STUDYING THE NATURAL HISTORY OF THE ENDANGERED FLORIDA PUMA: ECTOPARASITES, PIROPLASMS, AND GENETIC DIVERSITY

by

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(Under the Direction of Michael Yabsley)

ABSTRACT

The Florida puma population in southern Florida, United States is one of the best studied felid populations in the world. Since the 1980s, this population has been listed as an endangered subspecies or population of *Puma concolor*, and the conservation efforts since their rediscovery have allowed the population to rebound from ~20 individuals to ~200 individuals. Previously, a high prevalence of piroplasms was reported from Florida pumas (*Puma concolor coryi)* from southern Florida. In the current study, we provide biological, morphological, serological, and molecular data on a novel *Babesia* species. This study also describes the diversity and natural history of ectoparasites on Florida pumas. From January 1989 to May 1993 and January 2000 to April 2014, ectoparasites were collected from free-ranging and captive pumas. Ectoparasites from a total of 262 puma records included six ixodid tick species, one mite (*Lyxacarus* sp.), a Hippoboscid fly (*Lipoptena mazamae*), and a flea (*Ctenocephalides felis*). A live-engorged transmission study of ticks removed from pumas detected the transovarial transmission of a *Babesia* sp. The Major Histocompatibility Complex (MHC) is a component of the adaptive immune system involved in self/non-selfrecognition and disease susceptibility. Some endangered species maintain high genetic diversity at the MHC despite repeated population bottlenecks. Using

next-generation sequencing (Roche 454 and Illumina MiSeq), we determined the allelic diversity of the MHC I and II in Florida pumas respectively. It is currently unclear from our data if the Florida puma MHC was affected by the introgression event. Because most felid species are threatened or endangered, these collective data could have important implications for wild felid conservation.

INDEX WORDS: *Babesia*, ectoparasites, Florida puma, genetic diversity, MHC, *Puma concolor*

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DEDICATION

This dissertation is dedicated to my parents, Mark and Linda Shock. Mom and Dad, you have been so supportive of me every day. I just want to thank you for everything. I could not have written this dissertation without you. I love you.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

The Florida puma population in southern Florida, United States is one of the best studied felid populations in the world. Since the 1980s, this population has been listed as an endangered subspecies or population of *Puma concolor*, and the conservation efforts since their rediscovery have allowed the population to rebound from ~20 individuals to ~200 individuals. Although once widespread throughout the United States, the Florida puma population is the only breeding Eastern population that was not extirpated. Because of their low numbers which have been estimated to be as few as three breeding individuals over two generations and other factors such as habitat loss and diseases, the pumas were facing extinction. To alleviate inbreeding depression and the associated health consequences, the Florida Fish and Wildlife Conservation Commission released female *P. concolor* from Texas into Southern Florida. Within a single generation of cross-breeding with canonical Florida pumas, kitten survival increased and prevalence of inbreeding associated abnormalities (e.g., cowlicks and cryptorchidism) decreased (Johnson et al., 2010; Hostetler et al., 2011).

This intervention is one of the major success stories in conservation genetics. Although Florida pumas face challenges such as mortality from intraspecific aggression and roads, diseases, and habitat loss, this introgression resulted in increased genetic heterozygosity which has improved the overall health of this population. Because this population is so well-studied and because the Florida Panther Recovery Project has been ongoing for decades, there is a long-term

dataset which can be used to answer questions about the natural history of *P. concolor* in the Southeast. One major question in genetics and ecology is the effect population size has on genetic diversity and health (Lande, 1988). For the Florida puma, it has already been determined that genetic diversity at neutral loci (microsatellites) increased as a result of the introgression, but the same question has not been answered at non-neutral loci (Johnson et al., 2010).

The Major Histocompatibility Complex (MHC) is a group of genes which are an important component of the adaptive immune system. They are involved in antigen presentation and self/non-self recognition. Although there are several hypotheses concerning the maintenance of MHC diversity in mammals, the two relevant to this study are: 1)animals may mate with individuals with dissimilar MHC because allelic diversity is important as a function of immune health; or 2) selection drives diversity at the MHC to increase resistance to pathogens. The Florida puma provides an excellent organism to test these hypotheses in the future; however, we must first have data on the impact of the genetic introgression event had on the MHC diversity of the Florida puma population.

From an ecological standpoint, the Florida puma is the last remaining eastern population of *P. concolor* and, as such, is the last opportunity to understand the natural history of this species in the Eastern US. It is often discovered that charaismatic megafauna such as pumas serve as hosts for charaismatic parasites which are endemic to them. When their hosts become endangered, the parasites are also often endangered or extinct. Such may be the case for a parasite of Florida pumas discovered in 2006. The parasite is a *Babesia* sp., one of the tick-transmitted piroplasms, which can infect mammals and birds. So far, no other *Babesia* spp. has been reported from *P. concolor* and no other *Babesia* spp. has been reported from felids in the Americas. This piroplasms was not detected in the pumas from Texas, although *Cytauxzoon felis*,

a related parasite, was. It is possible that this *Babesia* spp. is only found in Florida pumas and as such, is endangered.

Babesia spp., with few exceptions, are transmitted by Ixodid ticks. It is more than likely that the Florida puma *Babesia*, which is genetically similar to *Babesia odocoilei*, is transmitted by an *Ixodes* sp. as *B. odocoilei* is transmitted by *I. scapularis*. Prior to the introgression, three studies examined the prevalence and abundance of ectoparasites such as Ixodid ticks on Florida pumas, however, there have been no studies to see how these ectoparasite populations have changed post-introgression. Studying the ectoparasites of the Florida puma may also help us to determine which tick is the vector for the Florida puma *Babesia.*

Although it does not appear that this *Babesia* sp. is negatively affecting the Florida puma population, gaining increased knowledge of its natural history may help us to prevent a *Babesia*-associated health event from occurring. Studying the ectoparasites of the Florida puma has twofold benefit. First, it allows us to gain insight into the changing ecology of ectoparasites in Florida, and second, it may help to determine the vector of the Florida pumas *Babesia* sp. which could be useful in understanding the transmission dynamics of *Babesia* spp. which infect domestic animals and humans.

With the establishment of *P. concolor* populations in Midwestern North America and the gradual movement of individuals farther east, *P. concolor* may eventually recolonize parts of eastern North America. From the microscopic to the charismatic, Florida puma natural history is an interesting and necessary topic of study.

In these studies, I evaluated parasites associated with and the genetic variability of the Florida puma, a species of ecological concern. Previously, a *Babesia* sp. was detected in Florida pumas that was presumably novel. It is unknown if other *Puma concolor* are infected with this

parasite. It is also unknown what the vector of this parasite is. First, I hypothesized that this is a novel parasite and is only found in the Florida puma population due the extirpation of *P. concolor* and the isolation of the Florida puma population. I also hypothesizde that the vector of the Florida puma *Babesia* would be an Ixodid tick. As a larger study on the genetic varability of the Florida puma, I designed a study to look at non-neutral loci in *P. concolor*, specifically focusing on the Florida puma. I hypothesized that, due to the genetic introgression event in 1995, Florida pumas born after this event would have greater MHC variability. I also hypothesized that pumas from other populations would have more variability. To test these hypotheses, I completed these four specific objectives:

1. Conducted a study to look at the prevalence, serologic cross-reactivity, and phylogeny of the Florida puma *Babesia*. I also determined if the parasite was present in *P. concolor* from other populations.
2. Determined the diversity and intensity of infestation of the ectoparasites, potential vectors of the Florida puma *Babesia*, of Florida pumas post-introgression.
3. Conducted a live-engorged transovarial transmission study of piroplasms in ticks removed from Florida pumas.
4. Determined the genetic variability of Florida pumas and other *P. concolor* at MHCI and MHCII DRB loci.

Literature Review

Natural history of the Florida puma: *Puma concolor* are apex predators which range from Canada to Chile. Genetic analysis suggests that there are six subspecies which include *P. c. cougar* in North America, *P. c. costaricensis* in Central America, *P. c. capricornensis* in eastern

South America, *P. c. concolor* in northern South America, *P. c. cabrerae* in central South America, and *P. c. puma* in southern South America (Culver et al., 2000).

The Florida puma (*Puma concolor coryi*) historically has been recognized by the USFWS and FWC to be an endangered subspecies of *Puma concolor* with a remnant population (estimated 150-200) in southern Florida. Currently there is a debate about the validity of the *Puma concolor* subspecies in North America, but regardless of the classification, the Florida puma represents an isolated and endangered population. Prior to 1972, the Florida puma was believed to be extirpated from its former range, but a road-killed puma was discovered in Moorhaven, Florida and an older female puma was captured in a World Wildlife Fund survey at Fish Eating Creek, Florida. Although the Florida puma was protected by both state and federal laws, it was not until the passage of the Endangered Species Act of 1973 that Florida puma research and conservation efforts were initiated. In 1981, radio telemetry and health assessments began and the genetic sustainability of the Florida puma population came into question (Johnson et al., 2010).

It is believed that Florida pumas underwent a genetic bottleneck in the 1960s-1980s which may have resulted in fewer than six breeding individuals (Culver et al., 2008). Inbreeding depression likely resulted in health issues such as atrial defects, cryptorchism, poor sperm quality, increased juvenile mortality, and low kitten survival (Rolke et al. 1993, Mansfield and Land 2002, Facemire et al. 1995, Hostetler et al., 2010). Studies estimated that, due to inbreeding, the Florida puma had a 95% likelihood of extinction within two decades (Johnson et al., 2010). To address this concern, a genetic restoration project was implemented in which eight female puma from Texas were released in 1995-1996 into the Florida puma range. Five of these Texas pumas ultimately produced offspring. Additionally, seven South American pumas (termed

Everglades Florida pumas) were released from a private collection (1957-1967) and eight western United States pumas escaped from the Seminole Indian reservation (1997-1999) and genetic testing indicates that some of these individuals successfully reproduced before recapture (Johnson et al., 2010).

Genetics of Florida pumas: Conservation genetics has garnered much attention in the field of wildlife diseases. Research has indicated that genetically isolated and inbred populations are more susceptible to infection and the expression of clinical disease than outbred, introgressed, or genetically robust populations (Trinkel et al. 2003; Acevedo-Whitehouse et al. 2003; Reid et al. 2003).

Ten years after the release of pumas from Texas, the genetic restoration project was assessed through several genetic and survival studies. Samples were collected from as many trapped and road-killed pumas as well as kittens of known damns. Each puma was genetically classified using twenty-three short-tandem-repeat (STR) loci and each loci was repeated 2-5 times for each individual (Johnson et al., 2010) which allowed for the identification of genetic individuals and the plotting of lineages and determination of the proportion of genetic contribution from different genetic groups. Using spatial and genetic data, probable parents were identified for each puma. First-order inbreeding was observed, even after the genetic introgression and among Texas offspring. The genetic introgression was deemed successful as canonical Florida pumas had a significantly higher incidence of cowlicks, tail kinks and cryptorchidism compared to the other lineages (Barone et al., 1994; Johnson et al., 2010). Although the incidence of atrial defects was not significantly different between the groups, it declined from 21% in births prior to 1995 to 7.5% in births from 1995 to the 2009. Overall, the presence of one or more genetic defects was higher in canonical Florida pumas (70% prevalence)

compared with admixed Florida pumas (<20%). Admixed males also had more, higher quality sperm and larger testicular volume compared with canonical Florida pumas. Finally, kitten, subadult, and adult survival have all increased since admixing (Hostetler et al., 2010; Johnson et al., 2010).

Internal pathogens and parasites of Florida pumas: Due to conservation efforts, infectious diseases are not currently a significant cause of morbidity and mortality for Florida pumas but there are several disease issues that still may be important. Historically, Barron et al. (2004) and Newman et al. (2004) found that mercury may have adversely affected Florida pumas, especially in the early 1990s, but currently, mercury exposure has decreased and no longer poses a threat. Viruses, especially novel ones associated with domestic cats (e.g., FeLV), can be significant pathogens (Luria et al., 2004; Cunningham et al., 2008). Many pumas that were captured during earlier studies and annual examinations were vaccinated against many of these viruses. In free-ranging pumas, antibodies to feline panleukopenia virus (78%), feline calcivirus (56%), feline immunodeficiency virus (37%), and feline infectious peritonitis virus (19%) have been reported (Roelke et al., 1993). In addition antibodies to pseudorabies virus have been detected and this virus can cause mortality (Glass et al., 1994; SCWDS reports). Numerous bacteria, fungi, and parasites have been reported from Florida pumas including *Dermatophilus congolensis*, *Bartonella*, *Spirometra mansonoides*, *Taenia omissa*, *Ancylostoma pluridentatum*, *Ancylostoma caninum*, *Molineus barbatus*, *Physaloptera rara*, *Strongyloides* sp., *Toxocara mystax*, *Alaria marcianae*, *Sarcocystis* sp., *Toxoplasma gondii*, *Babesia* sp., and *Cytauxzoon felis* (Forrester et al., 1985; Greiner et al., 1989; Roelke et al., 1993; Dunbar et al., 1994; Rotstein et al., 1999; Rotstein et al., 2000; Foster et al., 2006; Yabsley et al., 2006; Foster et al., 2009). Limited morbidity and mortality has been observed with these parasites: there have been kitten

mortalities due to *Alaria marcianae* and *Cytauxzoon felis* caused liver abnormalities and mild anemia in three panthers (Harvey et al., 2006; Foster et al., 2009). Prior to 1998, Florida puma females were often diagnosed with proliferative papillary vulvitis of unknown etiology (Rotstein et al., 2002). Currently, the greatest threats to Florida pumas are vehicle-related mortality and intraspecific aggression (Taylor et al., 2002; Schwab and Zandbergen, 2010).

Feline piroplasms: Wild and domestic felids are host to several *Babesia* spp. In domestic cats, several *Babesia* spp. have been reported including *Babesia felis* and *B. lengau* from southern African countries (Penzhorn et al., 2004; Bosman et al., 2007), *B. cati* from India (Mudaliar et al., 1950), *B. canis canis* from Spain and Portugal (Criado-Fornelio et al., 2003), *B. canis presentii* from Israel (Baneth et al., 2004), *Babesia canis vogeli* from Thailand (Simking et al., 2010), a *Babesia microti*-like species (previously referred to as *Theileria annae*) from Portugal (Criado-Fornelio et al., 2003) and the recently described *B. hongkongensis* from a feral cat in Hong Kong (Wong et al., 2012). In addition, uncharacterized small piroplasms have been reported from domestic cats from France, Germany, Thailand, and Zimbabwe (Stewart et al., 1980; Jittapalapong and Jansawan, 1993; Bourdeau, 1996; Moik and Gothe, 1997). Immunocompromised and older cats may be more susceptible to infection, especially if they are coinfected with pathogens such as feline immunodeficiency virus (FIV) or canine distemper virus (CDV) (Barr et al., 1989; Munson et al., 2008). Clinical babesiosis in domestic cats is primarily associated with *B. felis*, but severe disease has recently been associated with *B. lengau* (Bosman et al., 2013) In addition, clinical signs have also been documented in domestic cats infected with *B. canis* subsp. *presentii* and *B. herpailuri* (Stewart et al., 1980; Baneth et al., 2004). No clinical disease was noted in natural and experimental infection of domestic cats with

B. cati and *B. leo*, respectively (Futter et al., 1980; Lopez-Rebollar et al., 1999; Ayoob et al., 2010).

In contrast to domestic cats, wild felids rarely develop clinical disease; however, mortality has been reported in lions experiencing a CDV outbreak and stress due to drought (Munson et al, 2008). *Babesia* species reported from wild felids include *B. lengau* from cheetah (*Acinonyx jubatus*) from South Africa (Bosman et al., 2010), *B. felis* from African wild cats (*Felis silvestris*), caracals (*F. caracal*), cheetahs, lions (*Panthera leo)*, and servals (*Leptailurus serval*) from Africa (Penzhorn et al., 2004; Bosman et al., 2007; Williams et al., 2014), *B. leo* from lions and leopards (*Panthera pardus)* from South Africa and Zambia (Penzhorn et al., 2004; Bosman et al., 2007; Williams et al., 2014), *Babesia pantherae* from the African leopard (Dennig and Brocklesby, 1972), and *B. herpailuri* from the jaguarondi (*Herpailurus yaguarondi*) from Central America (Dennig, 1967). Undescribed species have been reported from the West African civet cat (*Viverra civetta*) (Wenyon and Hamerton, 1930), the Indian leopard (*Panthera pardus fusca*) (Shortt, 1940), pampas cats (*Leopardus pajeros*) and genets (*Genetta tigrina*) in Brazilian zoos (André et al., 2011), bobcats (*Lynx rufus*) (Shock et al., 2013), and the Florida puma (*Puma concolor*) (Yabsley et al., 2006).

Historically, the Florida puma was considered a reservoir host for *Cytauxzoon felis* based on the iatrogenic transmission of the parasite from a Florida puma in 1989 to a domestic cat (Butt et al., 1991). *Cytauxzoon felis* is an Apicomplexan piroplasm which is a significant pathogen of domestic cats in North America. Subsequent examination of blood smears detected a high prevalence of piroplasms which were initially all believed to be *C. felis* based on similar morphology (Rotstein et al., 1999). However, a molecular-based study revealed that the majority of Florida pumas were infected with a novel *Babesia* sp. while only a small percentage were

infected with *C. felis* (Yabsley et al., 2006). The prevalence of *Babesia* in Florida pumas was very high similar to data from raccoons in the US (prevalence >90%) (Birkenheuer et al., 2006; Birkenheuer et al., 2007). In introduced populations of raccoons in Japan, the prevalence was much lower (3 of 348 (0.9%)) (Jinnai et al. 2009).

In general, small piroplasms are morphologically similar; therefore, the use of molecular characterization has become increasingly important in the differentiation of these species. The Florida puma *Babesia* sp. is morphologically indistinguishable from *C. felis* and several small felid *Babesia* spp. (i.e., *B. leo*, *B. felis*, and *B. lengau*) but it is genetically unique (Davis, 1929; Penzhorn et al., 2001; Yabsley et al., 2006; Bosman et al., 2010). Florida pumas naturally infected with the Florida puma *Babesia* sp. had low parasitemias in a previous study (Rotstein et al., 1999) which supports that this is the natural host for this parasite. Similarly, cheetahs and lions naturally-infected with *B. lengau* and *B. leo*, respectively, have asymptomatic infections with low parasitemias which can easily be missed if blood-smear analysis alone is used for diagnosis (Bosman et al., 2010). Serologic cross-activity between *Babesia* spp. antigens of several piroplasm species has been noted in previous studies but no studies have been conducted on the Florida puma *Babesia* sp. (Herwaldt et al., 1996; Lopez-Rebollar et al., 1999; Herwaldt et al., 2003; Prince et al., 2010).

Phylogenetically, the Florida puma *Babesia* is included in a clade of *Babesia* species that have been reported from ticks (*I. ovatus*) from Japan and several carnivores (black bears, *Ursus thibetanus* and raccoons, *Procyon lotor*) from Japan and the United States. The *Babesia*-infected Japanese *I. ovatus* ticks were collected from dogs, thus the host of the *Babesia* spp. Fukui766 and Akita610 are unknown (Inokuma et al., 2003). The *Babesia* sp. Iwate248 (GenBank

AB586027) from the Japanese black bear was only detected in a single animal of unknown history (Ikawa et al, 2011).

Ixodid ticks serve as the vector for all mammalian *Babesia* for which the life cycle has been determined. However, a tick vector has not been identified for any of the feline *Babesia* species nor the *Babesia* from raccoons or the Japanese bear (Birkenheuer et al., 2006, 2007; Ikawa et al., 2011). The *Babesia*-positive *I. ovatus* nymphs and adults from Japan were collected from dogs but it is unknown if the ticks were infected prior to feeding on the dog or if they contained a positive canine blood meal (Inokuma et al. 2003). To date, six tick species have been reported from the Florida puma including *Dermacentor variabilis*, *Dermacentor nitens*, *Ixodes scapularis, Ixodes affinis, Amblyomma americanum*, and *Amblyomma maculatum* (Forrester et al., 1985; Forrester, 1992; Wehinger et al., 1995). But additional studies are needed to identify which tick species, if any, are involved in transmission of the Florida puma *Babesia* sp.

Although tick-borne transmission is the predominate method for *Babesia* spp. transmission, alternative transmission routes have been confirmed or suspected for several *Babesia* spp. (e.g., blood transfusion, vertical, or intraspecific aggression) (Stegeman et al., 2003; Fukumoto et al., 2005; Johnson et al., 2009; Georges et al., 2011). For example, vertical transmission has been confirmed for several piroplasm species including *B. gibsoni* in dogs (Fukumoto et al., 2005), *B. microti* in humans (Fox et al., 2006), a *B. microti*-like sp. in baboons (*Papio cynocephalus*) (Bronsdon et al., 1999), and *T. equi* in horses (Phipps and Otter, 2004; Georges et al., 2011). A previous study identified piroplasms in the blood smear of a 7-day-old Florida puma kitten, but unfortunately, the piroplasm was not identified using molecular techniques (Rotstein et al., 1999). This piroplasm could have been *C. felis* but a limited study of *C. felis* in two pregnant domestic cats failed to document vertical transmission (Lewis et al.,

2012); however, vertical transmission has been documented in *Theileria* spp. (Neitfeld and Pollock, 2002; Baek et al., 2003; Georges et al., 2011). Fighting has been suggested as an alternative route of transmission for *B. gibsoni* among traditional fighting dog breeds including pit-bull-type dogs in the United States and Tosa dogs in Japan (Miyama et al., 2005; Birkenheuer et al., 2005; Yeagley et al., 2009). One of the major causes of mortality (26%) among Florida pumas is intraspecific aggression (Taylor et al., 2002), but it is unknown if the Florida puma *Babesia* can be transmitted during fighting.

Outside of Florida, little work has been done on piroplasms of pumas. A previous study of Brazilian wild captive felids failed to detect *Babesia* spp.in nine pumas; although *Cytauxzoon felis* DNA was amplified from two pumas (André et al., 2009). An additional study of captive and wild felids in Brazil found antibodies to *B. canis* in 11% of 18 pumas, but *Babesia* DNA was not detected in pumas (André et al., 2011). In the United States, *Babesia* has not been reported from domestic cats and only a distinct *Babesia* species has been reported from a single bobcat from Georgia despite testing of nearly 800 bobcats from numerous states in the eastern US (including Florida) (Shock et al., 2013).

Besides the *Babesia* sp. in the Florida puma, there are other piroplasms circulating in wildlife in Florida. A study by Shock et al (2011) found that 44% (n=45) of bobcats from northern Florida were infected with *Cytauxzoon felis*. A study of piroplasms, specifically targeting *B. microti*, in rodents and raccoons in northeast Florida detected *Babesia microti*-like piroplasms in 68% of 31 cotton rats (*Sigmodon hispidus*) and 82% of 17 raccoons (Kerry et al., 2012) but did not detect *Babesia* spp. in 41 Cotton mice (*Permyscus gossypinus*), one flying squirrel (*Glaucomys volans*), three golden mice (*Ochrotomys nuttalli*), three rice rats (*Oryzomys plaustris*), two Virginia opossums (*Didelphis virginianus*), or two wood rats (*Neotoma*

floridana). However, this study had low samples sizes of most rodents and utilized *B. microti* specific primers so some piroplasms may have gone undetected. A study of white-tailed deer in Florida detected both *B. odocoilei* and *Theileria cervi* in 21 deer in northern Florida (Telford and Forrester, 1991a) but failed to find either parasite in 278 blood smears from 1984-1990 in deer from Collier, Monroe and Dade counties in southern Florida. The failure to detect parasites in deer from southern Florida could be to lack of infections or low parasitemias that could be easily missed with blood smear analysis only. A study of thin blood-smears from raccoons collected in Collier (n=170) and Duval (n=14) counties from 1972-1974 found that 41% were infected with piroplasms that were called *Babesia lotori* (Telford and Forrester, 1991b) but based on molecular data, we now know that raccoons are infected with two genetically distinct small piroplasms (Birkenheuer et al., 2008).

Ectoparasites of Florida pumas: *Ticks on pumas*: Ectoparasitic arthropods can cause disease by direct action (blood loss or secondary infections at bite site) or by pathogen transmission. For many fragmented or endangered populations of wildlife, ectoparasites can introduce or exacerbate disease or health issues (Millán et al., 2007; Roelke et al., 2007; Harris et al., 2014). Although ectoparasites can be problematic, they can be threatened or endangered as well, especially if their host(s) are threatened or endangered (Durden and Keirans, 1996; Perez and Palma, 2001; Mihalca et al., 2011). In recent years, there has been an attempt to recognize, understand, and conserve parasites for biodiversity and posterity (Whiteman and Parker, 2005; Pizzi, 2009; Mihalca et al., 2011; Pérez et al., 2013). Understanding ectoparasite natural history is important because zoonotic or veterinary important pathogens can be maintained or transmitted by some ectoparasite species (Shaw et al., 2001; Roelke et al., 2007; Goddard and Varela-Stokes, 2009; Dantas-Torres et al., 2012). The number and diversity medically and

veterinary important arthropod species of Florida have been increasing in recent years due to importation or introduction of exotic hosts with associated parasites and habitat alterations (Burridge, 2001; Keirans and Durden, 2001; Burridge and Simmons, 2003; Corn et al., 2011; Léger et al., 2013).

There have been five studies which focus on or reference ectoparasites removed from Florida pumas (Forrester et al., 1985; Forrester, 1992; Maehr, 1995; Wehinger et al., 1995; Harvey et al., 2007). These studies suggest that ectoparasites on Florida pumas are well studied compared to the only approximately fifteen studies which focus on or reference ectoparasites found on pumas from all of North and South America (Tables 1.1-1.3). Several species of Ixodid ticks have been reported from Florida pumas including *Dermacentor variabilis, Ixodes scapularis, Ixodes affinis, Amblyomma americanum, Amblyomma maculatum,* and *Dermacentor nitens* (Forrester et al., 1985; Wehinger et al., 1995; Harvey et al., 2007). These and other studies have reported: soft ticks (*Carios* sp.) [likely misidentified *Amblyomma* larvae], fleas (*Ctenocephalides felis),* mites (*Lynxacarus* sp., *Eutromibicula splendens* and *Notoedres cati),* and *Lipoptena mazamae* (Forrester et al., 1985; Forrester, 1992; Maehr, 1995; Harvey et al., 2007).

The American dog tick (*Dermacentor variabilis)* is common throughout the eastern US and parts of the western US. The tick is an important vector of several pathogens including *Rickettsia rickettsia* (causative agent of Rocky Mountain spotted fever in humans and dogs), *Cytauxzoon felis* in felids (Blouin et al., 1987; Fritzen et al., 2011).*Dermacentor variabilis* was the second most-common Ixodid tick collected from Florida pumas by Forrester et al. (1985) and Wehinger et al. (1995). *D. variabilis* also infests *P. concolor* in California (Foley et al., 1999) so this tick likely infests pumas throughout their overlapping ranges in North America. Currently *D.*

variabilis has a wide distribution throughout eastern and central United States. In general, ticks of the genus *Dermacentor* are common on pumas outside of the United States (Tables 1.2, 1.3).

Previous research on *D. variabilis* seasonality suggest that adults are active (questing) during late March to August in Georgia (Newhouse, 1983) and from April to July in Florida (McEnroe 1979b; Cilek and Olson, 2000). However, there have been many studies which document *D. variabilis* adults on domestic and wild animals in Florida during most months of the year. A 1984-1990 study of 300 white-tailed deer (*Odocoileus virginianus*) from Collier, Dade and Monroe counties collected *D. variabilis* during the months of August to December (Forrester et al., 1996). A 1979-1981 study of 645 feral hogs from Glades County, Florida found that 99.6% of the collected swine were infested with *D. variabilis* (Greiner et al., 1984). *Dermacentor variabilis* were collected from swine from all four seasons, with the most number of ticks per host being collected in the summer months (Greiner et al., 1984). In contrast, a study from 1997-1999 of *D. variabilis* on white-tailed deer and feral hogs found more ticks/host during the months of August to December (Allan et al., 2001). This tick utilizes smaller hosts for larval and nymphal stages and in a study conducted in Everglades National Park and Hillsborough County, Florida, these stages were found on cotton rats (*Sigmodon hispidus*), rice rats (*Oryzomys palustris*), cotton mice (*Peromyscus gossypinus palmarius*), roof rats (*Rattus rattus*), marsh rabbits (*Sylvilagus palustris paludicola*), raccoons (*Procyon lotor elucus*), and opossums (*Didelphis virginiana*) (Worth, 1950). Foster et al. (2003) did not find *D. variabilis* on coyotes from Collier and Hendry counties, but did collect them from Alachua, Levy, and Pasco counties in north central Florida. A study of feral cats from north central Florida found five female *D. variabilis* from three cats in the month of June (Akucewich et al., 2002). Due to the ambient and

consistent temperatures in the area of Florida where Florida pumas reside, *D. variabilis* is likely active year-round.

The genus Ixodes is highly diverse and includes numerous species in the United States. Many of these *Ixodes* species are competent vectors of *Borrelia burgorferi*, causative agent of Lyme disease. Other important pathogens transmitted by Ixodes spp. include *Anaplasma phagocytophilum* and *Babesia microti*, causative agents of human, equine, and canine anaplasmosis and human babesiosis, respectively (Adelson et al., 2004). Two *Ixodes* spp. have been reported from Florida pumas, *I. scapularis* and *I. affinis*. *Ixodes scapularis* is the most common Ixodid tick found on Florida pumas in terms of prevalence and intensity with Forrester et al. (1985) finding 3-222 ticks per puma (n=7) and Wehinger et al. (1995) finding 0-218 ticks per puma (n=76). Lower intensities were reported for *I. affinis* (Forrester et al., 1985; Wehinger et al., 1995). Outside of Florida, *Ixodes pacificus* has been collected from pumas in California and other *Ixodes* spp. have been collected from pumas in Central and South America (Tables 1.2, 1.3).

Previous research suggests that *I. scapularis* adults begin questing during October and continue questing when the temperatures are above freezing. Adults feed on large animals and once replete, females fall off and begin to lay eggs during May (Carey et al. 1980, Wilson et al. 1990; Cilek and Olson, 2000). Forrester et al. (1996), and Allan et al. (2001) both found that *I. scapularis* were the most abundant tick found on white-tailed deer in southern Florida. Interestingly, Greiner et al. (1984) only infrequently found *I. scapularis* on feral swine in southern Florida, but a subsequent study found that *I. scapularis* was the most abundant tick on feral swine (Allan et al., 2001).

Ixodes affinis is typically found in low numbers of pumas. Forrester et al. (1985) found from one to ten *I. affinis* from four pumas while Wehinger et al. (1995) collected zero to 37 *I. affinis* from 33 puma records. *Ixodes affinis* has a limited distribution and is found in Florida, Georgia, South Carolina, and recently North Carolina (Clark et al., 1998; Harrison et al., 2010). Fewer phenology studies have been conducted on *I. affinis*, but adult ticks have been found questing in January and March through July in North Carolina (Harrison et al., 2010). *Ixodes affinis* has been found on feral swine and white-tailed deer in southern Florida (Greiner et al., 1984; Forrester et al., 1996; Allan et al., 2001).

Amblyomma maculatum serves as a vector of *Rickettsia parkeri* to people and *Hepatozoon americanum* to domestic dogs (Ewing et al., 2002; Paddock et al., 2008). The range of *A. maculatum* is expanding in the Americas and is currently found within the range of several *Puma concolor* populations; however, it has only been collected from Florida pumas (Teel et al., 2010). Forrester et al. (1985) found two *A. maculatum* on one puma, while Wehinger et al. (1995) reported zero to 18 ticks per puma from 15 pumas. Although the range of *A. maculatum* is expanding in the Americas and occurs in the range of *Puma concolor*, it has only been collected from Florida pumas (Teel et al., 2010; Tables 1.1-1.3). The seasonality of *A. maculatum* varies from location, but in the Rio Grande plains and coastal prairie regions of Texas, adults have a peak for questing in September (Barker et al., 2004; Teel et al., 2010). In a study in northwestern Florida, adult *A. maculatum* were questing during August and September (Cilek and Olson, 2000). *Amblyomma maculatum* has been found on coyotes, white-tailed deer, and feral swine in southern Florida (Greiner et al., 1984; Forrester et al., 1996; Allan et al., 2001; Foster et al., 2003).

Amblyomma americanum has an expanding distribution in the United States (Springer et al., 2014). Because this tick transmits numerous pathogens of dog and people including *Ehrlichia* spp., establishment of this tick in southern Florida should be investigated (Paddock and Yabsley, 2007). *Amblyomma americanum* is a rare tick in southern Florida and most reports from pumas are from captive pumas in northern Florida. Forrester et al. (1985) and Wehinger et al. (1995) found *Amblyomma americanum* on pumas from Flagler, Highlands, and Alachua counties in northern Florida. Forrester et al. (1985) found eight ticks on one puma and Wehinger et al. (1995) found zero to six ticks from three pumas. More studies are necessary to determine the distribution of *A. americanum* in southern Florida (Springer et al., 2014). *Amblyomma americanum* has been found on feral swine during the fall in Glades County (Greiner et al., 1984) and on white-tailed deer from several counties throughout the state (Allan et al., 2001). From southern Florida counties where Florida pumas are frequently found, *A. americanum* has not yet been reported from Hendry, Monroe, Miami-Dade, or Lee counties. Because adult *A. americanum* quest from March through August (peak activity in May) and larvae and nymphs quest from June through November (peak July) and February through October (peak September), respectively, in northwestern Florida (Cilek and Olson, 2000), it maybe that *A. americanum* infestation of Florida pumas is under-reported because peak handling months for pumas is December to March. However, because *A. americanum* is so uncommon in southern Florida, there are no phenology studies to determine months of host-seeking activity.

Because of the subtropical climate and large number of introduced species, numerous exotic arthropod have become established in Florida. Historically, *D. nitens* was found on horses in Florida and is a vector of *Babesia caballi*, but it has since be eradicated from the United States (Strickland, 1976). Another exotic *Amblyomma* sp. that has been reported in southern Florida is

Amblyomma auricularum (Wilson and Reeder, 1993; Keirans and Durden, 2001; Guglielmone et al., 2003). This tick typically feeds on the family Dasypodidae, specifically *Dasypus novemcinctus*, but has been reported from numerous families including Myrmecophagidae, Didelphidae, Caviidae, Chinchillidae, Hydrochaeridae, Muridae, Canidae, Mustelidae, Procyonidae and also on several domestic animals such as cattle, dogs, horses (Guglielmone et al., 2003). In Florida, adult ticks were found on a single male armadillo (Mertins et al., 2011) and on a feral swine (Allan et al, 2001). Interestingly, it has not been reported on any felid species in the Americas (Guglielmone et al., 2003).

Other arthropods on pumas: Since 1974, a *Lynxacarus* sp. has been reported from Florida pumas in since 1974, (Whitaker et al., 2007). This parasite has been previously reported as *Lynxacarus morlani* (Forrester, 1992); however, no careful morphological analysis has yet been conducted. In the United States, *L. morlani* and *L. radovskyi* are commonly reported from bobcats (*Lynx rufus*) and domestic cats, respectively (Whitaker et al., 2007). *Lipoptena mazamae*, a hippoboscid fly, is a common parasite of white-tailed deer, but has been found on a wide range of mammals, including pumas and humans (Maa, 1969).

Population structure, genetics, and behavior of pumas: There are relatively few studies that address kinship in wild felids (Culver et al., 2010; Spong and Creel; 2004). Pumas are solitary, territorial animals that show sexual dimorphism in home range size and dispersal rates (Logan and Sweanor, 2002). Females pumas tend to show fidelity to their natal area (Logan and Sweanor, 2002; Biek et al., 2006). Male pumas disperse long distances from their natal areas, a behavior observed in many carnivore species (Biek et al., 2006; Perrin and Goudet, 2001). This behavior in females relates to genetic theories of kin selection while the long-range dispersal of male pumas is believed to limit inbreeding depression (Eizirik et al., 2001). In addition to male

dispersal, female choice to avoid inbreeding has been documented in feral cat populations (Ishida et al., 2000). Females have pre- and post-copulatory behaviors which can affect paternity (Eberhard, 1996; Gowaty, 1994). In non-felid taxa, there are numerous experimental studies which address kin recognition and inbreeding avoidances in female vertebrates (Keane, 1990; Simmons, 1991; Blouin and Blouin, 1988). An observational study in eight feral domestic cats showed that females avoid inbreeding with close kin (Ishida et al., 2000). It has been proposed that animals recognize kin based on major histocompatibility (MHC) genes (Brown and Eklund, 1994; Potts et al., 1994) and some work indicates that mate choice may be associated with MHC compatibility or variability which may be determined by olfactory receptors (Yamazaki et al., 1998). The area of MHC recognition is an area of intense research interest, especially in wild populations (reviewed in Penn, 2002).

The MHC is an important component of the adaptive immune system responsible for self/nonself recognition. Class I and II MHC are found on the surface of antigen-presenting cells (Apanius et al., 1997) and play a role in disease resistance. Interestingly, MHC genes are the most polymorphic genes found in vertebrates and it has been suggested that this is due to either parasite-mediated selection and disease resistances or because of disassortative mating (Potts and Wakeland, 1990; Penn and Potts; 1999; Brown and Eklund, 1994).

The Florida puma population underwent a severe bottleneck (Culver et al, 2000), which contributed to their lack of heterozygosity at microsatellites (Johnson et al., 2010). Researchers have hypothesized that lack of MHC diversity may adversely affect survival (Edwards and Potts, 1996; Mikko et al., 1999; Hedrick 2002) but some studies have shown that MHC diversity may be maintained despite bottleneck related loss in other genes (Aguilar et al., 2004). The link between MHC diversity and parasite-mediated selection is supported by some observational data

in humans (Thursa et al., 1997; Carrington et al., 1999) and feral sheep (Paterson et al., 1998). Some experimental studies have shown significant advantages to MHC heterozygotes in response to infectious diseases (e.g., *T. cruzi* in mice, Trischmann and Bloom; 1982), but more experimental studies are necessary to understand the role of parasite-mediated selection in MHC diversity and fitness.

Studies that examine MHC diversity in animals have increased during the recent decades (Winternitz et al., 2013). In Eurasian beavers (*Castor fiber*), MHC monomorphism was detected in several populations, suggesting that a recent genetic bottleneck may have contributed to less polymorphism (Babik et al., 2005). In a population of Soay sheep (*Ovis aries*), allelic variation was significantly associated with both survival of juveniles and resistance to parasites (Paterson et al., 1998). A study of the MHC class II, Fana-Dab*1 exon in lesser kestrels (*Falco naumanni*) found a role of balancing selection and spatial variation in selection affected MHC (Alcaide et al., 2008). The San Nicolas Island fox (*Urcyon littoralis dickey*), showed extreme monomorphism at neutral loci, but high MHC diversity which was attributed to a population bottleneck and balancing selection (Aguilar et al., 2004). Study of the MHC in the endangered Ethiopian wolf found that the largest population may have enough diversity for the population to persevere despite frequent rabies epidemics (Kennedy et al., 2011). There was no significant MHC diversity found in the class II alleles of the European mink (*Mustela lutreola*), however, many of the minks in the study were captive-bred (Becker et al., 2009). A study of *Coregonus* sp. lake whitefish however, did not find that balancing selection was acting on the MHC (Binz et al., 2001). In the Alpine newt (*Mesotriton alpestris*) two loci were found with different levels of variation due to both genetic drift and selection (Babik et al., 2008). In two hyena species, positive selection for allelic diversity was found in both species despite differences in their

mating systems and behavior (Califf et al., 2013). These studies suggest that there are major differences in the selective forces acting upon the MHC and affecting its diversity among different species.

Researchers have begun to address MHC I and II allelic diversity in wild felids because it is an effective tool to understand selection and disease susceptibility in these often threatened or endangered populations (Yuhki and O'Brien, 1990; Yukhi and O'Brian, 1997; Drake et al., 2004). Smith and Hoffman (2001) studied the diversity of a MHC I locus from the domestic cat, the caracal (*Felis caracal*), Asian golden cat (*Felis temminicki*), the Pallas's cat (*Octobulus manul*), jungle cat (*Felis chaus*), African sand cat (*Felis margarita*), Geoffroy's cat (*Felis geoffroyi*), margay (*Felis wiedi*), and ocelot (*Felis pardalis*). The study found a lot of genetic diversity at a single locus. A study of the MHC class II DRB exon-2 in Eurasian lynx (*Lynx lynx*) in China found a high level of diversity including 13 alleles with 46 variable sites suggesting some level of purifying selection (Wang et al., 2009). Wang et al. (2006) also examined the MHC class II DRB exon-2 locus in the clouded leopard (*Neofelis nebulosa*), the leopard (*Panthera pardus*), and the Amur tiger (*Panthera tigris altaica*) and found that balancing selection may be maintaining the variation of exon 2. Sachdev et al. (2005) looked at the diversity at exon 2 and exon 3 of the MHC class I in Asiatic (*Panthera leo persica*) and Afro-Asiatic hybrid lions. Their findings showed that captive Asiatic lions had lower levels of polymorphism than wild and hybrid lions and that wild lions may not be as inbred as previously believed. Pokorny et al. (2010) studied the allelic variation in MHC class I and MHC class II DRB genes from wild and captive tigers (*Panthera tigris tigris*). Although the sample size was limited, they did find a low number of alleles which may be due to the endangered status of Bengal tigers. Hendrickson et al. (2000) had previously examined an MHC class I loci in three

tiger subspecies including the Bengal (*Panthera tigris tigris*), the Siberian (*P. t. altaica*), and the Sumatran (*P. t. sumatrae*). Their results suggested that on average, captive individuals had higher variability than wild tigers. Castro-Prieto et al (2011) studied the MHC class I and the MHC class II-DRB genes in 25 free-ranging Namibian leopards (*Panthera pardus pardus*) and founder higher sequence variation than most felids and evidence of positive selection affected the MHC diversity. Cheetahs are the felid best studied with regards to MHC, and although captive cheetah disease susceptibility was associated with low MHC II diversity (O'Brien and Evermann, 1988), this was not found in free-ranging populations (Castro-Prieto et al., 2011). Currently there is no data on the MHC for *P. concolor*

Table 1.1. Ectoparasites found on *P. concolor* in the United States

Study	Location	Pumas in Study	Ectoparasites	Infested Pumas	Total (range)	Adults	Adult Female	Adult Male	Nymphs	Larvae
Nicholson and Krausman, 2011	Arizona, United States	4	*Pulex* sp.	3	< 15	*	*			
			Glyptina sp.	1	1	1				
			Argas (*Alveonasus*) *cooleyi*	1	1				1	
Foley et al., 1999	California, United States	47	*Ixodes pacificus*	35	NR	*			*	
			D. variabilis	35	NR	*			*	
Riley et al., 2007	California, United States	4	*Notoedres cati*	2	NR					
Castro and Wright, 2007	California, United States	NR	*I. pacificus*	NR	NR	*			*	
Keirans and Durden, 2001	Washington, United States	1	*Amblyomma ovale*	1						
Uzal et al., 2007	California, United States	2	*N. cati*	2	NR					

Table 1.2. Ectoparasites found on *P. concolor* in the Americas

Study	Location	Pumas in Study	Ectoparasites	Infested Pumas	Total (range)	Adults	Adult Female	Adult Male	Nymphs	Larvae
Young and Goldman, 1946	North America	NR	*Arctopsylla setosa*	NR	NR					
			Dermacentor variabilis	NR	NR					
			Ixodes ricinis§	NR	NR					
			Ixodes cookei§	NR	NR					
	South America	NR	*Amblyomma cajennense*	NR	NR					
			Boophilus microplus	NR	NR					
			Dermacentor cyaniventris	NR	NR					
			Trichodectes felis	NR	NR					
Durden et al., 2006	Paraguay (Chaco)	7	*Pulex simulans*	7	30	30	13	17		
			A. cajennense	6	220	3	3		30	187
			Amblyomma parvum	7	47	47	13	34		
			Amblyomma tigrinum	4	32	1	1			31
			Amblyomma triste	1	1	1	1			
Murgas et al., 2013	Panama	1	*A. ovale*	1	2	2				
Guglielmone et al., 2003	Central and South America	NR	*Amblyomma aureolatum*	NR	2	2	1	1		
		NR	*A. ovale*	NR	72	71	36	35	1	
Nava et al., 2008	Argentina and Brazil	NR	*A. parvum*	NR	>2	>2				

Table 1.3. Table 2 continued. Ectoparasites found on *P. concolor* in the Americas

Study	Location	Pumas in Study	Ectoparasites	Infested Pumas	Total (range)	Adults	Adult Female	Adult Male	Nymphs	Larvae
Marvulo et al., 2005	Brazil	33	*Ixodes aragaoi*	1	NR	*				
			A. aureolatum	3	NR	*				
			A. cajennense	4	NR	*				*
			Amblyomma coelebs	1	NR				*	
			A. ovale	13	NR	*				
			A. parvum	7	NR	*				
			A. tigrinum	3	NR	*				
			A. triste	1	NR	*				
			Amblyomma sp.	6, 5	NR				*	*
			Dermacentor nitens	4	NR	*				
			Boophilus microplus	5, 2	NR	*			*	
Labruna et al., 2002	Porto-Primavera, Brazil	2	*A. cajennense*	1	8					8
			A. coelebs	1	1				1	
			Amblyomma sp.	2	63					63
Autino and Lareschi, 1998	Salta Province, Argentina	NR	*Pulex irritans*	NR	NR					
Aragaõ, 1936; Aragaõ and Fonseca, 1961	Brazil	NR	*A. aureolatum*	NR	NR					
			A. cajennense	NR	NR					
			A. ovale	NR	NR					

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CHAPTER 2

BABESIA CORYI SP. NOV., A NOVEL PARASITE FROM FLORIDA PUMAS (*PUMA CONCOLOR*) FROM SOUTHERN FLORIDA, USA.

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Abstract: Previously, a high prevalence of piroplasms has been reported from Florida pumas (*Puma concolor coryi)* from southern Florida. In the current study, we provide biological, morphological, serological, and molecular data on this novel *Babesia* species. Ring-stage trophozoites were morphologically similar to trophozoites of numerous small babesids of felids including *B. leo, B. felis,* and *Cytauxzoon felis*. Parasitemias in naturally-infected Florida pumas were very low (<1%) and hematologic values of 25 *Babesia*-infected Florida pumas were within normal ranges for *P. concolor coryi*. Phylogenetic analysis of full-length 18S rRNA gene and β-tubulin sequences indicated that this *Babesia* species is a member of the *Babesia* sensu stricto clade and is closely related to *Babesia* spp. from ticks and carnivores in Japan and several species from bovids and cervids. Internal transcribed spacer (ITS)-1 region sequences from this *Babesia* sp. from 19 Florida pumas were 98.8-99.7% similar to each other and ~88% similar to *B. odocoilei.* Similarly, an ITS-2 sequence from one puma was 91% similar to *B. odocoilei*. Infected pumas were positive for antibodies that reacted with *B. odocoilei*, *B. canis,* and *B. bovis* antigens with titers of 1:256, 1:128, and 1:128, respectively. No serologic reactivity was noted for *Theileria equi*. No molecular evidence of congenital infection was detected in 24 kittens born to 11 *Babesia*-infected female pumas. Pumas from other populations in the United States [Louisiana (n=1), North Dakota (n=5) and Texas (n=28)], British Columbia, Canada (n=9), andCosta Rica (n=2) were negative for this *Babesia* sp. Collectively, these data indicate that this *Babesia* sp. is novel and herein we describe the species as *Babesia coryi* sp. nov.

Introduction

Florida pumas are an endangered population of *Puma concolor* which has persisted in southern Florida despite the extirpation of *P. concolor* from the rest of eastern North America.

Because of decreased genetic diversity and evidence of inbreeding depression, eight female Texas pumas were introduced into the range of the Florida pumas in 1995, five of which produced offspring (Johnson et al., 2010). This genetic introgression is credited with restoring population numbers and decreasing genetic defects (Johnson et al., 2010). This population is currently threated by many factors such as habitat loss, intraspecific aggression, and disease (Johnson et al., 2010). Although several pathogens, e.g., feline leukemia virus and pseudorabies virus, have been reported to cause mortalities, the Florida puma hosts several parasites of unknown pathogenicity, some of which may be host-specific (Glass et al., 1994; Miller et al., 2006; Yabsley et al., 2006; Cunningham et al., 2008). The host specificity is important ecologically since when a species becomes extinct or is extirpated, host-specific parasites will also be lost (Perez et al., 2001; Davis et al., 2013).

Worldwide, wild and domestic felids are host to several *Babesia* spp. In domestic cats, several *Babesia* spp. have been reported including *Babesia felis* and *B. lengau* from southern African countries (Penzhorn et al., 2004; Bosman et al., 2013), *B. cati* from India (Mudaliar et al., 1950), *B. canis canis* from Spain and Portugal (Criado-Fornelio et al., 2003), *B. canis presentii* from Israel (Baneth et al., 2004), *Babesia canis vogeli* from Thailand (Simking et al., 2010), a *Babesia microti*-like species (previously referred to as *Theileria annae*) from Portugal (Criado-Fornelio et al., 2003), and the recently described *B. hongkongensis* from a feral cat in Hong Kong (Wong et al., 2012). In addition, uncharacterized species have been reported from domestic cats from France, Germany, Thailand, and Zimbabwe (Stewart et al., 1980; Jittapalapong and Jansawan, 1993; Bourdeau, 1996; Moik and Gothe, 1997). Immunocompromised and older cats may be more susceptible to infection, especially if they are coinfected with pathogens such as feline immunodeficiency virus (FIV) or canine distemper

virus (CDV) (Barr et al., 1989; Munson et al., 2008). Clinical babesiosis in domestic cats is primarily associated with *B. felis*, but severe disease has recently been associated with *B. lengau* (Bosman et al., 2013) In addition, clinical signs have also been documented in domestic cats infected with *B. canis presentii* and *B. herpailuri* (Stewart et al., 1980; Baneth et al., 2004). No clinical disease was noted in natural and experimental infection of domestic cats with *B. cati* and *B. leo*, respectively (Futter et al., 1980; Lopez-Rebollar et al., 1999; Ayoob et al., 2010).

In contrast to domestic cats, wild felids rarely develop clinical disease due to *Babesia* infection; however, mortality has been reported in lions experiencing a CDV outbreak and stress due to drought (Munson et al, 2008). *Babesia* species reported from wild felids include *B. lengau* from cheetah (*Acinonyx jubatus*) from South Africa (Bosman et al., 2010), *B. felis* from African wild cats (*Felis silvestris*), caracals (*F. caracal*), cheetahs, lions (*Panthera leo)*, and servals (*Leptailurus serval*) from Africa (Penzhorn et al., 2004; Bosman et al., 2007; Williams et al., 2014), *B. leo* from lions and leopards (*Panthera pardus)* from South Africa and Zambia (Penzhorn et al., 2001; Bosman et al., 2007; Williams et al., 2014), *B. pantherae* from the African leopard (Dennig and Brocklesby, 1972), and *B. herpailuri* from the jaguarundi (*Herpailurus yaguarondi*) from Central America (Dennig, 1967). Undescribed species have been reported from the West African civet cat (*Viverra civetta*) (Wenyon and Hamerton, 1930), the Indian leopard (*Panthera pardus fusca*) (Shortt, 1940), pampas cats (*Leopardus pajeros*) and genets (*Genetta tigrina*) in Brazilian zoos (André et al., 2011), bobcats (*Lynx rufus*) (Shock et al., 2013), and the Florida puma (*Puma concolor*) (Yabsley et al., 2006).

Only a single report of *Babesia* infection of Florida pumas has been published, with 90% of 39 Florida pumas tested being positive (Yabsley et al., 2006). Genetically, the parasite was related to members of the *Babesia* sensu stricto; however, little is known about the basic biology

of this parasite. In the current study, we synthesize previous data and provide additional data on the morphologic, serologic, and molecular characteristics of this *Babesia* sp. Collectively, these data indicate the parasite is novel and we propose the name *Babesia coryi* sp. nov.

Materials and Methods

Morphologic Characteristics: Blood smears from 10 *Babesia*-infected Florida pumas were fixed in methanol for 3 minutes, stained with Giemsa, and examined with an Olympus CH30 light microscope (Olympus Optical Co., Japan) under oil immersion (1000x). The length and width of each parasite was measured with an ocular micrometer.

Hematological Evaluation: Hematologic data were compiled for 25 *Babesia*-infected free-ranging Florida pumas and compared with data from previous studies on Florida pumas and pumas from other populations (Currier and Russell 1982; Hawkey and Hart 1986; Dunbar et al., 1997; Rotstein et al., 1999; Foster and Cunningham, 2009). The samples were collected from 16 males and nine females from 2000-2005 from several locations in Hendry and Collier Counties in Florida.

Molecular Characterization: Sequences of the 18S rRNA gene from one puma and the β-tubulin gene of two Florida pumas was previously reported (Yabsley et al., 2006). In the current study, the entire 18S rRNA gene sequence and partial β-tubulin gene and the ITS-2 rRNA region were determined for a *Babesia* sample from an additional puma as described (Yabsley et al., 2006; Shock et al., 2011). The 18S rRNA *B. coryi* sp. nov. sequence from FP 44 (GenBank DQ329138) was aligned with piroplasm sequences in GenBank using Mega 6.0 (Tamura et al., 2013). We conducted a Bayesian phylogenetic analysis with a GTR substitution model (10,000

generations and 250 tree burn-in) with Mr. Bayes using Phylogeny.fr (DeReeper et al., 2008) (Figure 2.1).

To investigate intraspecific variation among *Babesia* from Florida pumas, we amplified and sequenced the internal transcribed spacer (ITS)-1 rRNA region from 23 Florida pumas (Shock et al., 2011). Samples originated from numerous counties throughout the Florida puma range (i.e., Collier, Broward, Dade, and Hendry Counties).

Distribution and Prevalence in Pumas: To investigate the prevalence of *Babesia* in Florida and the possible presence of *Babesia* in pumas outside of Florida, DNA extracted from blood or spleen samples from 162 *P. concolor* was tested using an ITS-1 PCR assay as previously described (Shock et al., 2011). Samples originated from 131 Florida pumas from southern Florida, a single puma from Georgia (n=1, genetically confirmed to be a Florida puma), and other subspecies of *P. concolor* from Texas (n=24) (Belden and McCown, 1996), Louisiana (n=1), North Dakota (n=5), British Columbia, Canada (n=9), and Costa Rica (n=2)

Serologic Analysis: Blood samples collected from six *B. coryi* sp. nov. positive pumas were tested for antibodies reactive to *Theileria equi* (n=2), *B. odocoilei* (n=4), *B. canis* (n=2) and *B. bovis* (n=2) antigens in an immunofluorescent antibody (IFA) assay as previously described (Lockhart et al., 1996; Yabsley et al., 2003). Briefly, whole blood was serially diluted from 1:2 to 1:256 with phosphate-buffered saline and placed onto prepared *Theileria equi*, *B. odocoilei*, *B. bovis*, and *B. canis* antigen slides (*B. odocoilei* slides provided by S. Telford and others were purchased from Fuller Laboratories, Fullerton, CA (*B. canis* and *T. equi* slides) and ProtaTek International St. Paul, MN (*B. bovis* slides)) and then washed with phosphate-buffered saline. Antibodies were then incubated with 1:50 FITC-labeled goat anti-cat immunoglobulin G

conjugate (Southern BioTech, Birmingham, Alabama). Slides were examined under a BH2 confocal microscope (Olympus, Japan).

Evaluate possible vertical transmission: Whole blood was collected from 24 kittens, all less than four months of age, born to 11 *Babesia*-positive mothers (range of 1-4 kittens/litter). DNA was extracted from 100 □l of whole blood samples using the DNAeasy extraction kit (Qiagen, Germantown, MD) following the manufacturer's protocol. DNA was tested for *Babesia* spp. using nested PCR targeting the ITS1 rRNA gene as described (Shock et al., 2011).

Results

Description of Babesia coryi sp. nov.

Host(s) and locations: The parasite has been detected in blood samples collected from Florida pumas sampled from 1989 to 2013. A previous survey of Florida pumas reported a prevalence of 95% (37 of 39 pumas) (Yabsley et al., 2006). In the current study, 72% of 100 Florida pumas were PCR positive for *B. coryi* sp. nov. To date, only Florida pumas in their southern Florida (Collier, Hendry, Broward, Monroe, and Dale Counties) range have been infected. Five cougars from Texas that were translocated to Florida were negative for *B. coryi* sp. nov. before their release and two pumas resampled after release remained negative (Rotstein et al., 1999; Yabsley et al, 2006). Additionally, 19 pumas from Texas, introduced into northern Florida in 1993 and removed in 1995 for a reintroduction feasibility study, were also blood-smear negative for piroplasms prior to their introduction to Florida (Belden and McCown, 1996; Rotstein et al., 1999). Our results confirm that they were negative for piroplams; however, some of the pumas did acquire *C. felis* infections while in Florida (Rotstein et al., 1999). All pumas tested from Georgia, Louisiana, North Dakota, Canada, and Costa Rica were negative for

piroplasms. Finally, during a previous study, 799 bobcats sampled from throughout the eastern US were negative for *B. coryi* sp. nov.; however, bobcats sampled in Florida were from northern counties (Shock et al., 2013).

Type specimens: The type host is a Florida puma from Collier Co., Florida, United States. A stained peripheral blood smear from a Florida puma was deposited in the U.S. National Parasite Collection (USNPC, accession number pending). Sequences of the 18S rRNA and β-tubulin genes and ITS-1 and ITS-2 regions for *B. coryi* sp. nov were submitted to GenBank (DQ329138, DQ329138, KJ592628 and KJ592629, respectively).

Description: All parasites observed were ring stage trophozoites and were morphologically similar to *Cytauxzoon*, *Theileria*, and small *Babesia* spp. All parasites measured ~1µm in diameter. No merozoites were observed.

Serologic characteristics: Samples from two *B. coryi* sp. nov.-positive Florida puma samples displayed fluorescence at a 1:128 dilution for *B. bovis* and *B. canis* antigens. Samples from four *B. coryi* sp. nov. positive Florida pumas displayed fluorescence at 1:256 dilution for *B. odocoilei* antigens. No sample displayed cross-reaction for *T. equi*.

Molecular analysis: The full-length 18S rRNA gene sequence of *B. coryi* sp. nov. from a Florida puma (FP40) from Big Cypress National Preserve (BCNP) was identical to a previously reported sequence (1,733 bases, DQ329138) (Yabsley et al., 2006). These sequences were most similar to *Babesia* spp. reported from two ticks removed from dogs in Japan (Fukui766 and Akita610) (98.3%, AY191023 and AY191024). The β-tubulin gene sequence from *B. coryi* from a Florida puma from BCNP was 99% similar to the previously reported sequence (933 bases, DQ329139). These sequence were 88% similar to *B. odocoilei* (AY144706) and 85% similar to *Babesia bovis* (L00978).

Bayesian phylogenetic analysis of an 18S rRNA sequence of *B. coryi* sp. nov. with related organisms (using overlapping 1586bp) included between *Babesia* species detected in two *Ixodes ovatus* ticks (Fukui766 and Akita610) from Japan, and *Babesia* species from various species of ungulates including white-tailed deer and reindeer from the USA and cattle, red deer, and chamois from Europe (Figure 2.1).

The ITS-1 rRNA sequences of *B. coryi* sp. nov. from 23 Florida pumas were all unique and percent identifies ranged from 98.8-99.7%. The sequences varied in size (641-657bp) due to numerous 1-15bp insertions/deletions. No apparent association was noted between ITS-1 sequences and county of origin or year of sampling (range from 1992 to 2005). The *B. coryi* sp. nov. ITS-1 sequences were 87.2-89.4% and 86.8-87.9% identical to *B. odocoilei* (AY339751) and *B. divergens* (EF458168), respectively. A representative sequence was submitted to GenBank (KJ592628, FP40).

The *B. coryi* sp. nov. ITS-2 rRNA sequence obtained from one Florida puma (GenBank KJ592629; FP40) was 244 bp and contained a single polymorphic base. The ITS-2 sequence was 91% similar to *B. odocoilei* from a Minnesota caribou (AY339758) and *Babesia* sp. RD61 from a captive reindeer (*Rangifer tarandus tarandus*) from California (GenBank AY339744).

Assessment of vertical transmission: All 24 kittens were PCR negative for piroplasms. During the year of sampling, all female mothers were confirmed positive for *B. coryi* sp. nov. by PCR.

Knowledge of vector(s): Currently, no vector has been identified for *B. coryi* sp. nov.

Hematological Evaluation: No hematological differences were detected between Florida pumas included in this study and the seven groups of pumas from the five other studies (Table 2.1).

Etymology: *Babesia coryi* is named in honor of Charles Barney Cory, an ornithologist and natural historian, who first described the Florida puma in late 1896 as *Felis concolor floridana* (Cory 1896). The Florida puma was later renamed in his honor, *Puma concolor coryi*.

Discussion

Historically, the Florida puma was considered a reservoir host for *C. felis* based on the iatrogenic transmission of the parasite from a Florida puma in 1989 to a domestic cat (Butt et al., 1991). Subsequent examination of blood smears detected a high prevalence of piroplasms which were initially all believed to be *C. felis* based on similar morphology (Rotstein et al., 1999). However, a molecular-based study revealed that the majority of Florida pumas were infected with a *Babesia* sp. while only a small percentage were infected with *C. felis* (Yabsley et al., 2006). Our data also indicate that a high percentage of pumas are infected. Although the prevalence we detected was lower than that reported by Yabsley et al., (2006), the results are not easily comparable because different PCR methods were used to test samples and pumas tested were from different years and age classes.

In general, small piroplasms are morphologically similar; therefore, the use of molecular characterization has become increasingly important in the differentiation of this group of parasites. *Babesia coryi* sp. nov. is morphologically indistinguishable from *C. felis* and several small felid *Babesia* spp. (i.e., *B. leo*, *B. felis*, and *B. lengau*) but it is genetically unique (Davis, 1929; Penzhorn et al., 2001; Yabsley et al., 2006; Bosman et al., 2010). Similar to other studies, we noted variable serologic cross-activity between *B. coryi* sp. Nov. and antigens of several piroplasm species (Herwaldt et al., 1996; Lopez-Rebollar et al., 1999; Herwaldt et al., 2003; Prince et al., 2010). The highest titers of reaction were noted for *B. odocoilei* antigens which

supports the closer relationship between *B. coryi* sp. nov. and *B. odocoileii* compared with other species tested for serologic cross-reactivity in the current study. Florida pumas naturally infected with *B. coryi* sp. nov. had low parasitemias (<1%) in this study and in a previous study (Rotstein et al., 1999) which supports that this is the natural host for this parasite. Similarly, cheetahs and lions naturally infected with *B. lengau* and *B. leo*, respectively, have asymptomatic infections with low parasitemias which can easily be missed if blood-smear analysis alone is used for diagnosis (Bosman et al., 2010).

For those pumas captured, all *B. coryi* sp. nov.-infected Florida pumas appeared healthy and exhibited normal behavior prior to captures. In general, hematological values of *B. coryi* sp. nov.-infected Florida pumas were within putative normal ranges of wild and captive *P. concolor* (Currier and Russell, 1982; Hawkey and Hart, 1986; Pospíšil et al., 1987; Paul-Murphy et al., 1994; Dunbar et al., 1997; Rotstein et al., 1999; Foster and Cunningham, 2009). Because anemia is a common clinical abnormality of clinical babesiosis (Schoeman et al., 2001), particular attention was given to various erythrocyte values (e.g., mean corpuscular Hb, PCV, and RBC counts), and although variability in data collection and analysis between studies precluded statistical analysis, no consistent differences were noted. Similarly, Rotstein et al (1999), found no differences between several hematologic values between Florida pumas positive for piroplasms based on blood smear and Florida pumas that were blood-smear negative for piroplasms.

Phylogenetically, *B. coryi* sp. nov. is included in a clade of *Babesia* species that have been reported from ticks (*I. ovatus*) from Japan and several carnivores (black bear and raccoons) from Japan and the United States (Yabsley et al., 2006). The *Babesia*-infected Japanese *I. ovatus* ticks were collected from dogs, thus the host of the *Babesia* spp. Fukui766 and Akita610 are

unknown (Inokuma et al., 2003). The prevalence of *B. coryi* sp. nov. in Florida pumas was very high which is similar to the prevalence of *Babesia* spp. from raccoons and skunks in the US (prevalence >90%) (Birkenheuer et al., 2006; Birkenheuer et al., 2007; Yabsley, unpublished data). In introduced populations of raccoons in Japan, the prevalence was much lower (3 of 348 (0.9%)) (Jinnai et al. 2009). The *Babesia* sp. Iwate248 (GenBank AB586027) from the Japanese black bear was only detected in a single animal of unknown history (Ikawa et al, 2011). Sequence analysis of other gene targets (β-tubulin gene, ITS-1, and ITS-2) indicated that *B. coryi* sp. nov. is also related to various cervid and bovid babesids, including *B. odocoilei*, *B. divergens*, *IB. capreoli*, and *B. bovis;* however, sequence data for additional gene targets, e.g., β-tubulin, from *Babesia* spp. from the raccoons, the bear, and the Japanese ticks are needed for comparison. The variability among the ITS-1 sequences was similar to data from other piroplasms (Holman et al., 2003; Aktas et al., 2007).

Ixodid ticks serve as the vector for all mammalian *Babesia* for which the life cycle has been determined. However, a tick vector has not been identified for any of the feline *Babesia* species nor the *Babesia* from raccoons or the Japanese bear (Birkenheuer et al., 2006, 2007; Ikawa et al., 2011). The *Babesia*-positive *I. ovatus* nymphs and adults from Japan were collected from dogs but it is unknown if the ticks were infected prior to feeding on the dog or if they contained a positive canine blood meal (Inokuma et al. 2003). To date, six tick species have been reported from the Florida puma including *Dermacentor variabilis*, *Dermacentor nitens*, *Ixodes scapularis*, *Ixodes affinis*, *Amblyomma americanum*, and *Amblyomma maculatum* (Forrester et al., 1985; Forrester, 1992; Wehinger et al., 1995). Two of these tick species are unlikely vectors because *A. americanum* is rarely found on Florida pumas in their native range and is primarily reported from pumas in northern Florida, and *D. nitens* has not been found on Florida pumas

surveyed from 1989-2014 (Wehinger et al., 1995; Shock unpublished). In previous studies, *D. variabilis* and *I. scapularis* were the two most common ticks detected on pumas (Forrester et al., 1985; Forrester, 1992; Wehinger et al., 1995). Of 12 pumas examined for ectoparasites from 1978-1983, nine had *D. variabilis* (range 1-26 ticks per puma) and seven had *I. scapularis* (3-222 ticks) (Forrester et al., 1985) and Wehinger et al. (1995) reported that of 104 samples from 53 pumas from 1983-1991, 73% had *I. scapularius* (0-218 ticks) and 92% had *D. variabilis* (0-94 ticks). Additional studies are needed to identify which tick species, if any, are involved in transmission of *B. coryi* sp. nov.

Although tick-borne transmission is the predominate method for *Babesia* spp. transmission, alternative transmission routes have been confirmed or are suspected for several *Babesia* spp. (e.g., blood transfusion, vertical, or intraspecific aggression) (Stegeman et al., 2003; Fukumoto et al., 2005; Johnson et al., 2009; Georges et al., 2011). In the current study, we found no evidence of vertical transmission; however, this route has been confirmed for several piroplasm species including *B. gibsoni* in dogs (Fukumoto et al., 2005), *B. microti* in humans (Fox et al., 2006), a *B. microti*-like sp. in baboons (*Papio cynocephalus*) (Bronsdon et al., 1999), and *T. equi* in horses (Phipps and Otter, 2004; Georges et al., 2011). A previous study identified piroplasms in the blood smear of a 7-day-old Florida puma kitten, but unfortunately, the piroplasm was not identified using molecular techniques (Rotstein et al., 1999). This piroplasm in the kitten could have been *C. felis* but a limited study of *C. felis* in two pregnant domestic cats failed to document vertical transmission (Lewis et al., 2012); however, vertical transmission has been documented in various *Theileria* spp. (Neitfeld and Pollock, 2002; Baek et al., 2003; Georges et al., 2011). Fighting has been suggested as an alternative route of transmission for *B. gibsoni* among traditional fighting dog breeds including pit-bull-type dogs in the United States

and Tosa dogs in Japan (Miyama et al., 2005; Birkenheuer et al., 2005; Yeagley et al., 2009). One of the major causes of mortality (26%) among Florida pumas is intraspecific aggression (Taylor et al., 2002), but it is unknown if *B. coryi* can be transmitted during fighting.

The historical distribution of *Babesia coryi* sp. nov. is unknown but testing of pumas from several populations in North and Central America was uniformly negative for *B. coryi* sp. nov. Historically, the puma was widespread throughout North, Central, and South America but the eastern population in North America was extirpated, except for an isolated population in southern Florida that is arguably classified as a separate subspecies by some researchers (Culver et al., 2000) and the US Fish and Wildlife Service. In addition to our data, a previous study of Brazilian wild captive felids failed to detect *Babesia* spp.in nine pumas; although *Cytauxzoon felis* DNA was amplified from two pumas (André et al., 2009). An additional study of captive and wild felids in Brazil found antibodies to *B. canis* in 11% of 18 pumas, but *Babesia* DNA was not detected in pumas (André et al., 2011). In the United States, *Babesia* has not been reported from domestic cats and only a distinct *Babesia* species has been reported from a single bobcat from Georgia despite testing of nearly 800 bobcats from numerous states in the eastern US (including Florida) (Shock et al., 2013).

In summary, we describe *B. coryi* sp. nov as a new species of *Babesia* that utilizes the Florida puma as a natural host. This parasite was found in a high prevalence of sampled pumas and was detected in pumas sampled from 1989 to 2013. Although we present data on the morphologic, molecular, and serologic characteristics of this new species, there are many questions about the natural history. For example, the susceptibility of other felids, including domestic cats, is unknown. Additionally, similar to other felid *Babesia*, the vector has not been determined nor have other alternative routes of transmission been examined. Worldwide, this

represents the sixth species of *Babesia* described from wild felids and only the second felid *Babesia* described from the New World. Because numerous uncharacterized piroplasms have been reported from felids, additional studies are needed to fully understand the diversity and ecology of felid *Babesia* species.

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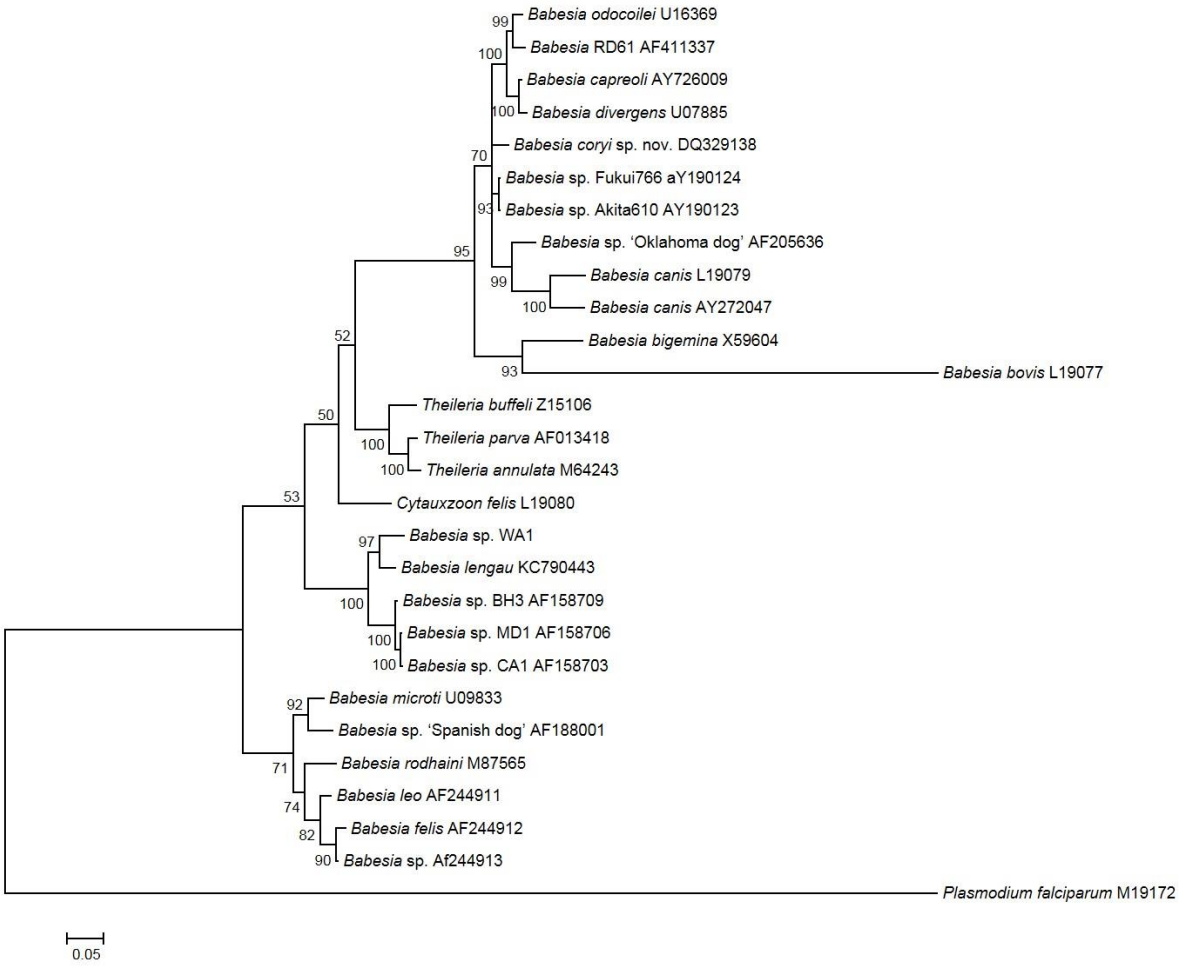

Figure 2.1. Bayesian phylogenetic analysis of 18S rRNA gene sequences of *Babesia coryi* sp. nov. and related piroplasms using a GTR substitution model (10, 000) generations and 250 tree burn-in)

Table 2.1. Hematological values for 25 *Babesia coryi* sp. nov.-infected Florida pumas (2000-2005) compared to hematological values for *Puma concolor* from previous studies.

	Florida (n=25)	Florida (n=63)	Texas (n=28)	Florida (n=32)	London zoo (n=29)	Florida (n=48)	Colorado (n=43)	Colorado (n=22)
Parameters:	Mean (Range)	Mean (SD)	Mean (SD)	Mean (Range)	Mean (Range)	Mean (SD)	Mean (95% CI)	Mean (95% CI)
Age (years)	7.2 (0.6-13.3)	5 (0.02-8)*	5 (0.02-8)*	(3-10)*	NR	(0.5- 13.0)*	non-kitten, 1+	non-kitten, 1+
MC Hb (mg/dl)	10.9 (7.2-14.8)	11.9 (1.7)	12.8 (2.5)	10.8 (3.6-14.8)	13.8 (10.5-17.8)	12.21 (1.70)	17.8 (15.6-20.0)	NR
HCT/PCV (%)	33.7 (22.5-45.7)	37.8 (5.7)	38.2 (8.3)	34.25 (12.9-47.7)	38.0 (27.0-48.0)	36.37 (5.30)	41.8 (38.9-43.7)	46.9 (44.9-48.8)
WBC ($x10^3/\mu L$)	9.5 (4.8-16.6)	11.5 (3.7)	7.8 (3.6)	9.31 (3.9-37.5)	6.4 (4.1-8.2)	12.2 (3.0)	9.6 (4.2-15)	NR
RBC ($x10^6/\mu L$)	7.2 (4.2-10.6)	7.8 (1.3)	7.9 (1.6)	7.11(2.72-10.6)	7.9 (6.0-9.7)	7.635 (1.033)	10.25 (9.04-11.46)	NR
MCV (fl)	47.2 (43-54)	48.6 (6.3)	48.7 (5.4)	48.3(43.0-54.0)	49 (46-54)	47.29 (2.89)	28.76 (25.51-32.01)	NR
MCH (pg)	15.3 (14-17.4)	15.3 (2.7)	16.2 (0.9)	15.26 (13.2-17.0)	17.6 (15.6-19.0)	16.07 (1.41)	NR	NR
MCHC (g/dl)	32.4 (29.8-36.6)	31.1 (3.5)	33.6 (2.3)	31.62 (27.9-34.4)	35.8 (32.0-38.2)	34.08 (3.26)	NR	NR
Platelets ($x10^3/\mu L$)	324.1 (139-642)	NR	NR	324.7 (99-642)	203 (145-280)	402.6 (131.5)	NR	NR
Neutrophils ($x10^3/\mu L$)	6.8 (3.4-14	7.7 (3.1)	4.8 (3.1)	7.1 (2.9-29.3)	4.5 (3.2-6.4)	8.0 (2.9)	NR	NR
Lymphocytes ($x10^3/\mu L$)	1.7 (.5-2.6)	2.8 (1.5)	2.4 (1.7)	1.4 (.4-6.4)	1.7 (.8-2.5)	3.4 (1.7)	NR	NR
Monocytes (cells/µL)	429.2 (121-1020)	563 (299)	298 (299)	300.5(42-744)	100 (0-300)	390 (340)	NR	NR
Eosinophils (cells/µL)	322.2 (0-774)	482 (227)	242 (220)	462.2(0-1570)	100 (0-400)	420 (310)	NR	NR
Basophils (cells/µL)	10.5 (0-124)	58 (49)	73 (111)	5.4(0-124)	NR	100 (60)	NR	NR
Neutrophils (%)	72.8 (50-88)	NR	NR	76.4(61-89)	71 (47-85)	64.3 (14.3)	60.7 (58.5-52.9)	60.7 (58.5-52.9)
Lymphocytes (%)	18.6 (5.0-26)	NR	NR	14.6(7-36)	25 (14-36)	28.8 (14.5)	35.1 (32.7-37.5)	35.1 (32.7-37.5)
Monocytes (%)	4.7 (1.0-12)	NR	NR	3.3(1-6)	(0-5)	3.2 (2.6)	1.9 (1.6-2.2)	1.9 (1.6-2.2)
Eosinophils (%)	3.6 (0-10.0)	NR	NR	5.5 (0-12)	(0-6)	3.4 (2.2)	2.3 (1.9-2.7)	2.3 (1.9-2.7)
Basophils (%)	0.1 (0-1.0)	NR	NR	0.1 (0-1)	0	0.89 (0.57)	NR	NR
Status	free-ranging	free-ranging	captive	free-ranging	captive	free-ranging	captive	free-ranging
Reference	Current study	Rotstein et al. (1999)	Rotstein et al. (1999)	Foster and Cunningham, 2009	Hawkey and Hart (1986)	Dunbar et al. (1997)	Currier and Russell (1982)	Currier and Russell (1982)

*range, SD = standard deviation, CI = confidence interval, NR = not reported, HCT/PCV = Hematocrit/Packed Cell Volume, WBC = white blood cells, RBC = red blood cells, MC= mean corpuscular, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC= mean corpuscular hemoglobin concentration

CHAPTER 3

ECTOPARASITES COLLECTED FROM PUMAS (*PUMA CONCOLOR*) FROM FLORIDA FROM 1989-2014 WITH THE FIRST REPORT OF *AMBLYOMMA AURICULARUM* ON A FELID HOST

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Abstract: The endangered Florida puma (*Puma concolor*) population is one of the world's most-studied felid populations. Although several ectoparasites surveys on the Florida pumas have been conducted, none were conducted after the genetic introgression event which occurred in the mid-1990s. Since this time, many vertebrate and invertebrate species have become established in Florida. This study was conducted to describe the diversity and natural history of ectoparasites on Florida pumas. From January 1989 to May 1993 and January 2000 to April 2014, ectoparasites were collected from free-ranging and captive pumas. Ectoparasites from a total of 262 puma records included six ixodid tick species, one mite (*Lyxacarus* sp.), a Hippoboscid fly (*Lipoptena mazamae*), and a flea (*Ctenocephalides felis*). Most records were from free-ranging pumas from southern Florida in Collier County (n=183) and fewer from Hendry (n=25), Miami-Dade (n=11), Monroe (n=2) and one record each from Lee, Highlands, and Palm Beach Counties. Four records were from pumas from the northern Florida counties of Nassau (n=3) and Flagler (n=1). The tick species were *Ixodes scapularis* (n= 2,014), *Dermacentor variabilis* (n= 771), *Ixodes affinis* (n= 48), *Amblyomma maculatum* (n= 35), *Amblyomma americanum* (n= 59), and *Amblyomma auricularium* (n= 3). This study represents the first report of *A. americanum* in Collier County, Florida and the first report of *A. auricularium* on a wild felid. The mite species, which was previously reported as *Lyxacarus morlani* was determined to be a novel *Lynxacarus* sp. Because many veterinary and medically important pathogens are vector-borne, additional studies should be conducted on pathogens potentially transmitted by ectoparasites commonly found on Florida pumas. This is the most comprehensive study of ectoparasites from Florida pumas which is an apex predictor in the southern Florida ecosystem.

Introduction

Ectoparasitic arthropods can directly cause morbidity or mortality or can transmit pathogens to their hosts. For many fragmented or endangered wildlife populations, significant health issues may arise if novel pathogens are introduced or if animals become more susceptible to disease due to coinfections, stress or immunosuppresion (Millán et al., 2007; Roelke et al., 2007; Harris et al., 2014). Furthermore, many of these ectoparasites also transmit pathogens of zoonotic or veterinary concern (Shaw et al., 2001; Roelke et al., 2007; Goddard and Varela-Stokes, 2008; Dantas-Torres et al., 2012). On the other end of the spectrum, some ectoparasites are host-specific and, if their host(s) are threatened or endangered, these parasites can be imperiled (Durden and Keirans, 1996; Perez and Palma, 2001; Mihalca et al., 2011; Harris et al., 2014). In recent years, there has been an increase in the recognition and understanding for the conservation of parasites for both biodiversity and posterity (Whiteman and Parker, 2005; Pizzi, 2009; Mihalca et al., 2011; Pérez et al., 2013).

Worldwide, almost all wild felids are known or suspected to be suffering population declines. Although there is still much to learn about the natural history of many wild felids, the Florida puma (*Puma concolor cougar* also known as *P. c. coryi*) population is a highly endangered and well-studied felid populations (Johnson et al., 2010; Casa-Marce et al., 2013). Florida pumas are the remaining population of breeding *P. concolor* in eastern North America. Prior to a genetic introgression with pumas from Texas, the Florida puma population suffered from inbreeding depression and low survival rates (Johnson et al., 2010). After the introgression, the population increased to ~150-200 individuals and fitness has increased (Johnson et al., 2010).

To date, the five studies on ectoparasites of Florida pumas reported six tick species (*Dermacentor variabilis*, *Ixodes scapularis*, *Ixodes affinis*, *Amblyomma americanum*,

Amblyomma maculatum, and *Dermacentor nitens)*, three mite species (*Notoedres cati*, *Eutromibicula splendens* and a *Lynxacarus* sp. [reported as *L. morlani*]), a flea (*Ctenocephalides felis*), and a Hippoboscid fly (*Lipoptena mazamae*) (Table 3.1) (Forrester et al., 1985; Forrester, 1992; Maehr et al., 1995; Wehinger et al., 1995; Harvey et al., 2007). These surveys were all conducted prior to the introgression event and in recent years, the diversity of medically and veterinary important arthropod species has been changing in Florida (Burridge, 2001; Keirans and Durden, 2001; Burridge and Simmons, 2003; Corn et al., 2011; Léger et al., 2013). Therefore, the goal of this study was to describe and quantify the ectoparasites collected from Florida pumas to determine if changes in the ectoparasite fauna have occurred over time.

Materials and Methods

From January 1989 to May 1993 and January 2000 to April 2014, ectoparasites were collected from free-ranging and captive Florida pumas in Florida as part of the Florida Fish and Wildlife Conservation Commission's Florida Panther Recovery Project. In addition, ectoparasites were collected from Texas cougars introduced to Florida as part of the genetic introgression program (Johnson et al., 2010). Because not all pumas were marked, individual pumas could have been sampled more than once during the study, thus data are analyzed by records which represent a separate sampling of an individual puma. Efforts were made to collect all ectoparasites from each pumas, however, it is likely that not all were collected, especially mites, fleas, and hippoboscid flies because of the emphasis on ticks. In the current study, all pumas were positive for ectoparasites, but this is likely because data from uninfested pumas were not recorded. Ectoparasites collected prior to 2011 were stored in 70% ethanol and sent to the National Veterinary Service Laboratory (Ames, Iowa) or the Southeastern Cooperative Wildlife

Disease Study (SCWDS, Athens, Georgia) for identification. Ectoparasites from 2011-2014 were shipped alive to SCWDS for identification and were subsequently used in a separate piroplasm (Apicomplexa) transstadial transmission trial (Shock, unpublished).

Data were analyzed by sex, month, year, and intensity. Chi-square statistics were calculated for *I. scapularis* and *D. variabilis* found on male and female pumas during the study using MiniTab 16. Demographic information was not collected on every puma, thus not all variables are analyzed for all 262 records.

Results

Ectoparasites were collected from a total of 262 individual records from pumas from Florida. A total of six ixodid tick species, one mite, a Hippoboscid fly, and a flea were identified from the pumas (Table 3.2). Only two of these records were from Texas cougars released in northern Florida during a previous study (Belden and McCown, 1996). For Florida pumas, most records were from free-ranging pumas from southern Florida with most from Collier County (n=183) and fewer from Hendry (n=25), Miami-Dade (n=11), Monroe (n=2) and one record each from Lee, Highlands, and Palm Beach Counties. Four records were available from captive pumas from the northern Florida counties of Nassau (n=3) and Flagler (n=1).

Two species of *Ixodes*, *Ixodes scapularis* and *I. affinis*, were detected. The most commonly detected tick in the study was *I. scapularis*, all of which were adults. *Ixodes scapularis* infested pumas were detected every year of the study with infestation rates ranging from 0 to 84 ticks per puma (Tables 3.1-3.4). They were found on pumas from every sampled county, except for Flagler. Male pumas (n=100) had 1001 *I. scapularis* over the course of the study, which was significantly higher than the 516 ticks collected from the 73 females (X^2= 690,

p<0.001) (Table 3.2). Seasonality of infestation was noted with *I. scapularis* primarily found on pumas from October to June with most ticks being found between November and March (Table 3.3). *I. affinis* was found in moderate numbers but reports were sporadic in regards to year and month (Tables 3.3, 3.4). Infestation rates were low (maximum of 14 on one puma) and no differences were noted between male and female pumas. Only pumas from Collier and Hendry counties were infested with *I. affinis*.

Dermacentor variabilis was the second most abundant tick collected from pumas (Table 3.2). Adult *D. variabilis* were collected from every month, year, and county sampled. Intensities were low (maximum of 23 ticks) and were lowest during the fall months (Table 3.2, 3.4). Male pumas (n=100) had 410 *D. variabilis*, which was significantly higher than the 203 ticks on the 73 females (X^2= 315, p<0.001)

Three species of *Amblyomma* were found on pumas. There were a total of 46 *A. maculatum* (28 reports) found on pumas from southern Florida (Table 3.2). Infestation rates were low with a maximum of three ticks per puma (Table 3.2). *Amblyomma americanum* was rare with only five infested pumas detected in Collier, Flagler, and Nassau counties. All collections of *A. americanum* were during the summer months (April-June) and occurred sporadically throughout the study in years 1992, 2002, 2005, and 2013 (Table 3.3). Two pumas were infested with *A. auricularum;* a single male puma from Hendry County sampled in July of 2004 had a single larval tick and a female puma from Collier County from January of 2005 was infested with an adult male and a nymph of *A. auricularum* (Table 3.3, 3.4).

Three other ectoparasites were detected on a small number of pumas (Table 3.1). Several life stages of a *Lynxacarus* sp. were present on four pumas from Collier and Hendry counties. Infested pumas were detected in June, November and December of 2004. Based on detailed

morphological examination, this *Lynxascarus* sp. was determined to be an undescribed species which will be described elsewhere. Three pumas from Collier County were infested with the Neotropical deer ked, *Lipoptena mazamae*. In 2013, one flea (*Ctenocephalides felis*) was detected on a captive puma from Nassau County.

Discussion

Previously, there were only five reports of ectoparasites on Florida pumas which collectively reported six species of ticks, three species of mites, a single flea species, and a hippoboscid fly (Forrester et al., 1985; Forrester, 1992; Maehr et al., 1995; Wehinger et al., 1995; Harvey et al., 2007). Only two of these reports were surveys and both specifically targeted Ixodid ticks. The other reports reflected case reports of one or two pumas infested with *Notoedres cati* (clinical case of a neonate) (Maehr et al., 1995), *Lynxacarus morlani* (presumably the novel *Lynxacarus* sp.), *Eutrombicula splendens*, or *Lipoptena mazame* (Forrester, 1992). Since these studies were published, the Florida puma population was supplemented with cougars from Texas as a way to increase genetic diversity within the southern Florida population (Johnson et al., 2010) and numerous exotic vertebrate species have been introduced and/or become established in Florida which could have resulted in the introduction of novel ectoparasites (Burridge et al., 2003; Corn et al., 2011). In the current study, we report the first infestation of a felid with *A. auricularum*, the most southern report of *A. americanum* in Florida (Collier County), and confirm that Florida pumas are important hosts of several tick vectors of veterinary and medically important pathogens.

The majority of ticks we collected from pumas were *I. scapularis*. The maximum intensity and average ticks per puma in our study were slightly lower than reported by Forrester

et al. (1985) and Wehinger et al. (1995) which was probably related to sampling effort. One limitation of our study was that it is likely that not all field personnel collected all ticks from the pumas. Similar to Wehinger et al. (1995), we noted seasonality for *I. scapularis* infestation rates with few adult ticks being found on pumas from May to November. This corresponds with questing activity of adult ticks which begins in October and continues while the temperatures are above freezing and when female begin to lay eggs during May (Carey et al. 1980, Wilson et al. 1990; Cilek and Olson, 2000). Data from two studies of feral swine suggested that *I. scapularis* numbers were increasing in southern Florida between 1979-1981 and 1997-1999 (Greiner et al., 1984; Allan et al., 2001); however, we detected high infestation rates for *I. scapularis* on Florida pumas during each year of our study beginning in 1989 (when >3 pumas examined). Infestation rates were similar between pumas in the current study and white-tailed deer and feral swine reported by Allan et al., (2001).

The second most common tick detected on pumas was adult *D. variabilis* which was also very common in the two previous surveys of ticks from pumas (Forrester et al., 1985; Wehinger et al., 1995). Overall, the intensities were lower compared to the two previous surveys but this difference is likely due to sampling effort in the current study. Similar to data from pumas and feral swine in Florida, we found no seasonal difference in the infestation rates for *D. variabilis* but mean numbers of ticks per puma were highest in summer (Greiner et al., 1984; Wehinger et al., 1995). Despite phenology studies of *D. variabilis* which indicate that adults are most active during late March to August in Georgia (Newhouse, 1983) and from April to July in Florida (McEnroe 1979b; Cilek and Olson, 2000), surveys of numerous wild and domestic animals in southern Florida have documented *D. variabilis* during most months of the year (Greiner et al., 1984; Forrester et al., 1996). This tick is a vector of several important pathogens including

Rickettsia rickettsii to people and dogs and *Cytauxzoon felis* to cats (Blouin et al., 1984; Dantas-Torres, 2007). Based on our data, *D. variabilis* appears to be active year-round, which is likely due to the subtropical climate of southern Florida. Thus, the risk of acquiring *D. variabilis*-transmitted pathogens remains present year-around.

Ixodes affinis has a limited distribution in the United States and is currently found in Florida, Georgia, South Carolina, Virginia, and North Carolina (Clark et al., 1998; Harrison et al., 2010; Nadolny et al., 2014). We detected adult *I. affinis* on 44 pumas, most of which were sampled in February and March which corresponds with host seeking activity of adults in January in North Carolina (Harrison et al., 2010) but contrasts with the finding of adult *I. affinis* on white-tailed deer in southern Florida only in August and October (Forrester et al., 1996). In Florida, *I. affinis* has been found on several large mammals including pumas, bobcats, white-tailed deer, and black bears (*Ursus americanus floridanus*) but they were absent from feral swine (Forrester et al., 1985; Greiner et al., 1985; Wehinger et al., 1995; Allan et al., 2001; Yabsley et al., 2009). The primary hosts of larval and nymphal *I. affinis* are rodents and these ticks and rodents maintain a sylvatic cycle of *Borrelia burgdorferi* sensu stricto (Clark, 2004).

Amblyomma maculatum was the fourth most common tick collected from Florida pumas. This tick has been detected on numerous hosts throughout Florida, including pumas (Greiner et al., 1984; Forrester et al., 1985; Wehinger et al., 1995; Forrester et al., 1996; Allan et al., 2001; Foster et al., 2003). The intensity of *A. maculatum* was low but these studies indicate that pumas are important hosts for this tick which serves as a vector of *Rickettsia parkeri* to people and *Hepatozoon americanum* to domestic dogs (Ewing et al., 2002; Paddock et al., 2008). The range of *A. maculatum* is expanding in the Americas and is currently found within the range of several *Puma concolor* populations; however, it has only been collected from Florida pumas (Teel et al.,

2010). The seasonality of *A. maculatum* questing varies between locations, but in the Rio Grande plains and coastal prairie regions of Texas, adult questing peaks in September (Barker et al., 2004; Teel et al., 2010) and in northwestern Florida, adult *A. maculatum* quest during August and September (Cilek and Olson, 2000). Similar to these studies, the peak numbers of *A. maculatum* were found on pumas in the fall, but adult ticks in the current study were found on pumas sporadically throughout the year suggesting that *A. maculatum* may be host-seeking year-around in southern Florida.

We detected *A. americanum* on pumas from two northern counties (Flagler and Nassau) and one southern Florida county (Collier) which represents the most southern report of this tick in Florida (Springer et al., 2014). Previously, Forrester et al. (1985) and Wehinger et al. (1995) detected low numbers of *A. americanum* on pumas from Flagler, Highlands, and Alachua counties. Other surveys of potential vertebrate hosts in Florida reported *A. americanum* on feral swine as far south as Glades County (Greiner et al., 1984), and bobcats and pumas in Highlands County (Wehinger et al., 1995). The three *A. americanum* ticks detected on a female puma from Collier County were adults which is the first report of *A. americanum* in Collier County (Springer et al., 2013). However, female puma home ranges average 93km^2 but can be larger or smaller given reproductive status so this puma may have acquired the ticks in another county (Dickson et al., 2002). Because this tick transmits numerous pathogens of dog and people, establishment of this tick in southern Florida should be investigated (Paddock and Yabsley, 2007). In addition, in northern Florida, *A. americanum* adults are host seeking during the summer months which represents the season that the fewest Florida pumas have been examined for ticks; however, in southern Florida adult ticks have been collected in winter so continued surveillance

of potential hosts throughout the year is needed to determine if this tick is expanding into southern Florida (Forrester et al., 1985; Wehinger et al., 1995; Cilek and Olson, 2000).

Interestingly, two pumas were infested with larvae, nymphs, and an adult male *Amblyomma auricularium.* This tick has been previously reported from Texas and Florida, but it is considered an exotic tick in the United States (Wilson and Reeder, 1993; Keirans and Durden, 2001; Guglielmone et al., 2003). This tick typically feeds on hosts in the family Dasypodidae (armadillos), specifically the nine-banded armadillo (*Dasypus novemcinctus*), but has been found on representatives of the families Myrmecophagidae, Didelphidae, Caviidae, Chinchillidae, Hydrochaeridae, Muridae, Canidae, Mustelidae, Procyonidae and also on several domestic animals such as cattle, dogs, horses (Guglielmone et al., 2003). Our report represents the first report of this tick on a felid species. In Florida, there have only been three previous reports of this tick species with 13 adult ticks detected on a single armadillo (of 140) sampled from Glades County, 23 adult ticks detected on a single male armadillo from Hendry County and a single tick on a feral pig (of 166) sampled from Collier County (Lord and Day, 2000; Allan et al, 2001; Mertins et al., 2011). The detection of all three mobile stages of this tick on Florida pumas in the current study suggests the tick is established in southern Florida. Because this tick has a wide host range and is a possible vector of '*Candidatus* R. amblyommii', a rickettsial species suspected to cause mild disease in people (Apperson et al., 2008; Nicholson et al., 2009; Saraiva et al., 2013), more research is warranted into the natural history of this tick in the United States.

A novel *Lynxacarus* sp. was found on Florida pumas in this study and it has been reported from pumas in Florida since 1974 (Whitaker et al., 2007). This mite was previously reported as *Lynxacarus morlani* (Forrester, 1992), but careful morphological analysis during this study determined that the parasite collected from Florida pumas is a novel, undescribed species

of *Lynxacarus*. In the United States, *L. morlani* and *L. radovskyi* are commonly detected on bobcats (*Lynx rufus*) and domestic cats, respectively (Whitaker et al., 2007). Because it has been reported from numerous pumas previously and we only detected the parasite on four pumas in a single year, it was likely overlooked in our surveys. A morphologic description of this novel species is needed. *Lipoptena mazamae*, a hippoboscid fly, is a common parasite of white-tailed deer but has been found on a wide range of wildlife species as well as humans (Maa, 1969). Fleas, including *Pulex simulans* in Paraguay, *Polyenis tripopsis* in Brazil, *Pulex porcinus* in Mexico, *Pulex* sp. in Arizona and *Ctenocephalides felis* in Florida have been reported from pumas (Forrester et al, 1985; Linardi and Guimarães, 2000; Eckerlin, 2004; Durden et al., 2006; Nicholson et al., 2011). We only report one flea from 262 records, and Forrester et al. (1985) found one flea on 1 of 12 pumas thus the prevalence of flea infestation of *P. concolor* is poorly understood.

In summary, this is the most comprehensive study of ectoparasites from Florida pumas which is an ecologically important population of pumas and an apex predictor in the southern Florida ecosystem. Because this population of pumas faces many threats, understanding disease risks is very important. Because many pathogens are vector-borne, additional studies should be conducted on pathogens potentially transmitted by ectoparasites commonly found on Florida pumas. Our data suggest that additional work is needed in southern Florida to evaluate the possible southern expansion of *A. americanum*, an important vector of numerous pathogens to people and animals. In addition, these data suggest that the 'exotic' tick, *A. auricularum*, is established in southern Florida. Finally, the seasonality of several tick species present on pumas was similar to data on questing ticks in northern Florida but differences highlight the need for phenology studies on these medically important ticks in southern Florida.

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Table 3.1. Ectoparasites previously reported from *Puma concolor* from Florida, United States.

Study	Location	Years	Puma Status	Pumas in Study	Ectoparasites	No. Infested Pumas	Total No. of ticks if provided (range)
Forrester et al., 1985	Southern Florida, United States	1978-1983	free-ranging	12	*Dermacentor variabilis*	9	(1-26)
					Ixodes scapularis	7	(3-222)
					Ixodes affinis	4	(1-10)
					Amblyomma americanum	1	(8)
					Amblyomma maculatum	1	(2)
					Dermacentor nitens	1	(1)
					Ctenocephalides felis	1	(1)
Wehinger et al., 1995	Southern Florida, United States	1983-1991	free-ranging	53 (104)*	*I. scapularis*	76	1788 (0-218)
					I. affinis	33	228 (0-37)
					D. variabilis	96	1170 (0-94)
					A. maculatum	15	33 (0-18)
					A. americanum	3	32 (0-6)
Forrester, 1992	Southern Florida, United States	1985	free-ranging	1	*Lynxacarus morlani**	1	NR
					Eutrombicula splendens	1	NR
				2	*Lipoptena mazamae*	2	(1-2)
Maehr et al., 1995	Southern Florida, United States	1992	free-ranging	2	*Notoedres cati*	2	NR
Harvey et al., 2007	Northern Florida, United States	1989, 1995	captive	3	*Amblyomma americanum*	1	numerous
					unidentified soft ticks*	1	numerous

*Subsequent samples of Lynxacarus from Florida pumas have been identified as a novel *Lynxacarus* sp. so this identification is likely in error. *These ticks were not identified and were noted on a clinical case record. No ticks were saved for identification and given the time of year and because adult *A. americnum* was on the puma, these ticks were likely engorged larvae or nymphs of *A. americanum*.
*There were 53 individual pumas in the study, but some were captured more than once, resulting in 104 records

Table 3.2. Ectoparasites identified from 262 records from Florida pumas

Ectoparasite species	County	No. records (% of 262 records)	Total number of ectoparasites	Mean No. of ectoparasites per puma (Range)
Acarina: Ixodidae				
Dermacentor variabilis adults	Collier, Miami-Dade, Flagler, Hendry, Highlands, Lee, Monroe, Nassau, and Palm Beach	204 (77.9)	990	3.8 (0-23)
Ixodes scapularis adults	Collier, Miami-Dade, Hendry, Highlands, Monroe, and Nassau	203 (77.5)	2,339	8.9 (0-84)
Ixodes affinis adults	Collier, and Hendry	44 (16.8)	88	0.3 (0-10)
Amblyomma maculatum adults	Collier, Hendry, and Monroe	28 (10.7)	46	0.2 (0-3)
Amblyomma americanum				
Nymphs	Flagler and Nassau	2 (0.8)	13	0.05 (0-12)
Adults	Collier, Flagler, and Nassau	5 (1.9)	46	0.2 (0-20)
Amblyomma auricularum				
Larva	Hendry	1 (0.4)	1	0.004 (0-1)
Nymphs	Collier	1 (0.4)	1	0.004 (0-1)
Adults	Collier	1 (0.4)	1	0.004 (0-1)
Acarina: Astigmata				
Lynxacarus sp.	Collier and Hendry	4 (1.5)	33*	0.13 (0-24)
Diptera: Hippoboscidae				
Lipoptena mazamae	Collier	3 (1.1)	4	0.02 (0-2)
Insecta: Siphonapera				
Ctenocephalides felis	Nassau	1 (0.4)	1	0.004 (0-1)

*Five adult males, 11 adult females, 11 nymphs, and 6 larvae.

Table 3.3. Seasonality of adult ticks collected from 220 Florida puma records for which month of collection was available.

Season	Month	No. Records	Tick Species Total No. of Ticks [Range] (Mean number of ticks/puma) *Dermacentor variabilis*	*Ixodes scapularis*	*Ixodes affinis*	*Amblyomma maculatum*	*Amblyomma americanum*	*Amblyomma auricularium*
Winter	December	18	49	375	2	1	0	0
	January	33	71	311	5	5	0	1 [0-1] (3)
	February	66	260	635	17	5	0	0
	Total	**117**	**380 [0-21] (3.2)**	**1321 [0-84] (11.3)**	**24 [0-6] (0.2)**	**11 [0-2] (0.09)**	**0**	**1 [0-1] (0.9)**
Spring	March	35	125	386	13	2	0	0
	April	13	28	68	2	0	5 [0-5] (7.7)	0
	May	12	57	2	2	0	3 [0-3] (8.3)	0
	Total	**60**	**210 [0-12] (3.5)**	**456 [0-64] (7.6)**	**17 [0-8] (0.3)**	**2 [0-2] (0.03)**	**8 [0-5] (0.1)**	**0**
Summer	June	10	56	1	0	0	38 [0-20] (3.8)	0
	July	7	23	0	0	5	0	0
	August	3	31	0	2	4	0	0
	Total	**20**	**110 [0-21] (5.5)**	**1 [0-1] (0.05)**	**2 [0-1] (0.1)**	**9 [0-3] (0.5)**	**38 [0-18] (1.9)**	**0**
Fall	September	3	7	0	0	1	0	0
	October	6	4	14	3	2	0	0
	November	14	60	222	2	9	0	0
	Total	**23**	**71 [0-8] (3.1)**	**236 [0-38] (10.3)**	**5 [0-2] (0.2)**	**13 [0-3] (0.6)**	**0**	**0**
TOTAL		**220**	**771 (3.5)**	**2014 (9.2)**	**48 (0.2)**	**35 (0.2)**	**46 (0.2)**	**1 (0.5)**

Table 3.4. Species and stage of Ixodid ticks on Florida pumas from 1989-1993 and 2000-2014.

		Tick species and stage Number of ticks collected [range] (percent of positive records)								
		Dermacentor variabilis	*Ixodes scapularis*	*Ixodes affinis*	*Amblyomma maculatum*	*Amblyomma americanum*		*Amblyomma auricularium*		
	No. Records	adult	adult	adult	adult	adult	nymph	adult	nymph	larva
1989	5	36 [0-12] (60)	24 [0-20] (80)	2 [0-2] (20)	0	0	0	0	0	0
1990	9	45 [0-12] (78)	31 [0-10] (89)	2 [0-1] (22)	1 [0-1] (11)	0	0	0	0	0
1992	4	1 [0-1] (25)	32 [0-19] (100)	0	0	7 [0-7] (25)	0	0	0	0
1993	10	21 [0-5] (80)	54 [0-2] (80)	5 [0-2] (40)	0	0	0	0	0	0
2000	1	1 [0-1] (100)	0	1 [0-1] (100)	0	0	0	0	0	0
2001	6	14 [0-8] (67)	35 [0-15] (67)	0	3 [0-3] (17)	0	0	0	0	0
2002	14	55 [0-11] (86)	271 [0-114] (93)	11 [0-10] (14)	4 [0-2] (21)	20 [0-20] (7)	0	0	0	0
2003	20	72 [0-21] (70)	149 [0-52] (65)	6 [0-2] (20)	1 [0-1] (5)	0	0	0	0	0
2004	37	155 [0-24] (78)	343 [0-139] (65)	7 [0-2] (14)	10 [0-4] (11)	0	0	0	0	1 [0-1] (3)
2005	29	114 [0-14] (86)	393 [0-88] (69)	4 [0-2] (10)	6 [0-2] (14)	14 [0-11] (7)	12 [0-12] (6)	1 [0-1] (3)	1 [0-1] (3)	0
2006	13	60 [0-12] (92)	90 [0-35] (92)	7 [0-6] (15)	1 [0-1] (8)	0	0	0	0	0
2007	1	4 [0-4] (100)	0	0	0	0	0	0	0	0
2008	2	3 [0-2] (100)	0	0	0	0	0	0	0	0
2009	13	55 [0-22] (77)	131 [0-28] (62)	1 [0-1] (8)	3 [0-2] (15)	0	0	0	0	0
2010	22	46 [0-8] (86)	172 [0-29] (82)	2 [0-1] (9)	0	0	0	0	0	0
2011	2	3 [0-3] (50)	9 [3-6] (100)	0	0	0	0	0	0	0
2012	13	37 [0-11] (62)	71 [0-18] (92)	0	1 [0-1] (8)	0	0	0	0	0
2013	7	22 [0-11] (71)	78 [0-49] (100)	0	4 [0-2] (29)	5 [0-5] (14)	1 [0-1] (14)	0	0	0
2014	12	27 [0-12] (50)	131 [0-46] (92)	0	1 [0-1] (8)	0	0	0	0	0
TOTAL	220	771 [0-24]	2,014 [3-139]	48 [0-10]	35 [0-4]	46 [0-20]	13 [0-12]	1 [0-1]	1 [0-1]	1 [0-1]

CHAPTER 4

TRANSOVARIAL TRANSMISSION OF A *BABESIA* SP. BY *IXODES SCAPULARIS* COLLECTED FROM FLORIDA PUMAS (*PUMA CONCOLOR CORYI*)

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Scientific Note

The Florida puma population (*Puma concolor*) is a highly endangered felid population (Johnson et al., 2010). Recently, a novel *Babesia* sp. was identified in Florida pumas (Yabsley et al., 2006) and the high prevalence and extensive ITS-1 variation suggests that this *Babesia* sp. is endemic to Florida pumas, and to date, no health issues have been associated with this parasite (Shock et al. submitted). However, coinfection with *Babesia* and canine distemper virus during climate extremes caused significant mortality in South African lion (*Panthera leo*) populations and because endangered or fragmented populations of animals may be at increased risk of disease or stochasitic events, research on seemingly nonpathogenic parasites is important (Munson et al., 2008).

Ixodid ticks serve as the vector for all mammalian *Babesia* for which the life cycle has been determined; however, a tick vector has not been identified for any of the feline *Babesia* species. We hypothesize that the Florida puma *Babesia* sp. is transmitted by an Ixodid tick and based on data from a recent ectoparasite study, *Dermacentor variabilis* and *Ixodes scapularis* are the two most common and abundant ticks on Florida pumas. This study was conducted to determine if the Florida puma *Babesia* sp. is transovarially transmitted by either of these two tick species that had been removed from Florida pumas.

From February 2012 to April 2014, ticks were collected from free-ranging and captive Florida pumas as part of the Florida Fish and Wildlife Conservation Commission's Florida Panther Recovery Project. Ticks were carefully removed with forceps to preserve mouthparts and placed in sterile polypropylene ventilated 50ml vials (Corning Inc. Life Sciences, Acton, MA) with several grass blades placed inside to maintain moisture during shipment. Ticks were shipped alive to the Southeastern Cooperative Wildlife Disease Study (SCWDS), Athens,

Georgia for identification and processing. Partially and fully engorged female ticks were placed in individual sterile ventilated 50ml polypropylene vials which were maintained at 94% humidity in chambers containing saturated potassium nitrate (Sigma- Aldrich, St. Louis, MO). Chambers were covered with transparent cloth to stimulate presence of leaf litter. Temperature was monitored daily and the average temperature was 23 C. Once the females laid eggs or died prior to laying, they were placed in 70% ethanol until identification. Approximately ¼ of each egg mass was placed in 70% ethanol while the remainder of the eggs were placed in sterile ventilated 50ml polypropylene vials which were maintained in a humidity chamber.

Once larvae hatched, they were placed on ice for 30 minutes and then were placed in 70% ethanol. From each batch of larvae, three random subsets of ~25 larvae were placed in sterile 1.5mL microcentrifuge tubes (VWR Scientific, Suwanee, GA) which were dipped into liquid nitrogen until ticks were frozen. Contents of the vial were then macerated with a sterile plastic pestle and DNA was extracted with a Qiagen Blood and Tissue DNA kit (Germantown, MD). Subsets of eggs from each mass were treated similarly to larvae and DNA extracted as described.

The internal transcribed spacer (ITS)-1 rRNA region of *Cytauxzoon* spp., *Theileria* spp., and *Babesia* spp. was amplified as previously described (Shock et al., 2014). All amplicons >200 bp were purified with a Qiagen gel extraction kit (Germantown, MD) and bi-directionally sequenced at the Georgia Genomics Facility (Athens, GA). All chromatogram data were analyzed with Sequencher 5.1 (Ann Arbor, MI). Sequences were aligned in MEGA 5.2 and compared to sequences in GenBank. Sequences were aligned with those from related organisms obtained from GenBank using a basic local alignment search tool (BLAST) search (National Center for Biotechnology Information, Bethesda, MD, USA) (Altschul et al., 1990).

A total of 390 Ixodid ticks were submitted for this study, of which 46 were morphologically identified as *D. variabilis* females and 180 were *I. scapularis* females. The ticks were in various states of engorgement, but if they appeared to be at least half engorged, they were maintained for egg laying. The 30 *I. scapularis* female ticks which were placed in the humidity chamber came from 18 collections from 11 free-ranging pumas in Collier and Hendry Counties and captive pumas in Nassau County. Of the 11 pumas, blood samples were available for five of them and one of the pumas was positive for a *Babesia* sp. (Shock et al submitted).

Only 10 *Ixodes scapularis* females (30% of *I. scapularis* females) laid eggs, after an average of 17.9 days. Larvae hatched between 48 to 61 days. Seven of the 10 (70%) ticks had at least one PCR positive batch of eggs or larvae. Two ticks (29%) had PCR positive eggs only, four (57%) had PCR positive larvae only, and one tick (14%) had positive egg and larvae batches. Eight of the positive amplicons from five of the ticks were sequenced (352bp) and they were 97.5% similar to each other and a representative sequences was 70% similar to *Babesia rodhaini* (GenBank AF510201). Only minimal similarity (~23%) was noted with *B. microti*, a very close relative of *B. rodhaini*. A representative sequence was submitted to GenBank (pending). None of the *D. variabilis* females laid eggs.

Although we did not detect the Florida puma *Babesia* sp. in *I. scapularis* egg or larvae batches, we did identify sequences of a different *Babesia* sp. related to *B. rodhaini*. These data do not necessarily exclude *I. scapularis* as a vector of the Florida puma *Babesia* sp. because the pumas may not have been infected when the ticks were removed or the parasitemias may have been very low resulting in few infected ticks. In addition, the sample size for this study is small; however, many of the ticks were not engorged. Unfortunately, none of the five *D. variabilis* placed in the humidity chamber deposited eggs during this study, probably because they were not

fully engorged. Another common tick on Florida pumas is *Ixodes affinis* (Wehinger et al., 1995); however, none were submitted for the current study. Finally, the *Babesia* sp. of Florida pumas may not be vector-borne, alternative transmission routes have been confirmed or are suspected for several *Babesia* spp. (e.g., vertical, or intraspecific aggression) (Matsuu et al., 2004; Fukumoto et al., 2005; Georges et al., 2011). A recent study on this parasite failed to find evidence of vertical transmission but possible transmission by intraspecific aggression has not be studied (Shock et al., submitted).

Based on ITS-1 sequences, the *Babesia* sp. we detected was most similar to *Babesia rodhaini*, a rodent parasite. *Babesia rodhaini* was originally isolated from a wild rodent in Africa, and despite being used worldwide in numerous laboratory experiments, it has not been detected in free-ranging rodents since its original description (Kawabuchi et al., 2005), although it has been detected in *Ixodes ricinus* and *Rhipicephalus turanicus* which had fed on roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*), respectively, from Italy (Iori et al., 2010). Based on numerous gene targets, *B. rodhaini* is closely related to *B. microti* and *B. microti*-like species (Zahler et al., 2000; Yamasaki et al., 2007; Nakajima et al., 2009). Currently, *B. rodhaini* is classified as a strain of *B. microti* by the American Type Culture Collection (ATCC® 30222™), although these species have not been formally synonymized. *Babesia microti* is a species complex that includes known zoonotic strains and others that are considered rodent-specific (Vannier et al., 2008; Nakajima et al., 2009). In addition, there are numerous *B. microti*-like species reported from various wild mesomammals such as raccoons (*Procyon lotor*), striped skunks (*Mephitis mephitis*), red fox (*Vulpes vulpes*), and river otters (*Lontra canadensis)* (Birkenheuer et al., 2007; Birkenheuer et al., 2008; Birkenheuer et al., 2010; Kerry et al., 2012; Cardoso et al., 2013; Yabsley, unpublished). Unfortunately, we were unable to obtain sequences

of other gene targets and there are no ITS-1 sequences available for the mesomammal *B. microti*-like parasites, thus a more precise phylogenetic relationship of the parasite we detected in *I. scapularis* with other *Babesia* species was not possible. However, our ITS-1 sequences were distinct from rodent- and human-infecting *B. microti* strains.

Although there is very high diversity of piroplasms that infect wildlife, most of these *Babesia* spp. have unknown vectors (Yabsley and Shock, 2013). Screening of questing ticks for piroplasms can suggest potential vectors for pathogens but, unfortunately, many surveillance studies on ticks rely on ticks that have been removed from hosts, thus they are contaminated with host blood (Shock et al., 2014). In addition, the prevalence of *Babesia* in questing ticks is usually very low (e.g., Svehlová et al., 2014; Shock et al., 2014). Collection of partially and fully engorged female ticks from wildlife and allowing them to oviposit followed by testing of larvae is an inexpensive strategy to detect transovarially transmitted pathogens. If possible, collection times for ticks should overlap with the peak time of attachment to hosts to maximize the number of engorged ticks; however, the phenology of many tick species is poorly understood, especially across their entire geographic range. Similar work has been done with engorged larvae being removed from potential reservoir hosts to evaluate reservoir competence and ability of certain ticks to maintain pathogens transstadially (Hersh et al., 2012). Combining these two techniques would increase our understanding of both vector and reservoir competence for *Babesia* spp. of wildlife, many of which have poorly understood life cycles.

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CHAPTER 5

DIVERSITY OF THE MHC CLASS I AND MHC CLASS II DRB LOCI IN THE ENDANGERED FLORIDA PUMA AND OTHER *PUMA CONCOLOR*

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Abstract: The Major Histocompatibility Complex (MHC) is a component of the adaptive immune system involved in self/non-selfrecognition and disease susceptibility. Some endangered species maintain high genetic diversity at the MHC despite repeated population bottlenecks. Florida pumas, an endangered population of Puma concolor (approx. 150 individuals) in southern Florida, underwent a bottleneck in the 1980s with as few as three breeding individuals over two generations. To reverse inbreeding depression, in 1995 the population was introgressed with five female Texas cougars. We hypothesized that the Florida puma MHC diversity increased as a result of this introgression event. Functionally important antigen-binding sites from the MHCI and MHCII loci were amplified with a touchdown PCR using high-fidelity polymerase. Additionally, piroplasm prevalence was detected using nested PCR. Using next-generation sequencing (Roche 454 andIllumina MiSeq), we are determining allelic diversity of the MHC I and II in Florida pumas respectively. It is currently unclear from our data if the Florida puma MHC was affected by the introgression event. The MHC I was highly conserved and we only found 39 variable sites. The techniques used to assemble and demultiplex the MHC II gene did not yield as many sequences as necessary for this study; but we did see some potential spatial clustering of related pumas. Future studies with these data will address parasite- and sexual-mediated selection of MHC genotypes. Because most felid species are threatened or endangered, these data could have important implications for wild felid conservation.

Introduction

The Florida puma (*Puma concolor coryi*), recognized by the USFWS and FWC to be an endangered subspecies of *Puma concolor,* has a remnant population (estimated 150-200) in

southern Florida. It is believed that Florida pumas underwent a genetic bottleneck in the 1960s-1980s which may have resulted in fewer than six breeding individuals (Culver et al. 2008). Inbreeding depression led to issues such as atrial defects, cryptorchism, poor sperm quality, increased juvenile mortality, and low kitten survival (Rolke et al. 1993, Mansfield and Land 2002, Facemire et al. 1995, Hostetler et al., 2010). Studies estimated that, due to inbreeding, the Florida puma had a 95% likelihood of extinction within two decades (Johnson et al., 2010). To address the genetic issues plaguing the Florida puma, a genetic restoration project was implemented

The major histocompatibility complex is an important component of the adaptive immune system responsible for self/nonself recognition. Class I and II MHC are found on the surface of antigen-presenting cells (Apanius et al., 1997) and play a role in disease resistance. Interestingly, MHC genes are the most polymorphic genes found in vertebrates and it has been suggested that this is due to either parasite-mediated selection and disease resistances or because of disassortative mating (Potts and Wakeland, 1990; Penn and Potts; 1999; Brown and Eklund, 1994). Researchers have hypothesized that lack of MHC diversity may adversely affect survival (Edwards and Potts, 1996; Mikko et al., 1999; Hedrick 2002). The Florida puma population underwent a severe bottleneck (Culver et al, 2008), which contributed to their lack of heterozygosity at microsatellites (Johnson et al., 2010). Some studies have suggested that MHC diversity may be maintained despite bottleneck related loss in other genes (Aguilar et al., 2004).

Studies that examine MHC diversity in animals have increased during the recent decades (Winternitz et al., 2013). The San Nicolas Island fox (*Urcyon littoralis dickey*), showed extreme monomorphism at neutral loci, but high MHC diversity which was attributed to a population bottleneck and balancing selection (Aguilar et al., 2004). Researchers investigate MHC I and II

allelic diversity in wild felids because it is an effective tool to understand selection and disease susceptibility in these often threatened or endangered populations (Yuhki and O'Brien, 1990; Yukhi and O'Brian, 1997; Drake et al., 2004). Cheetahs are the felid best studied with regards to MHC, and although captive cheetah disease susceptibility was associated with low MHC II diversity (O'Brien and Evermann, 1988), this was not found in free-ranging populations (Castro-Prieto et al., 2011). Currently there is no data on the MHC genes for *P. concolor*.

This study was designed to investigate the allelic diversity of the MHC I and the MHC II in Florida pumas before and after the introgression event. We compared these data with data on other *P. concolor* as well to understand the diversity of the Florida puma population compared to other pumas. We utilized a next-generation sequencing approach (Illumina MiSeq and Roche 454) to increase our sequence coverage. With the large number of sequences generated in these methods, total allelic diversity should be available without the use of Sanger sequencing and cloning. This is the largest study of MHC in a felid population to date and the first study in *P. concolor*.

Methods

Samples for this study were obtained from previous studies (Johnson et al., 2010; Shock et al., 2011; André et al., 2009; Shock et al. submitted). Because of a longitudinal study, we had over 200 Florida puma samples from 1980-present, including the five Texas females. Additionally, we had samples from puma populations in Brazil (n=5), Canada (n=9), Costa Rica (n=2), and the western United States (n=26). Genomic DNA was extracted using the Qiagen DNA blood and tissue kit (Germantown, MD), following the manufacturers protocol.

We used oligonucleotide forward primer AJDRBa ln1Ex2_F (5'-CCTGTSYCCACAGCACATTTCYT-3') and reverse primer AJDRB Ex2ln2_R (5'-GCTCAMCTCGCCGSTGCAC-3') described by Castro-Prieto et al. (2011a) to amplify the MHC II-DRG gene that contains the second exon (280bp). We also used the oligonucleotide primers Acju_Ex2Mhcl_cF (5'-CCTGTSYCCACAGCACATTTCYT-3') and Acju_Ex3Mhcl_eR (5'-CCTGTSYCCACAGCACATTTCYT-3') to amplify a region of the MHC I gene which contains Exon 2, intron 2, and exon 3 (720bp). These primers have amplified these gene targets from Namibian cheetahs (*Acinonyx jubatus*) and leopards (*Panthera pardus pardus*) (Castro-Prieto, 2011a, 2011b).

All PCRs were performed in a reaction volume of 25 μl, each containing 0.5 mM of each primer (50mM, Sigma-Aldrich), 13.5uL of RNase-Free Water, 5ul of 5X HotStar HiFidelity PCR Buffer (incl. dNTPs), and 0.5ul of Qiagen HotStar HiFidelity DNA Polymerase. PCR was performed with an initial denaturation step at 96°C for 10mins followed by 15 cycles of 95°C for 30s, 60°C down 0.5°C each cycle, and an extension step of 72°C for 1min and then 25 cycles of 95°C for 30s, 50°C for 1min and 72°C for 1min with a final extension step of 72°C for 5mins. All bands of appropriate size were excised with a scalpel and extracted with a Qiagen gel extraction kit (Germantown, MD). A subset of each were bi-directionally Sanger sequenced at the Georgia Genomics Facility (Athens, GA) and their chromatogram data were analyzed with Sequencher 5.1 (Ann Arbor, MI) to check primers. Sequences were aligned in MEGA 6.0 and compared to sequences in GenBank. Sequences were aligned with those from related felids and mammals obtained from GenBank using a basic local alignment search tool (BLAST) search (National Center for Biotechnology Information, Bethesda, MD, USA) (Altschul et al., 1990).

To assign reads to specific individuals, 6-8-bp tags were used to create 12 forward and 12 reverse 5' tagged primers that resulted in 144 unique forward-tagged and reverse-tagged primer pairs (tags provided by UGA Genomics, Table 5.1). These tags sequences were developed to have an edit (Levenshtein) distance of five, whereby five mutations are required for one tag to transform into another sequence (Faircloth & Glenn 2011). Individual PCR products (20uL) were plated into two libraries per sequencing type (288 individuals) and submitted to Georgia Genomics (Athens, GA) for library prep and sequencing. Samples were pooled into two libraries and the MHCI amplicons were sequenced in a 454 Roche Junior run and the MHCII-DRB amplicons were sequenced in a MiSeq PE150 run at the Georgia Genomics Facility.

Reads from the MHC I were were assembled with the program Geneious 7.0. Consensus sequences for each individual were assembled using the de-novo function. All sequences were viewed with MEGA 6.0 and aligned using Clustal-W. A tree was created of the sequences using a neighbor-joining method (1000 bootstraps, Kimura 2-parameter model). A subset of sequences were selected to create a phylogenetic tree (neighbor-joining, 1000 bootstraps, Kimura 2-parameter model) aligned with those from related felids and mammals obtained from GenBank using a basic local alignment search tool (BLAST) search (National Center for Biotechnology Information, Bethesda, MD, USA) (Altschul et al., 1990). Reads from the MHCII-DRB were assembled with the program PandaSeq (http://www.biomedcentral.com/1471-2105/13/31). Samples were demultiplexed using the commercially available NextGene software (Softgenetics). Sequences were aligned in MEGA 6.0 with MUSCLE. A subset of sequences were selection to create a phylogenetic tree aligned with those from related felids and mammals obtained from GenBank using a basic local alignment search tool (BLAST) search (National Center for Biotechnology Information, Bethesda, MD, USA) (Altschul et al., 1990).

Results

The MHC I analysis resulted in reads from 247 pumas in the study including seven of the Seminole pumas, three pumas from British Colombia, four pumas from Brazil, two pumas from North Dakota, two pumas from Costa Rica, 21 pumas from Texas including six of the eight introduced pumas, and 208 Florida pumas. Each sequence was a contig of over 200 reads per puma. There were 39 variable sites in the 740 bases. Phylogenetically, none of the sequences were distinguishable. Florida puma sequences were compared with MHC I sequences from other mammals (Figure 5.1). The MHC II resulted in much fewer reads per animal (1-10). A subset of 28 individuals were selected for analysis based on their spatial or temporal importance. Of these 28, there were 96 variable sites out of 235 bases. Phylogenetically, the pumas did not differentiate from other wild felids, but they separated from other mammal groups (Figure 5.2). Pumas from Costa Rica grouped with pumas from Texas, and pumas from Brazil grouped with the Seminole Reservation pumas which had South American lineage (Johnson et al., 2010).

Discussion

This is the largest study of MHC in wild felids to date. Previously, Castro-Prieto et al. (2011a) had examined these same genes in 148 Namibian cheetahs (*Acinonyx jubatus*). Castro-Prieto et al. (2011b) had also examined the variability of these genes in Namibian leopards (*Panthera pardus pardus*). Our study examined the variability of 247 individual pumas at the MHC I gene and 28 pumas with the MHC II-drb genes. Previous studies have also examined 25 Asiatic lions (*Panthera leo persica*) with MHC I, 14 Bengal tigers (*Panthera tigris tigris*) with MHC I, 16 Eurasian lynx (*Lynx lynx*) with MHC II-drb, and 36 domestic cats (*Felis* catus) with

MHC II-drb (Sachdev et al. 2005; Pokorny et al., 2010, Wang et al., 2009, Yuhki and O'Brien, 1997).

We detected three alleles in the MHC I gene, which is much lower than the ten alleles found in the 108 Namibian cheetahs (Castro-Prieto et al, 2011a). A previous study of cheetahs had found two alleles (Yuhki and O'Brien, 1994). These numbers are very low compared to the 52 alleles found in the Asiatic lion population (Sachdev et al., 2005) and the 14 alleles detected in the Bengal tiger populations (Pokorny et al., 2010). While we found 39 variable sites in the MHC I, Castro-Prieto et al (2011b) found 46 variable sites in the Namibian leopard population. Due to the high number of reads, we feel confident that our results reflect true alleleic diversity in *P. concolor* MHC I.

The phylogenetic analysis of the MHC I gene clearly distinguished *P. concolor* from other felids and mammals, however, phylogenetic reconstruction of the MHC II-drb was poorly resolved in relation to other felids as has been seen previously (Castro-Prieto et al., 2011b). Interestingly, however, there was clustering of *P. concolor* sequences which indicate that these data deserve further analysis. *Puma concolor* sequences from Brazilian pumas clustered with Seminole pumas which were believed to have a South American origin (Johnson et al., 2010). Additionally, pumas from Texas and Costa Rica clustered together as well which may be due to their geographic proximity.

Unfortunately, the yield of the Illumina MiSeq was much lower than expected: one to 10 reads per individual as opposed to 300 reads per individual as had been expected. As seen by the phylogenetic analysis (Figure 5.2), there is considerable variation in the MHC II-drb of *P. concolor*. Although we cannot comment on the number of alleles, this diversity was not found in

the Namibian cheetah population, which only had four alleles (Castro-Prieto et al., 2011a). Resequencing of this target with Roche 454 may yield more consisten results.

Although this study is preliminary, the results are very interesting because they suggest that the introgression event did not affect the MHC I and that diversity at the MHC II drb loci may be maintained. It may be that the previous lack of diversity observed at the MHC I in felids is due to evolutionary conservation of this as opposed to inbreeding depression or population bottlenecks. The MHC I in Florida pumas was conserved across continents while the MHC I diversity was high. Our data contribute to the understanding of MHC diversity in wild felids.

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Table 5.1. 5’ primer sequence tags.

5' Tag	Tag_ID	tag_seq	5' Tag		Tag_ID	Tag_seq
Tag set 1	1	TGCATAC	Tag set 4	13MHCX	13	ATCTGG
	2	ACCGAC		15MHCX	15	GAAGTCTA
	3	CCTATTGG		16MHCX	16	ACGTC
	4	GTCAA		25MHCX	25	CTAGG
Tag set 2	5	TCAGC	Tag set 5	26MHCX	26	AGTATA
	6	AACACCAG		27MHCX	27	TTGTTG
	7	CATGAG		28MHCX	28	AACCGA
	8	GGATGAT		29MHCX	29	AATACT
Tag set 3	9	TTGACA		30MHCX	30	AATTAA
	10	ACAAGGC		31MHCX	31	ACCGCC
	11	CGGTT		32MHCX	32	AAGCTT
	12	GGTACTAC		33MHCX	33	AGAACC

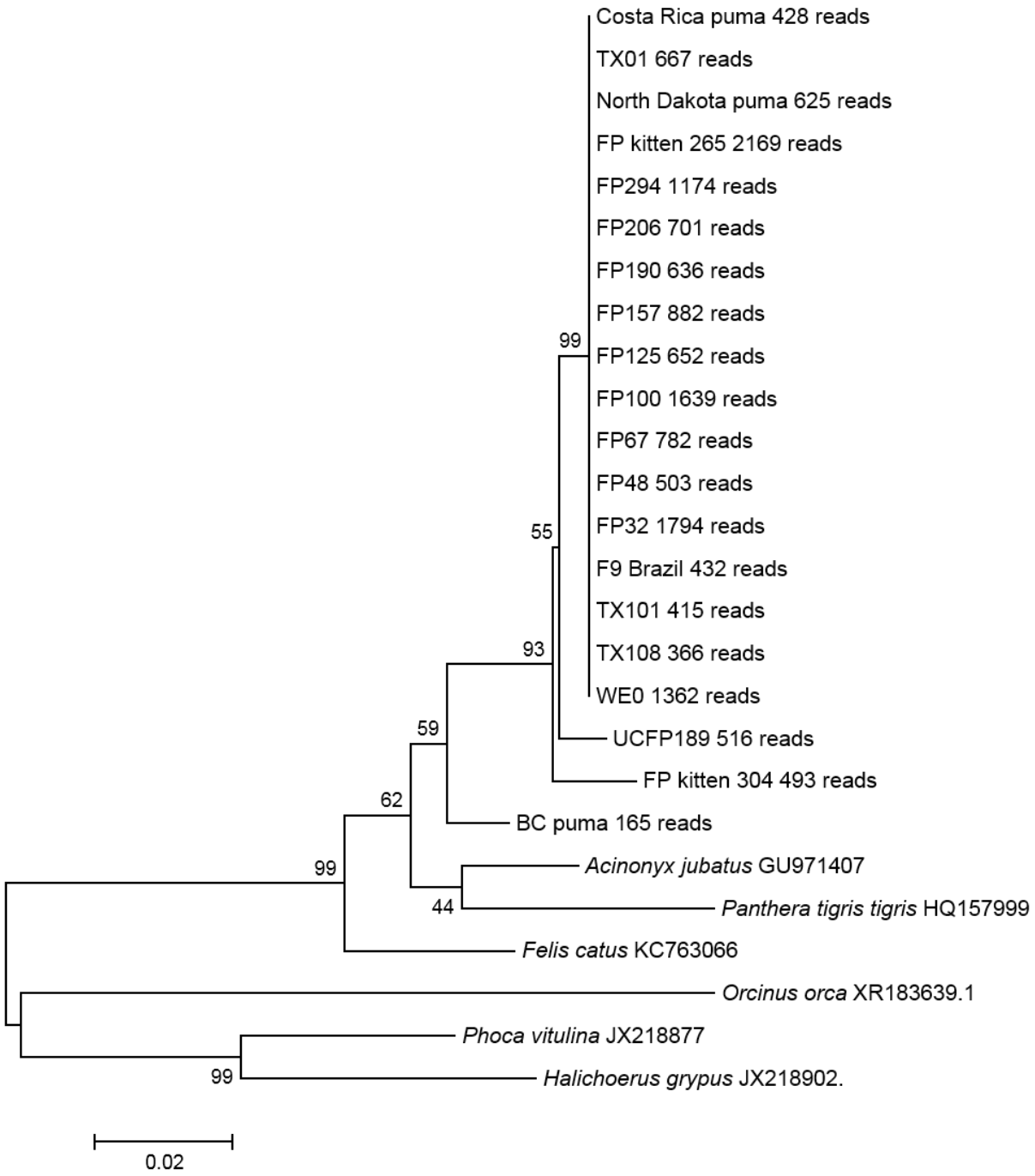

Figure 5.1. Neighbor-joining tree of Florida puma MHC 1 sequences (1000 bootstraps, Kimura-2)

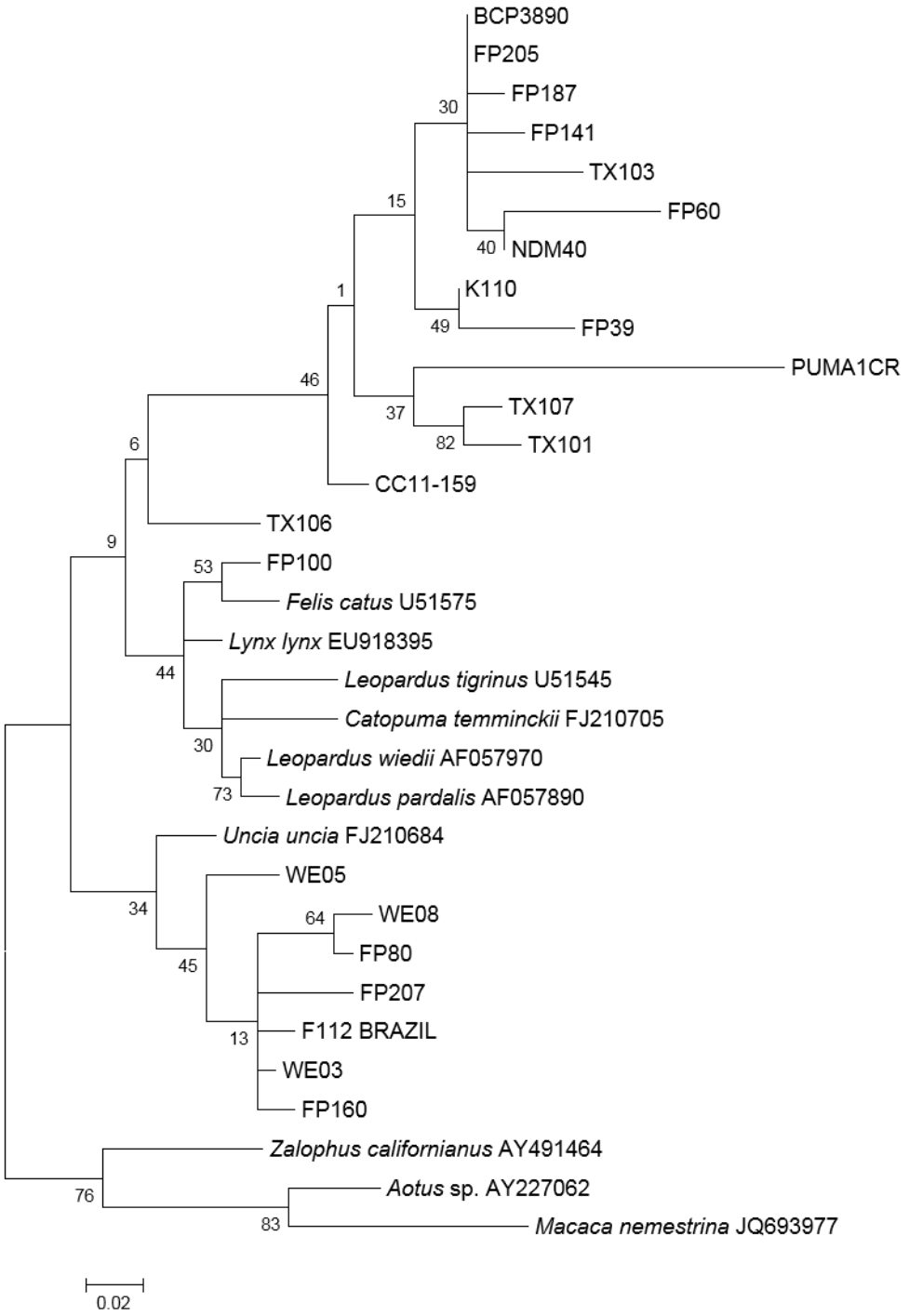

Figure 5.2. Maximum-likelihood tree of Florida puma MHC2 sequences (1000 bootstraps, Kimura-2)

CHAPTER 6

CONCLUSIONS

The Florida puma population in southern Florida, United States is one of the best studied felid populations in the world. Since the 1980s, this population has been listed as an endangered subspecies or population of Puma concolor, and the conservation efforts since their rediscovery have allowed the population to rebound from ~20 individuals to ~200 individuals. Although once widespread throughout the United States, the Florida puma population is the only breeding Eastern population that was not extirpated.

Previously, a high prevalence of piroplasms has been reported from Florida pumas (*Puma concolor coryi)* from southern Florida. In the current study, we provide biological, morphological, serological, and molecular data on this novel *Babesia* species. Ring-stage trophozoites were morphologically similar to trophozoites of numerous small babesids of felids including *B. leo, B. felis*, and *Cytauxzoon felis*. Parasitemias in naturally-infected Florida pumas were very low (<1%) and hematologic values of 25 *Babesia*-infected Florida pumas were within normal ranges for *P. concolor coryi*. Phylogenetic analysis of full-length 18S rRNA gene and β-tubulin sequences indicated that this *Babesia* species is a member of the *Babesia* sensu stricto clade and is closely related to *Babesia* spp. from ticks and carnivores in Japan and several species from bovids and cervids. Internal transcribed spacer (ITS)-1 region sequences from this *Babesia* sp. from 19 Florida pumas were 98.8-99.7% similar to each other and ~88% similar to *B. odocoilei.* Similarly, an ITS-2 sequence from one puma was 91% similar to *B. odocoilei*. Infected pumas were positive for antibodies that reacted with *B. odocoilei*, *B. canis*, and *B. bovis*

antigens with titers of 1:256, 1:128, and 1:128, respectively. No serologic reactivity was noted for *Theileria equi*. No molecular evidence of congenital infection was detected in 24 kittens born to 11 *Babesia*-infected female pumas. Pumas from other populations in the United States [Louisiana (n=1), North Dakota (n=5) and Texas (n=28)], British Columbia, Canada (n=9), and Costa Rica (n=2) were negative for this *Babesia* sp. Collectively, these data indicate that this *Babesia* sp. is novel and herein we describe the species as *Babesia coryi* sp. nov.

The endangered Florida puma (*Puma concolor*) population is one of the world's most-studied felid populations. Although several ectoparasites surveys on the Florida pumas have been conducted, none were after the genetic introgression event which occurred in the mid-1990s. Since this time, many vertebrate and invertebrate species have become established in Florida. This study was conducted to describe the diversity and natural history of ectoparasites on Florida pumas. From January 1989 to May 1993 and January 2000 to April 2014, ectoparasites were collected from free-ranging and captive pumas. Ectoparasites from a total of 262 puma records included six ixodid tick species, one mite (*Lyxacarus* sp.), a Hippoboscid fly (*Lipoptena mazamae*), and a flea (*Ctenocephalides felis*). Most records were from free-ranging pumas from southern Florida in Collier County (n=183) and fewer from Hendry (n=25), Miami-Dade (n=11), Monroe (n=2) and one record each from Lee, Highlands, and Palm Beach Counties. Four records were from pumas from the northern Florida counties of Nassau (n=3) and Flagler (n=1). The tick species were *Ixodes scapularis* (n= 2,014), *Dermacentor variabilis* (n= 771), *Ixodes affinis* (n= 48), *Amblyomma maculatum* (n= 35), *Amblyomma americanum* (n= 59), and *Amblyomma auricularium* (n= 3). This study represents the first report of *A. americanum* in Collier County, Florida and the first report of *A. auricularium* on a wild felid. The mite species, which was previously reported as *Lyxacarus morlani* was determined to be a novel *Lynxacarus* sp. Because

many veterinary and medically important pathogens are vector-borne, additional studies should be conducted on pathogens potentially transmitted by ectoparasites commonly found on Florida pumas. This is the most comprehensive study of ectoparasites from Florida pumas which is an apex predictor in the southern Florida ecosystem.

We did not identify any potential vectors of the Florida puma *Babesia*, however we did document transovarial transmission of a different *Babesia* sp. in female ticks removed from Florida pumas. Live-engorged tick studies such as this may be a cost effective way to evaluate transovarial or transstadial transmission of pathogens.

The Major Histocompatibility Complex (MHC) is a component of the adaptive immune system involved in self/non-selfrecognition and disease susceptibility. Some endangered species maintain high genetic diversity at the MHC despite repeated population bottlenecks. Florida pumas, an endangered population of Puma concolor (approx. 150 individuals) in southern Florida, underwent a bottleneck in the 1980s with as few as three breeding individuals over two generations. To reverse inbreeding depression, in 1995 the population was introgressed with five female Texas cougars. We hypothesized that the Florida puma MHC diversity increased as a result of this introgression event. Functionally important antigen-binding sites from the MHCI and MHCII loci were amplified with a touchdown PCR using high-fidelity polymerase. Additionally, piroplasm prevalence was detected using nested PCR. Using next-generation sequencing (Roche 454 andIllumina MiSeq), we are determining allelic diversity of the MHC I and II in Florida pumas respectively. It is currently unclear from our data if the Florida puma MHC was affected by the introgression event. The MHC I was highly conserved and pumas had nearly identical sequences, although we did detect three alleles. The techniques used to assemble and demultiplex the MHC II gene did not yield as many sequences as necessary for this study. Future studies with these data

will address parasite- and sexual-mediated selection of MHC genotypes. Because most felid species are threatened or endangered, these data could have important implications for wild felid conservation.

With the establishment of *P. concolor* populations in Midwestern North America and the gradual movement of individuals farther east, *P. concolor* may eventually recolonize parts of eastern North America. These data represent an attempt to understand the natural history of the Florida puma and its associated parasites after a conservation genetic introgression event.