe more recent occurrences for *L. passim* in the USA. The difference in what kind of colonies sampled for determining seasonality may impact results. Commercial beekeepers are more likely to come in contact with parasites than those that keep their colonies stationary year-round. Therefore, colony management and maintenance may have an impact on when to expect trypanosome infections peak during the year. Location of colonies may also impact when peak infections occur. What may be considered seasonal in one state may not be considered the same for another since cooler

weather occurs more often in the northern part of the USA compared to southern states where weather tends to be warmer year-round.

It was determined that at this time there can be no conclusion made concerning how honey bee trypanosomes are being distributed across the USA. There were not enough positive *L. passim* samples to determine if queen breeding facilities and packaged bees are distributing honey bee trypanosomes across the USA. Future research should look into buying queen breeding stocks to determine if honey bee trypanosomes are still present in breeding facilities and being distributed across the USA.

This study has exemplified the importance of one key item of research for the future of studying honey bee diseases. The preservation and maintenance of a large database collection of DNA samples allows one to go back several years and document infections of parasites or pathogens that were previously not present in the country. Preserving a database will also allow the user to go back several years into the past to understand infection incidence as the infections may increase, decrease, or fluctuate over time.

There are many ways to build on this research. Honey bee trypanosomes have not been screened in several states within the USA and should be to create a consensus of prevalence at a larger nationwide level. How uninfected bees acquire parasite infections has not been determined. Honey bees ingest trypanosomes but as far as what types of contaminated materials bees ingest, such as pollen or nectar, to obtain infections has not been determined. A vector for honey bee trypanosomes remains a mystery. Honey bee trypanosomes have been documented to infect other species such as orchard bees, yellowjackets, and bumble bees but at what rate these other organisms are sharing the same environment and acting as vectors for honey bee trypanosomes is not fully understood. The research concerning honey bee trypanosomes and

their impact is still far from being concrete. Further analysis will allow researchers to better understand disease dynamics among honey bees and try to develop a plan of action to prevent colony health from deteriorating.

Although internal parasites have been overlooked as causative agents for honey bee health decline it is important to keep them in mind as populations continue to fluctuate. This research provides insight as to how little we understand about internal parasites we cannot visually see and the importance of using molecular techniques to properly identify the presence and species of honey bee trypanosomes in the USA.