

TRANSMISSION OF *CYTAUXZON FELIS* BY
AMBLYOMMA AMERICANUM: ENGORGEMENT
WEIGHT OF NYMPHS AND ATTACHMENT TIME OF
ADULTS FOR TRANSMISSION TO DOMESTIC CATS

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Abstract:

Cytauxzoon felis causes the most severe tick-borne disease of domestic cats in the United States. The geographic distribution and prevalence of cytauxzoonosis seem to be increasing as the range of *Amblyomma americanum* expands. However, disease prevalence and life cycle have not been completely understood, and diagnosis and treatment remain challenging. My research focused on determining the prevalence of *C. felis*, a better understanding of life cycle, and developing supportive diagnostic techniques.

To contribute establishing the prevalence of *C. felis* in domestic cats, blood was collected from 380 free-roaming cats in 2014 in Oklahoma and 292 in 2012–2014 in Iowa. PCR detected *C. felis* DNA in 3 of 380 (0.8%; 0.22–2.3%) feline samples from OK and 0 of 292 (0.0%; 0.0–1.3%) from IA. Prevalence of *C. felis* infection was examined in *C. felis*-exposed *A. americanum* using staining technique and PCR. *Cytauxzoon felis*-infected salivary glands were not observed in 350 fed ticks (0.0%; 0.0–1.0%), whereas 54 of 352 (15.3%; 11.7–19.5%) salivary glands from flat ticks were infected with *C. felis*. PCR detected *C. felis* DNA in 4 of 358 (1.1%; 0.30–2.84%) ticks. Transmission of *C. felis* from *A. americanum* to a domestic cat was demonstrated in ≤ 72 hours. Tick morphometric method was examined as a supportive diagnostic technique and shown to be less practical to predict probability of transmission. Additionally, nymphal sexual dimorphism was examined with *A. americanum*, *A. maculatum*, *Dermacentor andersoni*, *D. variabilis*, and *Rhipicephalus sanguineus*. The mean body weight of engorged nymphs that became females was significantly greater than that of nymphs that molted to males in *A. americanum* ($t=32.3$; $P<0.0001$), *A. maculatum* ($t=-9.70$; $P<0.0001$), *D. variabilis* ($t=15.7$; $P<0.0001$), and *R. sanguineus* ($t=5.17$; $P<0.0001$), whereas, the mean body weight of *D. andersoni* engorged nymphs that became males was significantly greater ($t=8.71$; $P<0.0001$) than that of nymphs that molted to females.

Overall, these studies have contributed to a better understanding of the prevalence of *C. felis* and life cycle with timing of transmission. Additionally, the findings in nymphal sexual dimorphism may help explain potential differences in prevalence of tick-borne pathogens between female and male ticks.

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CHAPTER I

INTRODUCTION

Ticks on domestic cats are often overlooked as parasites and vectors of disease agents compared with those on dogs. This oversight results, in part, from the belief that cats are fastidious groomers and remove these ectoparasites before attachment. It is also possible that some cats spend a majority of their time indoors, leading owners to believe that the cats are not exposed to ticks. A limited number of studies report feline tick infestations and occurrence of feline tick-borne diseases in the United States. However, *Amblyomma americanum* (Reichard et al., 2009; Bowman, 2002), *Dermacentor andersoni* (Greene, 2012; Bowman, 2002), *D. occidentalis* (Greene, 2012; Bowman, 2002), *D. variabilis* (Blouin et al., 1979; Bowman, 2002), *Ixodes scapularis* (Krupka and Straubinger, 2010; Little, 2010; Bowman, 2002), *I. pacificus* (Krupka and Straubinger, 2010; Little, 2010; Bowman, 2002), and *Rhipicephalus sanguineus* (Lappin, 2012; Little, 2010; Bowman, 2002) have been commonly found and reported on cats. Furthermore, those tick species can transmit disease agents to domestic cats, including *Cytauxzoon felis* (Blouin et al. 1984, Reichard et al. 2009, Reichard et al. 2010), *Anaplasma phagocytophilum* (Little, 2010; Lappin, 2012), *Francisella tularensis* (Lappin, 2012)

Borrelia burgdorferi (Krupka and Straubinger, 2010; Greene, 2012), and *Ehrlichia canis*-like agent (Lappin, 2012; Little, 2010).

Cytauxzoon felis is a protozoan parasite that causes the most severe tick-borne disease of domestic cats in the United States (Sherrill and Cohn, 2015). Transmission of *C. felis* to domestic cats has been demonstrated with *A. americanum* (Reichard et al., 2009; Reichard et al., 2010) and *D. variabilis* (Blouin et al., 1984). Although cytauxzoonosis has been predominantly reported in the central, southeastern, and south-central U.S. (Sherrill and Cohn, 2015), it is difficult to state with confidence the true prevalence of *C. felis* infection since only a few reports of prevalence exist.

The complete life cycle of *C. felis* is not known. Historically bobcats (*Lynx rufus*) were thought to be the only vertebrate reservoirs of *C. felis* (Blouin et al., 1987; Cowell et al., 1988). However, more recent reports have indicated domestic cats that survived acute cytauxzoonosis become chronically infected with *C. felis* and can also serve as a source of *C. felis* (Birkenheuer et al., 2006; Reichard et al., 2009; Reichard et al., 2010; Rizzi et al., 2015). Ticks become infected when they feed on a *C. felis*-infected cat. Gametogony is thought to occur within the tick, leading to zygote and then sporozoite formation (Tarigo et al., 2013). When an infected tick feeds on a naïve cat, sporozoites are inoculated into the cat. *Cytauxzoon felis* then undergoes schizogony in mononuclear phagocytic cells forming schizonts. The infected macrophages often clog venules in organs such as liver, spleen, lungs, and lymph nodes, which is usually associated with clinical illness. *Cytauxzoon felis* multiplies within schizonts eventually rupturing the host cell releasing merozoites. Merozoites then invade erythrocytes where they appear as piroplasms (intraerythrocytic merozoites). Although *C. felis* piroplasms can rupture

erythrocytes, erythrocytic disease is not as pathologically important as schizogonous replication in macrophages (Kier et al., 1987, Susta et al., 2009, Sherrill and Cohn, 2015). Infected cats typically develop clinical signs about 2 weeks after being bitten by an infected tick. Clinical signs progress rapidly and may include fever, inappetence, anorexia, listlessness, icterus, dehydration, dark urine, and dyspnea. Many cats die within 24 hours of presentation to a veterinary clinic regardless of treatment. Piroplasms persist for years in cats that survive cytauxzoonosis and serve as a source of *C. felis* infection (Wagner, 1976; Kier et al., 1987; Hoover et al., 1994; Meinkoth et al., 2000; Birkenheuer et al., 2006; Haber et al., 2007; Brown et al., 2008; Brown et al., 2010; Sherrill and Cohn, 2015). According to the study conducted by Motzel and Wagner, 1990, cats that survived acute disease develop immunity against *C. felis* preventing reinfection.

Cytauxzoonosis is a top differential diagnosis for any acutely ill, febrile and/or icteric cat during spring through early fall (i.e., when ticks are active) in enzootic areas. Definitive diagnosis is made based on demonstration of *C. felis* in blood samples. Demonstration of piroplasms on peripheral blood smear slides may be the simplest diagnosis; however, a certain level of training is required to identify the organism in erythrocytes since it is small ($\leq 3 \mu\text{m}$) and often confused with Howell-Jolly bodies and *Mycoplasma haemofelis* (Meinkoth and Kocan, 2005). In addition, it may be difficult to find piroplasms on blood smear especially if it is still in the early stage of disease course. Another method of diagnosing the infection is demonstration of schizonts by performing fine needle aspirates or impression smears of infected tissues, such as lung, spleen, liver, lymph nodes, and bone marrow. Conventional and real-time polymerase chain reaction (PCR) using primers that amplify the 18S rRNA gene or Internal Transcribed Spacer

(ITS) regions of *C. felis* have been developed and are commercially available. PCR for *C. felis* is the most sensitive and specific method for recognition of infection (Birkenheuer et al., 2006). Serologic tests to detect antibodies to *C. felis* have been developed but are not considered useful for detecting acute cytauxzoonosis since antibodies are not likely produced by the time clinical signs develop (Cowell et al., 1988).

Historically infection with *C. felis* in domestic cats had a grave prognosis, and mortality was almost 100% (Meinkoth and Kocan, 2005). Currently a combination of atovaquone (15 mg/kg PO q8h for 10 days) and azithromycin (10 mg/kg PO q24h for 10 days) is recommended to treat cytauxzoonosis. However, even with this combination therapy, the survival rate of cytauxzoonosis was still only about 60% (Cohn et al., 2011). Before atovaquone and azithromycin, imidocarb dipropionate was used to treat cytauxzoonosis but is no longer recommended due to its lack of efficacy and side effects (Cohn et al., 2011). In addition to antiprotozoal therapy, aggressive supportive care is necessary.

Cytauxzoonosis is a life-threatening hematoprotzoal disease in domestic cats with rapidly progressive clinical disease course. The geographic distribution and range of cytauxzoonosis seem to be increasing as the range of vector tick expands. However, disease prevalence and life cycle have not been completely established, and diagnosis and treatment remain challenging. Therefore, my research focused on determining the prevalence of *C. felis* infection, a better understanding of life cycle with timing of *C. felis* transmission, and developing supportive diagnostic techniques.

The major objectives for the described research were as follows:

1. Estimate prevalence of *C. felis* infection in healthy free-roaming cats in northcentral Oklahoma and central Iowa
2. Determine prevalence of *Cytauxzoon felis* in salivary glands of *Amblyomma americanum* and determine if tick morphometrics are related to duration of attachment and probability of *C. felis* transmission to cats
3. Determine if nymphal engorgement weight predicts sex of adult *Amblyomma americanum*, *Amblyomma maculatum*, *Dermacentor andersoni*, *Dermacentor variabilis*, and *Rhipicephalus sanguineus*

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CHAPTER II

LITERATURE REVIEW

RESERVOIR HOSTS AND TICK VECTORS

Historically bobcats (*Lynx rufus*) were thought to be the only reservoir hosts for *Cytauxzoon felis*, and domestic cats (*Felis catus*) were considered aberrant hosts (Birkenheuer et al., 2008; Blouin et al., 1984; Glenn et al., 1983; Kier et al., 1982). However, several studies have demonstrated transmission of *C. felis* from chronically infected domestic cats to naïve cats via *Amblyomma americanum* (Reichard et al., 2010; Reichard et al., 2009). In addition, DNA of *C. felis* was detected in 56 of 902 (6.2%) blood samples collected from healthy domestic cats in Arkansas, Missouri, and Oklahoma (Rizzi et al., 2015), demonstrating domestic cats are competent reservoirs for *C. felis*. Besides bobcats, *C. felis* infection has been reported in other wild felids: Bengal tiger (*Panthera tigris tigris*) (Jakob & Wesemeier, 1996), tiger (*Panthera tigris*) (Garner et al., 1996; Lewis et al., 2012), lion (*Pathera leo*) (Peixoto et al., 2007), cougar (*Puma concolor stanleyana*) (Harvey et al., 2007; Rotstein et al., 1999; Yabsley et al., 2006), and Florida panther (*Puma concolor coryi*) (Rotstein et al., 1999; Yabsley et al., 2006). All tigers and lions were captive-reared and died acutely, except 4 tigers in Missouri that

were PCR positive for *C. felis* without clinical signs (Lewis et al., 2012). *Cytauxzoon felis* has been detected in free-roaming cougars (Harvey et al., 2007; Rotstein et al., 1999; Yabsley et al., 2006) and Florida panthers (Rotstein et al., 1999; Yabsley et al., 2006). Cytauxzoonosis has not been reported in non-felid animals.

Transmission of *C. felis* has been demonstrated experimentally by *A. americanum* (Reichard et al., 2010; Reichard et al., 2009) and *Dermacentor variabilis* (Blouin et al., 1984). However, Reichard et al. (2010) demonstrated differences in the ability of *A. americanum* and *D. variabilis* to transmit *C. felis*; transmission of *C. felis* with *A. americanum* was successful, but unsuccessful with *D. variabilis* in the same experiment. Reichard et al. (2009) also demonstrated the occurrence of cytauxzoonosis coincided with the distribution and seasonal activity of *A. americanum*. Reichard et al. (2009) also discussed that differences in ability of *C. felis* transmission between *A. americanum* and *D. variabilis* were possibly related to immune status of donor and principal hosts and strain variation of *C. felis*.

Brown et al. (2009) demonstrated 3 different genotypes of *C. felis*, ribosomal internal transcribed spacer regions (ITS) A, B, and C, in infected domestic cats in Arkansas and Georgia. A total of 88 blood samples from acutely ill cats diagnosed with cytauxzoonosis from 2005 to 2007 were collected in Arkansas (n = 57) and Georgia (n = 31) and analyzed. ITSA was detected in 48 of 57 Arkansas samples (84.2%) and 1 of 31 Georgia samples (3.2%). ITSB was only detected in 21 of 31 Georgia samples (67.7%), and ITSC was only detected in 5 of 57 Arkansas samples (8.8%). This study also demonstrated an association between particular ITS genotypes and the outcome of infection; 38 of 48 (79.2%) of infections identified as having the ITSA genotype

survived, while only 4 of 21 (19.0%) of cats infected with the ITSB genotype survived. None of the 5 cats identified as infected with the ITSC genotype survived. However, a following study reported by the same author (Brown et al., 2009) showed there was no significant difference in survival between *C. felis* genotypes. Genetic variation of *C. felis* is still inconclusive.

In addition, several surveys have been done to demonstrate and compare prevalence of *C. felis* infection in wild-collected *A. americanum* and *D. variabilis*. Bondy et al. (2005) analyzed 839 *A. americanum*, 298 *D. variabilis*, and 160 *R. sanguineus* that were collected from dogs and cats in Missouri, and *C. felis* DNA was detected only in *A. americanum*. Reichard et al. (2010) collected a total of 928 flat *A. americanum* (n = 768) and *D. variabilis* (n = 160) from the wild and tested for *C. felis* infection. DNA of *C. felis* was discovered only in *A. americanum*. Lastly, Shock et al. (2014) collected a total of 1,631 ticks from wild, animals, and humans in Georgia (n = 486), Kentucky (n = 103), Pennsylvania (n = 1), Tennessee (n = 626), and Texas (n = 414). Besides *A. americanum* (n = 743) and *D. variabilis* (n = 702), *A. cajennense* (n = 99), *A. maculatum* (n = 16), *Ixodes scapularis* (n = 4), *I. woodi* (n = 1), and unidentified *Amblyomma* spp. nymphs (n = 64) were also collected and tested for tick-borne parasites. Interestingly, *C. felis* DNA was detected only in *D. variabilis* (n = 9; 8 from TN and 1 from GA), and not from *A. americanum*.

Amblyomma americanum, lone star tick, is the most commonly identified in the south-central and southeastern U.S. (Barrett et al., 2015). Geographic distribution and range of *A. americanum* has been expanding. Populations of this tick have been established throughout the majority of the eastern half of the U.S., extending as far north

as Maine (Barrett et al., 2015; Childs & Paddock, 2003). *Amblyomma americanum* is found predominantly in woodland habitats, particularly young forests with dense underbrush (Childs & Paddock, 2003). Seasonal peaks in the populations of *A. americanum* have been reported in Georgia and Missouri; adults peak April to June, nymphs have a bimodal distribution during May to July and August to September, whereas larvae peak July to September (Kollars et al., 2000; Semtner & Hair, 1973). *Amblyomma americanum* can be a non-specific, aggressive feeder while seeking hosts (Goddard & Varela-Stokes, 2009) although some host preferences are exhibited by each life stage. Adult *A. americanum* prefer to feed on medium- and large-sized mammals, and larvae and nymphs primarily infest various ground-dwelling birds, medium- and large-sized mammals, and occasionally small mammals (Kollars, 1993). White-tailed deer seem to be a preeminent host for *A. americanum* because they can provide blood meal sources for all three stages of *A. americanum* (Mount et al., 1993). In addition to *C. felis*, *A. americanum* is an important vector for *Francisella tularensis* (Green, 2012) and *Ehrlichia* species (Lappin, 2012) to domestic cats.

Dermacentor variabilis, American dog tick, is also a three-host tick commonly found on grassy meadows or other low vegetation and widely distributed in the U.S. east of the Rocky Mountains (McNemee et al., 2003). Adult *D. variabilis* are most active from around mid-April to early September, while nymphs and larvae are often found from June to early September and from March through July, respectively (Goddard, 1996). Adult *D. variabilis* usually feed on dogs or other large animals when available (Matheson, 1950). Nymphs prefer to feed on medium-size animals, while larvae often infest on small mammals, such as mice (Matheson, 1950). Just like *A. americanum*, *D.*

variabilis can be a vector for *C. felis* (Blouin et al., 1984), *F. tularensis* (Greene, 2012), and *Ehrlichia* species (Lappin, 2012) in domestic cats.

It is still unknown what factors account for the differences in the ability of *A. americanum* and *D. variabilis* to transmit *C. felis*; however, *A. americanum* seems to play a more primary role in transmission and dissemination of *C. felis* and thus was the focus of the current thesis.

GEOGRAPHIC DISTRIBUTION

Cytauxzoon felis was first reported in 1976 from domestic cats in southwestern Missouri (Wagner, 1976). Although cytauxzoonosis was once considered as a rare disease in a small geographic area, it is now confirmed in domestic cats from 17 states in U.S., including Alabama (Shock et al., 2011), Arkansas (Ferris, 1979), Florida (Haber et al., 2007), Georgia (Ferris, 1979), Illinois (MacNeill et al., 2015), Kansas (Meier & Moore, 2000), Kentucky (Jackson & Fisher, 2006), Louisiana (Hauck et al., 1982), Mississippi (Shock et al., 2011), Missouri (Wagner, 1976), North Carolina (Birkenheuer et al., 2006), Oklahoma (Glenn & Stair, 1984), South Carolina (Birkenheuer et al., 2006), Tennessee (Haber et al., 2007), Texas (Bendele et al., 1976), Virginia (Birkenheuer et al., 2006), and West Virginia (Cohn and Birkenheuer 2012). *Cytauxzoon felis* has been discovered in bobcats in Pennsylvania and North Dakota where infected domestic cats have not been reported (Birkenheuer et al., 2008).

Prevalence of *C. felis* infection in healthy free-roaming cats in Florida, North Carolina, and Tennessee was investigated by Marion et al. (2007). Blood from 961 free-

roaming cats (494 from Florida; 392 from North Carolina; 75 from Tennessee) was analyzed by PCR using *C. felis*-specific primers found 3 (0.3%; 0–0.8%, 99% confidence interval) of the cats, two from Florida and one from Tennessee, were positive for *C. felis* DNA. Additionally, Rizzi et al. (2015) examined prevalence of *C. felis* infection in healthy domestic cats in Arkansas, Missouri, and Oklahoma by sampling 902 blood samples from domestic cats collected in private veterinary clinics, animal shelters, spay/neuter programs, and client cared for feral cats. DNA from *C. felis* was detected in 56 of 902 (6.2%; 4.7–7.9%, 95% confidence interval) blood samples. The highest prevalence of *C. felis* infection (15.5%; 10.3–21.7%) was observed in cats from Arkansas, followed by cats from Missouri (12.9%; 6.1–24.0%) and Oklahoma (3.4%; 2.2–5.1%). Interestingly, Rizzi et al. (2015) showed a difference in prevalence of *C. felis* infection depending on geographic locations within OK; 13 of 77 (16.9%) cats were infected with *C. felis* in eastern OK while 10 of 602 (1.7%) cats were infected with *C. felis* in northcentral OK.

Although cytauxzoonosis seems to be increasing as the geographic distribution and range of *A. americanum* expands (Barrett et al., 2015; Sherrill & Cohn, 2015), it is difficult to determine prevalence of *C. felis* infection because cytauxzoonosis is not reportable, and infected cats may not receive veterinary care or proper diagnosis especially in non-enzootic areas. Temporal occurrence and environmental risk factors associated with infection with *C. felis* in domestic cats from Oklahoma were examined (Reichard et al., 2008). Cytauxzoonosis cases were retrospectively searched in the electronic medical records from Oklahoma Animal Disease Diagnostic Laboratory (OADDL) and Boren Veterinary Medical Teaching Hospital (BVMTH). A total of 232

cytauxzoonosis cases from OADDL (n = 180) in 1995–2006 and BVMTH (n = 52) in 1998–2006 were analyzed. The number of cytauxzoonosis cases was relatively consistent from year to year. The number of cytauxzoonosis cases increased between April and September. The majority of cases (n = 72) were diagnosed in May. Only a few cases (n = 10) were diagnosed from November through March, and no cases were diagnosed in December. To determine environmental risk factors, geographic coordinates were assigned and landscape characteristics were quantified within a 100-m radius of each cytauxzoonosis case location (n = 41). A majority (n = 28) occurred in low density residential areas and more cases (n = 8) were found in urban edge habitat. The study concluded that generally outdoor or outdoor/indoor cats living near heavily wooded, low-density residential areas particularly close to natural or unmanaged habitats where both ticks and bobcats may be in close proximity are at higher risk of infection.

Since free-roaming cats live outside and have more contact with the tick vectors of *C. felis*, they can be an excellent population to monitor the expansion of *C. felis* distribution in the United States.

TIMING OF TRANSMISSION AND TICK MORPHOMETRICS

Prior to the work described in this thesis, the minimum time required for transmission of *C. felis* from an infected tick to a cat was not known. Transmission studies conducted to date have not examined the minimum time necessary for transmission as ticks were allowed to feed to repletion (Blouin et al., 1984; Cohn et al., 2011; Kier et al., 1982; Reichard et al., 2010; Reichard et al., 2009). Transmission of

Theileria spp. and *Babesia* spp., which are closely related to *C. felis* (Criado-Fornelio et al., 2004; Lack et al., 2012), usually takes at least a few days of tick-attachment on animals (Piesman & Spielman, 1980; Shaw and Young, 1994). Transmission of *B. microti* from an infected *Ixodes dammini* to hamsters experimentally demonstrated in <36–54 hours of tick attachment (Piesman & Spielman, 1980). Ochanda et al. (1988) demonstrated a transmission of *T. parva parva* from an infected *R. appendiculatus* to a naïve cow in <24–72 hours. They also showed that rate of transmission of *T. parva parva* transmission was more rapid to cattle by ticks kept at high ambient temperatures compared to those kept at low ambient temperatures. The minimum length of tick attachment on a host to inoculate disease agents varies widely depending on tick species and type of pathogens (Fourie et al., 2013; Piesman et al., 1987; Piesman et al., 1991; Piesman & Spielman, 1980). Since commercially available acaricides for cats in the U.S. take about 24 hours of initial administration to kill *A. americanum* (Reichard et al., 2013), it is important to know how quickly *C. felis* can be transmitted to a cat from infected ticks.

Since duration of tick-attachment is an important factor to determine the probability of infection, previous studies developed tick engorgement indices to describe the duration of tick feeding in relation to transmission of parasites to animals (Obenchain et al., 1980; Piesman et al., 1987; Yeh et al., 1995; Williams et al., 2015). Yeh et al. (1995) created tick engorgement indices using *I. scapularis*, describing the time course of *I. scapularis* feeding in relation to *B. burgdorferi* development and transmission to animals or humans. Two groups of 50 laboratory-reared adult *I. scapularis* matching pairs were placed on the ears of a New Zealand white rabbit. Ten ticks were removed

from the rabbit's ears at time intervals of 12, 24, 36, 48, 60, and 72 hours. Similarly, a total of 200 *I. scapularis* nymphs were allowed to feed on three hamsters. At time intervals of 12, 24, 36, 48, and 60 hours following their attachment, a total of 30 nymphs were removed from the hamsters. Body measurement was immediately performed on the removed ticks. The 4 areas of measurements included (1) total body length, defined as the distance on the midline between the anterior edge of the scutum and the posterior tip of the opisthosoma; (2) scutal length, defined as the midline distance between the anterior edge and the posterior tip of the scutum; (3) maximum scutal width, measured at the widest point on the scutum; and (4) maximum alloscutal width, measured at the widest point on the alloscutum. Tick engorgement indices were then calculated comparing between total body length and scutum length (Index-1), total body length and scutum width (Index-2), and body width and scutum width (Index-3). Both nymphal and adult engorgement indices increased with increasing duration of attachment to hamsters and rabbits. Moreover, mean engorgement indices of nymphal ticks attached ≤ 24 -hour were significantly different from those attached $\geq 36, 48,$ and 60-hour attachment. For adult ticks, mean engorgement index-1 and -3 showed 0, 12, and 24-hour attachment were significantly different from those at 36, 48, 60, and 72-hour attachment. In addition, Index-1 was measured on 128 adult female and 377 nymphal *I. scapularis* collected from people with ticks in Rhode Island and Pennsylvania in order to predict the duration of attachment of *I. scapularis* and showed that most people found and removed both adult and nymphal *I. scapularis* before 36-hours of attachment.

Since clinical symptoms of cytauxzoonosis progress quickly, prompt diagnosis and treatments are necessary for infected cats. As a help for clinicians to predict

probability of *C. felis* infection, tick engorgement indices may be a convenient, reliable, and objective measure to determine the duration of tick attachment.

TICK SEXUAL DIMORPHISM AND PROBABILITY OF INFECTION

As with most other ixodid ticks commonly found in the U.S., sex of *A. americanum* cannot be determined morphologically until adult stages (Bowman, 2014). However, heavier engorged nymphs are known to molt to females and lighter nymphs molt to males in *Amblyomma neumanni* (Aguirre et al., 1999), *Amblyomma triguttatum triguttatum* (Guglielmone, 1985), *Amblyomma variegatum* (Centurier, 1979), *Dermacentor variabilis* (Hu & Rowley, 2000), *Hyalomma anatolicum* (Arthur & Snow, 1966), *Ixodes ricinus* (Dusbabek, 1996; Dusbabek et al., 1994; Kahl et al., 1990), *Ixodes rubicundus* (Belozarov et al., 1993), *Ixodes scapularis* (Hu & Rowley, 2000), and *Rhipicephalus bursa* (Yeruham et al., 2000).

These previous studies were conducted to help elucidate biology of those tick species that are important vectors and have economic significance. Laboratory-reared ticks were used in most of the studies (Aguirre et al., 1999; Belozarov et al., 1993; Hu & Rowley, 2000; Yeruham et al., 2000). Dusbabek (1996) used both laboratory-reared and field-collected *I. ricinus* and demonstrated the body weight limits for sex determination can be different in different tick populations; engorged body weights of laboratory-derived *I. ricinus* nymphs (4.22±0.50 mg for female 2.61±0.32 mg for male) were significantly lighter than those of field-collected nymphs (4.94±0.76 mg for female and 2.97±0.44 mg for male). The number of nymphs used in these studies varied widely with

as many as 476 nymphs (Aguirre et al., 1999) to as few as 30 nymphs (Dusbabek, 1996) that were weighed, separated individually or allocated into different containers based on their body weights, kept under laboratory conditions (~24°C, >95% relative humidity, and a photoperiod of 14-hour light and 10-hour dark), and monitored daily. Similarly a variety of vertebrate hosts were used to conduct the studies, including mice (Dusbabek, 1996; Hu & Rowley, 2000), rabbits (Guglielmone, 1985), rats (Guglielmone, 1985), and sheep (Yeruham et al., 2000).

Although Belozarov et al. (1993) and Hu and Rowley (2000) concluded that lighter nymphs molted into males and heavier nymphs molted into females for *I. rubicundus* and *I. scapularis*, respectively, other studies reported no definite way to differentiate sex of engorged nymphs by body weights due to overlap of engorgement weight of nymphs that became males and females (Aguirre et al., 1999; Arthur & Snow, 1966; Dusbabek, 1996; Dusbabek et al., 1994; Hu & Rowley, 2000; Kahl et al., 1990; Yeruham et al., 2000).

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CHAPTER III

PREVALENCE OF *CYTAUXZOOM FELIS* INFECTION IN HEALTHY FREE-ROAMING CATS IN NORTHCENTRAL OKLAHOMA AND CENTRAL IOWA¹

¹Yoko Nagamori, Jennifer E. Slovak and Mason V. Reichard, 2016. *Journal of Feline Medicine and Surgery Open Reports*. 1–4. Reprinted here with permission of publisher.

ABSTRACT

Cytauxzoonosis is a tick-borne disease of cats, and Oklahoma is considered an enzootic state. To determine the prevalence of *C. felis*, blood was collected from free-roaming cats since they are frequently exposed to tick vectors. Our study objective was to determine the prevalence of *C. felis* infection in free-roaming cats in northcentral Oklahoma and central Iowa. Infection with *C. felis* was determined using DNA extracted from blood and PCR amplification. Blood was collected from 380 free-roaming cats between January and May in 2014 in OK. DNA from *C. felis* was detected in 3 of 380 (0.8%; 95% confidential interval, 0.22–2.3%). In IA, 292 blood samples were collected between 2012 and 2014. No *C. felis* infected cats were detected (0.0%; 0.0–1.3%). The prevalence of *C. felis* (0.8%) in northcentral OK in our study was lower than the previously reported state average 3.4% in domestic cats in Oklahoma. Our study supports the prevalence in a given enzootic area can vary by location and from the pool of cats sampled. Zero of 291 (0.0%) cats were infected with *C. felis* in central IA. To date, only one case of cytauxzoonosis in a domestic cat has been reported in IA. It is important to monitor cats for *C. felis* infections in northern states, as geographic distribution of *A. americanum* expands northward. Since free-roaming cats have more contact with the tick vectors of *C. felis*, this population allows us to monitor the expansion of *C. felis* distribution.

INTRODUCTION

Cytauxzoon felis is a tick-transmitted protozoan parasite that can cause cytauxzoonosis in wild and domestic felids. Transmission of *C. felis* has been demonstrated with *Amblyomma americanum* (Reichard et al., 2009; Reichard et al., 2010) and *Dermacentor variabilis* (Blouin et al., 1984). Cytauxzoonosis in domestic cats has been reported throughout the central, southeastern, and south-central U.S. (Meinkoth and Kocan, 2005; Reichard et al., 2008). Oklahoma is considered enzootic for *C. felis* and Iowa is a non-enzootic state but borders other enzootic states (Rizzi et al., 2015). Domestic cats infected with *C. felis* often show severe, acute clinical signs characterized by fever, inappetence, anorexia, dyspnea, and icterus. Onset of disease typically occurs 10–14 days after *C. felis*-infected ticks feed on naïve cats and progresses quickly with fatalities reported 1–7 days after onset of clinical signs. A recent study demonstrated 60% survival in *C. felis*-infected cats that received a combination therapy of azithromycin and atovaquone with supportive care (Cohn et al., 2011).

A free-roaming cat is a domestic cat that has been born and raised without or with only limited contact to humans and is unsocialized. Since free-roaming cats live outdoors and are exposed to ticks, they are favorable populations to examine and monitor the distribution and range of cytauxzoonosis (Centonze and Levy, 2002). Our objective was to determine the prevalence of *C. felis* infections in free-roaming cats in enzootic northcentral Oklahoma and non-enzootic central Iowa.

MATERIALS AND METHODS

Blood samples were collected from free-roaming cats in Stillwater, Oklahoma and Ames, Iowa as part of community trap-neuter-return (TNR) programs. Cats were trapped mainly in northcentral Oklahoma and in central Iowa. In OK, all cats were sedated with a mixture of tiletamine hydrochloride and zolazepam hydrochloride (Telazol; Zoetis), ketamine hydrochloride (Ketamine; Putney), and xylazine (AnaSed; Akorn). In IA, a mixture of ketamine hydrochloride (Ketamine; Putney), dexmedetomidine hydrochloride (Precedex; Orion Pharma), buprenorphine (Simbadol; Zoetis), and butorphanol tartrate (Torbugesic-SA; Zoetis) was used for sedation in 2012–2013. In 2014, butorphanol tartrate (Torbugesic-SA; Zoetis) was added to the mixture only for fractious cats. The approximate ages of cats were determined based on dentition (The Humane Society of the United States, 1996), and cats that were 4–6 months or older were selected for blood collection to increase the chance of finding *C. felis* infected individuals. Cats were placed in a dorsal recumbency, hair around neck was clipped, and 70% isopropyl was sprayed in the area. Approximately 1 ml of blood was collected from jugular vein and was immediately placed in an EDTA collection tube.

Genomic DNA was extracted from peripheral whole blood samples using GeneJET Whole Blood DNA Purification Mini Kit (Thermo Scientific, Grand Island, New York). Briefly, 200 μ l of whole blood was mixed with 20 μ l of Proteinase K Solution and 400 μ l of Lysis Solution. After incubating the sample at 56 °C for 10 minutes, 200 μ l of ethanol (96–100%) was added into the sample. The prepared mixture was transferred to the spin column and centrifuged for 1 minute at 6,000 x g (~8,000 rpm). The column was then washed with 500 μ l of Wash Buffer I and centrifuged again for 1 minute at 8,000 x g (~10,000 rpm). The column was washed again with Wash

Buffer II and centrifuged for 3 minutes at 20,000 x g (14,000 rpm). DNA was eluted with 200 µl of preheated (approximately 56 °C) PCR-quality water added to the center of the column membrane. The sample was incubated for 2 minutes at room temperature and centrifuged for 1 minute at 8,000 x g (~10,000 rpm).

A conventional polymerase chain reaction (PCR) was performed to amplify the *Cytauxzoon felis* small subunit rRNA (ssu-rRNA). A nested PCR amplification was performed using primers (CfnestF. 5'-TCGCATTGCTTTATGCTGGCGATC-3' and CfnestR. 5'-GCCCTCCAATTGATACTCCGGAAA-3') that amplify 289 bp of the 18S rRNA gene of *C. felis* (Reichard, 2010). Cycling conditions of nested PCR were as follows: denaturation at 95 °C for 5 minutes, annealing at 54 °C for 1 minute, and extension at 72 °C for 1 minute. The cycle was repeated 34 times. To determine success of genomic DNA extraction, PCR using general primers that amplify conserved regions of the small subunit rRNA (ssu-rRNA) across hosts and parasite phyla were performed (Bondy et al., 2005). PCR products were separated on 1.75% agarose gel and viewed with ultraviolet light. Positive control templates consisted of DNA extracted from whole blood of a cat that died from an infection of *C. felis*, whereas negative control reactions used DNA isolated from purified water. PCR products were purified using QIAGEN, QIAquick PCR purification kit (Germantown, MD) and amplicons were sequenced by Eurofin Genomics (Huntsville, AL). Sequences from infected cats were compared using BLAST in GenBank.

The prevalence of *C. felis* was calculated according to Bush et al. (Bush et al., 1997). 95% confidence intervals were calculated according to Sterne's exact method (Reiczigel et al., 2003) using Quantitative Parasitology 3.0 (Rozsa et al., 2000).

RESULTS

A total of 380 blood samples (179 males and 201 females) were collected from January through May 2014 in OK. Of these, 20 cats were in 3–5 months of age, 93 cats were in 6–12 months, and 267 cats were in > 12 months. Three of 380 (0.8%; 0.22–2.3%) samples showed approximately 250 base pair bands, which were targeted product size for *C. felis*. BLAST comparison of these three-sequences showed 100% identity to *C. felis* (e.g., L19080, AY531524, AY679105, AF399930, GU903911). All *C. felis* infected cats were male and older than 12 months of age. Two infected cats were from Stillwater, OK and caught in March and April separately. One *C. felis* infected cat originated from Drumright, OK in March (Figure 1). A total of 292 blood samples were collected from August 2012 through April 2014 in IA. Genomic DNA extraction from 1 of 292 blood samples was not successful. There were no positive samples (0.0%; 0.0-1.3%).

DISCUSSION

The prevalence of *C. felis* (0.8%) in northcentral OK in the current study was lower than the previously reported state average 3.4% in domestic cats in Oklahoma (Rizzi et al., 2015). Rizzi et al. reported a difference in prevalence of *C. felis* infection depending on geographic locations within OK; 13 of 77 (16.9%) cats were infected with *C. felis* in eastern OK while 10 of 602 (1.7%) cats were infected with *C. felis* in northcentral OK. Rizzi et al. postulated differences in the prevalence of *C. felis* within OK could include strain variation in virulence of *C. felis*, differences in immunologic responses of cats to infection with *C. felis*, and differences in *C. felis* inoculation from

ticks. Although genetic variation of *C. felis* is still inconclusive (Brown et al., 2009), 3 different genotypes of *C. felis*, ribosomal internal transcribed spacer regions (ITS) A, B, and C, have been demonstrated, and ITSB and ITSC seem to be more pathogenic with higher mortality rate than ITSA (Brown et al., 2009). In OK, it has not been established which genotype is more prevalent or pathogenic than others; however, genotypic variations could influence prevalence because cats infected with more pathogenic genotype are likely to succumb to infection. Since cats become infected with *C. felis* through tick bites, prevalence of *C. felis* infection in domestic cats is likely affected by geographic variation of the abundance and activity of ticks and reservoir hosts. Our study supports the previous report (Rizzi et al., 2015) demonstrating that the prevalence of *C. felis* in a given enzootic area can vary from location to location and from the population of cats sampled.

DNA of *C. felis* was not detected in 291 blood samples collected from free-roaming cats in central IA, a non-enzootic state that borders enzootic states. One case of *C. felis* infection in a domestic cat has been reported in southwestern IA along the Missouri river (Raghavan et al., 2014). Unfortunately, this report did not provide details regarding the travel history, age, or sex of the infected cat. In our study, blood samples were collected from relatively young cats, and that might have influenced the results since those cats had less time being exposed to tick vectors. As geographic distribution and range of *A. americanum* expands northward (Lingren et al., 2005), it is important to keep monitoring free-roaming cat populations where cytauxzoonosis has not been considered enzootic.

Epizootiology of *C. felis* has not been completely elucidated. Historically only bobcats were thought to be reservoirs for *C. felis*; however, reports have indicated domestic cats that survived acute cytauxzoonosis become chronically infected with *C. felis* and can also serve as a source of *C. felis* infection (Reichard et al., 2009; Reichard et al., 2010; Rizzi et al., 2015; Kier et al., 1982; Birkenheuer et al., 2006; Ferris, 1979; Meinkoth et al., 2000; Haber et al., 2007). Since free-roaming outdoor cats encounter ticks, the source of *C. felis* infections, they are an excellent population to monitor the expansion of *C. felis* distribution in the United States.

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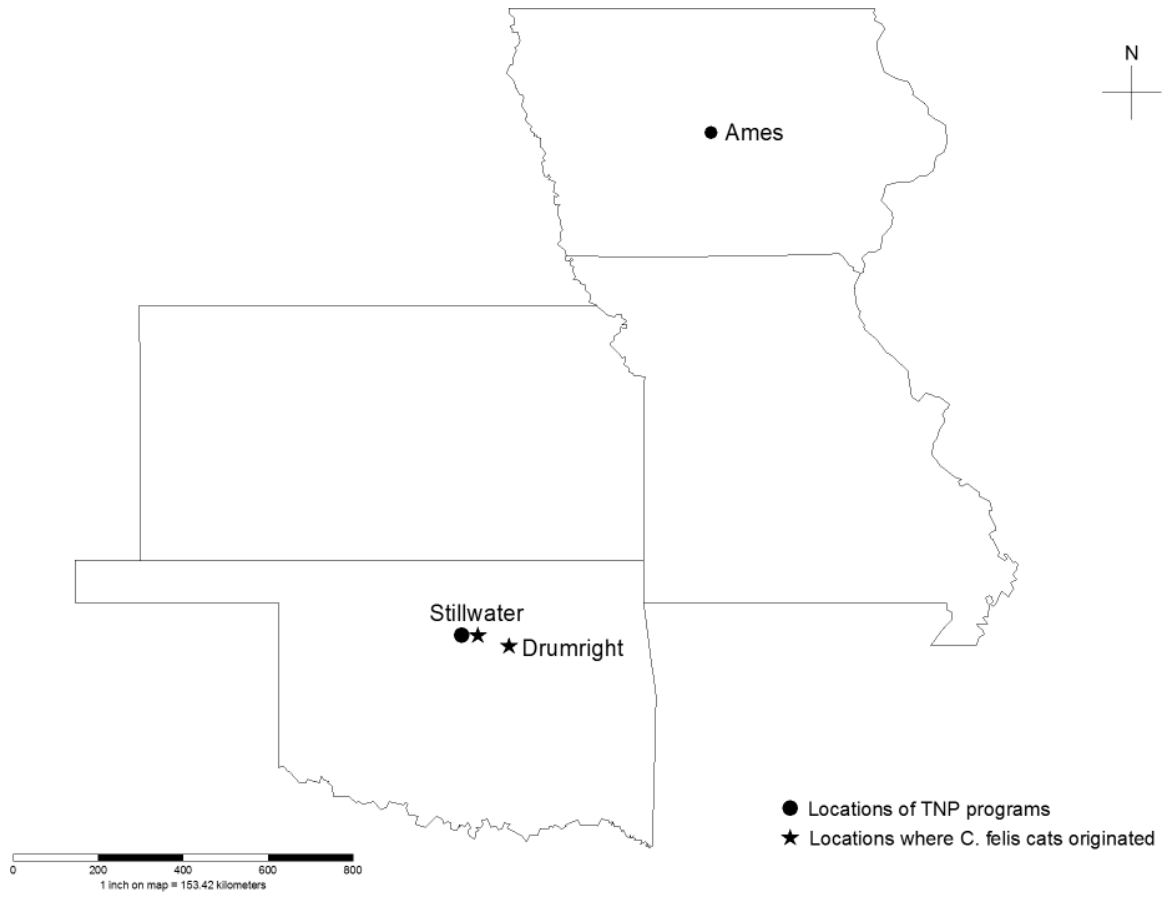
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Figure 1. Trap-neuter-return (TNR) programs were conducted in Stillwater, OK and Ames, IA. Cats infected with *Cytauxzoon felis* were found in Stillwater and Drumright, OK.



CHAPTER IV

PREVALENCE OF *CYTAUXZOOM FELIS* IN SALIVARY GLANDS OF *AMBLYOMMA AMERICANUM* AND APPLICATION OF TICK MORPHOMETRICS TO PREDICT DURATION OF ATTACHMENT AND PROBABILITY OF TRANSMISSION TO CATS

ABSTRACT

Cytauxzoonosis is a life-threatening hematoparasitosis in domestic cats caused by infection with *Cytauxzoon felis*. The geographic distribution of *C. felis* seems increasing as the range of *Amblyomma americanum* expands. However, the life cycle of *C. felis* is not completely understood, and diagnosis and treatment remain challenging. Objectives of this study included establishing supportive diagnostic technique using tick morphometrics, determining prevalence of *C. felis* infection in *A. americanum*, and developing a better understanding of life cycle with timing of *C. felis* transmission. Laboratory-reared *A. americanum* nymphs were acquisition fed on a *C. felis* infected donor cat to repletion, molted to adults, grouped as 25 or 30 mating pairs, and then transmission fed on *C. felis* naïve cats. Ticks were removed from principal cats at 24, 48, 72, 96-hours, or after repletion. Tick engorgement indices obtained from morphometric measurements of partially or fully engorged female ticks were calculated to predict the

risk of *C. felis* transmission. Ticks were then dissected longitudinally for *C. felis* detection; salivary glands removed from the right-halves were stained with methyl green pyronin (MGP), and the left-halves were used for DNA extraction followed by polymerase chain reaction (PCR). Determination of *C. felis* infection in principal cats was made by PCR on blood samples. Statistically significant differences in engorgement indices were observed at different intervals between 0, 1, 2, 3, and 4 day post-infestation ($P < 0.0001$). The salivary glands infected with *C. felis* were not observed in 350 fed ticks (0.0%; 0.0–1.0%). Flat ticks were also dissected for staining examination, and 54 of 351 (15.4%; 11.8–19.6%) were infected with *C. felis*. DNA from *C. felis* was detected in 4 of 358 ticks (1.1%; 0.30–2.84%); 2 females were recovered from 2 cats that had ticks on for 24 and 48 hours, respectively. *Cytauxzoon felis* infection was confirmed in 4 cats. One cat had ticks on for 72 hours and 3 cats had ticks on until repletion. The current study is the first report demonstrating salivary glands infected with *C. felis* in *A. americanum* and timing of *C. felis* transmission in ≤ 72 hours. Although morphometrics were statistically significant, it is difficult to apply practically because of overlaps between engorgement indices.

INTRODUCTION

Cytauxzoon felis is an intracellular protozoan parasite that can cause severe, often fatal, disease in domestic cats. Transmission of *C. felis* has been experimentally demonstrated with *Amblyomma americanum* (Reichard et al., 2010a; Reichard et al., 2009) and *Dermacentor variabilis* (Blouin et al., 1984). When a tick vector feeds on an infected feline, the tick acquires the organism. Although a sexual production phase,

called gametogony, is thought to occur within a tick (Tarigo et al., 2013), *Cytauxzoon felis* has not been visually demonstrated yet. Previous study demonstrated the salivary glands of *Rhipicephalus appendiculatus* infected by *Theileria parva*, which is closely related to *C. felis* (Criado-Fornelio et al., 2004; Ketz-Riley et al., 2003; Lack et al., 2012), with various staining techniques (Voigt et al., 1995). *Theileria parva* migrates from gut to salivary glands via hemolymph in *R. appendiculatus* after parasitized erythrocytes from an animal host were ingested (Dolan, 1989). Sporozoites develop in salivary glands and occupy most of the greatly enlarged glandular cells (Fawcett, 1985; Dolan, 1989). Voigt et al. (1995) demonstrated the salivary-gland acini infected by *T. parva* in 3 types of *R. appendiculatus*: unfed, attached to a host for 1–3 days, and attached for 4 days.

Clinical signs of cytauxzoonosis progress rapidly and may include fever, inappetence, anorexia, listlessness, icterus, dehydration, dark urine, and dyspnea (Blouin et al., 1984). Infected cats usually develop clinical signs about 2 weeks after being bitten by an infected tick (Blouin et al., 1984; Reichard et al., 2010a; Reichard et al., 2009), and many cats die within 24 hours of presenting to a veterinary clinic regardless of treatment (Cohn et al., 2011). Timing of *C. felis* transmission from an infected tick to cat remains unknown even though transmission of *Theileria* spp. and *Babesia* spp. usually takes at least 36–72-hours of tick-attachment on animals (Shaw & Young, 1994; Piesman & Spielman, 1980).

Since duration of tick-attachment is an important factor to determine the probability of infection, previous studies developed tick engorgement indices to describe the duration of tick feeding in relation to parasite development and transmission to animals (Obenchain et al., 1980; Piesman et al., 1987; Yeh et al., 1995). Tick

engorgement indices were created from ratios comparing total body length and scutum length, total body length and scutum width, and body width and scutum width, and were applied to predict the duration of attachment of ticks removed from people (Yeh et al., 1995). Since cytauxzoonosis progresses rapidly, prompt diagnosis and treatments are necessary to increase the survival infected cats. The purpose of the present study was to quantitate infection of *C. felis* in the salivary glands of *A. americanum*, determine the transmission time of *C. felis* from *A. americanum* to domestic cats, and develop an engorgement index to predict the risk of transmission of *C. felis* to domestic cats.

MATERIALS AND METHODS

Experimental Design

Laboratory-reared nymphs of *A. americanum* were acquisition fed on a *C. felis* infected donor cat to repletion. The nymphs were collected, stored in a humidity chamber, and allowed to molt to adults. These *C. felis* exposed ticks were grouped as 25–30 adult mating pairs and then fed on principal *C. felis* naïve cats. All ticks were removed from each principal cat at 24 (n=2), 48 (n=2), 72 (n=2), 96 (n=1)-hours, or until repletion (n=3). Partially or fully engorged ticks were measured for the total body length and width as well as the length and width of the scutum to the nearest millimeter. Ticks were then dissected longitudinally, and salivary glands were collected and separated to examine to sporogenous stages of *C. felis*. The right-half of salivary glands was stained with methyl green pyronin (MGP), and the left-half of ticks or salivary glands was used for DNA extraction followed by a conventional polymerase chain reaction (PCR) amplification using primers specific to *C. felis*.

Donor and Principal Cats

The *C. felis* donor cat remains persistently infected with *C. felis*. The parasitemia of *C. felis* in the donor cat at the time of acquisition feeding before group 1 and group 3 were 1.0% and 0.6%, respectively. Nine domestic cats were used as principals for transmission of *C. felis* from the donor cat. All principal cats were tested by blood smears and DNA extraction followed by conventional PCR amplification using primers specific to *C. felis* confirming free of *C. felis* infection. All animal procedures were approved by the Oklahoma State University Institutional Animal Care and Use Committee prior to initiation of the study.

Acquisition Feeding

The *C. felis* donor cat was sedated via intramuscular injection with 4.0 mg/kg Telazol (Fort Dodge, USA), and hairs in the trunk region were clipped to facilitate nymphal attachment. Approximately 1,000 nymphs of *A. americanum*, purchased from the Oklahoma State University Entomology Tick Rearing Facility (Stillwater, OK), were placed on the trunk region of the donor cat. A Surgi-Sox (DogLeggs; Reston, VA) was used to help confine and minimize escape of the ticks while feeding on the cat (Reichard et al., 2010b). The cat was monitored daily for any clinical change. Replete nymphs were collected and stored in a humidity chamber (90–95% humidity at 25 °C with a 14-hour light-dark photophase) until they molted to adult stage. Adult ticks were separated by sex and used for transmission feeding on principal cats.

Partial Feeding and Tick Engorgement Indices

The principal cats were randomly assigned to one of three groups (Figure 1). Thirty paired adult ticks (*i.e.* 30 females and 30 males) were allowed to feed on each of four principal cats (Group 1). All ticks were removed from principal cat A at 24 hours

post-infestation and from principal cat B at 48 hours post-infestation. Ticks on principal cats C & D were allowed to feed till repletion, and replete ticks were collected. Twenty-five paired adult ticks were placed on each of four principal cats (Group 2). All ticks were removed from principal cat E at 24 hours post-infestation, from principal cat F at 48 hours post-infestation, from principal cat G at 72 hours post-infestation, and from principal cat H at 96 hours post-infestation. Twenty-five paired adult ticks were placed on each of two cats (Group 3). All ticks were removed from principal cat E at 72 hours post-infestation. All ticks on principal cat I were allowed to feed until repletion, and replete ticks were collected.

Female ticks removed from the principal cats were immediately measured to the nearest millimeters with a digital schieblehre (Marathon; Ontario, Canada) with aid of a dissecting stereo microscope (VWR; Radnor, PA). The four areas of measurements included (1) total body length, (2) scutal length, (3) maximum scutal width, and (4) maximum alloscutal width as described in the previously published paper (Yeh et al., 1995). After measurements were completed all ticks were dissected longitudinally. Salivary glands were removed from right halves for MGP staining technique and the left halves were used for PCR amplification.

MGP Staining of Salivary Glands

The right-half of salivary glands in fed ticks were stained with methyl green pyronin (MGP; Irvin et al., 1981). Additionally, as a control, the salivary glands in flat *A. americanum* adults, acquisition fed as nymphs on the *C. felis* survivor, were removed and stained with the same method. Briefly, salivary glands were air dried and fixed in Carnoy's fixative for 2–5 minutes. After rinsing for 2 minutes each in 70% alcohol and in

distilled water, the salivary glands were stained in MGP for 7 minutes. After the slides were rinsed in distilled water and air dried, the slides were mounted in synthetic resin for microscopic examination with 100 and 400 magnification. Pictures of stained salivary-gland acini of *R. appendiculatus* infected with *T. parva* (Voigt et al., 1995) were used to identify infected from non-infected salivary glands.

Genomic DNA Extraction and PCR Amplification

Salivary glands were removed from left half of replete ticks and used for phenol-chloroform gDNA extraction technique (Halos et al., 2004). For partially engorged ticks, the entire left halves were used for gDNA extraction. The conventional PCR was used to confirm the presence of *C. felis* in salivary glands by amplifying the *C. felis* small subunit rRNA (ssu-rRNA). A nested PCR amplification was performed using primers (CfnestF. 5'-TCGCATTGCTTTATGCTGGCGATC-3' and CfnestR. 5'-GCCCTCCAATTGATACTCCGGAAA-3') that amplify 289 bp of the 18S rRNA gene of *C. felis* (Reichard, et al., 2010). Cycling conditions of nested PCR were as follows; denaturation at 95 °C for 5 minutes, annealing at 54 °C for 1 minute, and extension at 72 °C for 1 minute. The cycle was repeated 34 times. To determine success of genomic DNA extraction, PCR was performed for the gene encoding the small subunit rRNA (ssu-rRNA) (Bondy et al., 2005). PCR products were separated on 1.75% agarose gel and viewed with ultraviolet light. Positive control templates consisted of DNA extracted from whole blood of a cat that died from an infection of *C. felis*, whereas negative control reactions used DNA isolated from purified water.

Determination of *C. felis* Infection in Principal Cats

Cats were observed for any clinical signs of cytauxzoonosis and given routine physical examinations. Temperature was also monitored via an implanted temperature transponder by using a handheld scanner (Bio Medic Data Systems; Seaford, DE) daily or every 12 hours for all principal cats. Blood of principal cats was collected into a purple-top tube including ethylenediaminetetraacetic acid (EDTA; Monoject, Convivial, Minneapolis, MN) around 30-day post-infestation (dpi) if they did not show clinical signs. If principal cats showed clinical signs of cytauxzoonosis, blood samples were collected into an EDTA tube and then they were euthanized via intravenous administration of Beuthanasia-D (0.20 ml/kg; Schering-Plough, Summit, NJ). Genomic DNA was extracted from peripheral whole blood samples using GeneJET Whole Blood DNA Purification Mini Kit (Thermo Scientific, Grand Island, New York). Briefly, 200 μ l of whole blood was mixed with 20 μ l of Proteinase K Solution and 400 μ l of Lysis Solution. After incubating the sample at 56 °C for 10 minutes, 200 μ l of ethanol (96-100%) was added into the sample. The prepared mixture was transferred to the spin column and centrifuged for 1 minute at 6,000 x g (~8,000 rpm). The column was then washed with 500 μ l of Wash Buffer I and centrifuged again for 1 minute at 8,000 x g (~10,000 rpm). The column was washed again with Wash Buffer II and centrifuged for 3 minutes at 20,000 x g (14,000 rpm). DNA was eluted with 200 μ l of preheated (approximately 56 °C) PCR-quality water added to the center of the column membrane. The sample was incubated for 2 minutes at room temperature and centrifuged for 1 minute at 8,000 x g (~10,000 rpm) and a conventional PCR was performed as previously mentioned.

Data Analyses

Engorgement indices were calculated as the relationships between total body length and scutal length (index 1), total body length and scutal width (index 2), and body width and scutal width (index 3; Yeh et al., 1995). Regression analysis was used to determine relationships between duration of tick attachment and engorgement index. One-way analysis of variance (ANOVA) was performed to determine whether there are significant differences in engorgement indices at various time intervals. To determine prevalence of *C. felis* infection in *A. americanum*, 95% confidence intervals were calculated according to Sterne's exact method (Reiczigel, 2003) using Quantitative Parasitology 3.0 (Rozsa et al., 2000). Chi-square test was used to determine if prevalence of *C. felis* infection in salivary glands of ticks was different between sexes.

RESULTS

Tick Attachment

After 24-hour attachment, 29 female and 29 male *A. americanum* were recovered from cat A. After 48-hour attachment, 28 female and 30 male ticks were recovered from cat B. From 11–14 dpi, 52 female and 53 male replete adult *A. americanum* were recovered from two control cats, C and D. Unfortunately, the majority of ticks placed on cats in Group 2 did not attach and were found dead at the end of infestation periods. In addition, Surgi-Sox on cat E was torn by the cat within 4 hours after infestation allowing ticks to escape. During recovery from sedation, cat E was fractious preventing the application of a new Surgi-sox or mending of the old one. From cat E, 10 female and 12 male *A. americanum* were recovered after 24-hour attachment. After 48-hour attachment,

4 female and 8 male ticks were recovered from cat F. After 72-hour attachment, 14 female and 12 male ticks were recovered from cat G, and 15 female and 13 male ticks were recovered from cat H after 96-hour attachment. After 72-hour attachment on cat E, 23 female and 22 male *A. americanum* were recovered. On cat I, many ticks were found dead or not attaching on the cat, and only 9 females and 8 males were recovered on 15–16 dpi.

Tick Morphometrics

Both the total body length and width of *A. americanum* adult females increased with duration of feeding until 12 dpi, and their corresponding scutal length and width stayed relatively constant throughout the duration of attachment (Figure 3). Body length and width decreased after 12 dpi. Regression test showed statistically significant increase in all indices with duration of attachment even from 0 to 4 dpi: Index 1 ($t=13.76$; $P<0.0001$), Index 2 ($t=11.54$; $P<0.0001$), and Index 3 ($t=11.29$; $P<0.0001$). Additionally ANOVA showed statistically significant differences in all engorgement indices at different intervals between 0, 1, 2, 3, and 4 dpi: Index 1 ($F=50.20$; $df=4$; $P<0.0001$), Index 2 ($F=35.02$; $df=4$; $P<0.0001$), and Index 3 ($F=32.88$; $df=4$; $P<0.0001$).

MGP Staining of Salivary Glands

Right-half of salivary glands were removed from 168 female and 182 male fed *A. americanum* and entire salivary glands were removed from 198 female and 153 male unfed *A. americanum*. In fed ticks, infected salivary gland acini were not observed (0.0%; 0.0–1.0%), whereas 36 of 198 (18.2%; 13.1–24.3%) female and 18 of 153 (11.8%; 7.12–18.0%) male of salivary gland acini in flat *A. americanum* acquisition fed as nymphs were infected with *C. felis* (Figure 2). There was no statistically significant difference in

infected salivary glands between unfed female and male ticks ($X^2=0.0985$; $df=1$, $P=0.754$).

Genomic DNA Extraction and PCR Amplification

Genomic DNA extraction and conventional PCR were performed on left-half of 172 female and 186 male *A. americanum*. DNA from *C. felis* was detected in 4 of 358 ticks (1.1%; 0.30–2.84%); 2 females were recovered from cat A and 2 females were from cat B.

Determination and Confirmation of *C. felis* Infection in Principal Cats

Body temperatures of cat C and D began increasing around 12–13 dpi although they seemed still bright, alert, and responsive and had good appetite. Blood samples were collected from cat C and D on 14, and 13 dpi, respectively, and DNA extraction and a conventional PCR were performed. Approximately 250 base pair bands, which were targeted product size for *C. felis*, were detected from cat C and D. No clinical signs of cytauxzoonosis were observed on cat A and B throughout the study period. Blood samples of cat A and B were collected on 46 dpi, and no DNA of *C. felis* was found.

Body temperature of cat G began increasing on 14 dpi with no other significant clinical signs. Blood sample was collected from cat G on 18 dpi, and DNA of *C. felis* was detected. No clinical signs of cytauxzoonosis were observed for cats E, F, and H through 31 days post-infestation. Blood samples were collected and tested; none were infected with *C. felis*.

The body temperature of cat I started to increase around 14 dpi without other clinical signs. Blood samples were collected on 14 and 17 dpi. DNA of *C. felis* was found

in both samples. Cat E did not show any clinical signs of cytauxzoonosis through 32 dpi; PCR on collected blood sample confirmed that cat E was not infected by *C. felis*.

DISCUSSION

The current study is the first report demonstrating infection of *C. felis* in salivary glands of *A. americanum*. Although previous studies (Ochanda et al., 1996; Voigt et al., 1995) demonstrated *T. parva* in both fed and flat *R. appendiculatus*, we detected infection of *C. felis* in the salivary glands only in flat *A. americanum*. It remains unknown why we did not detect infected salivary glands in fed *A. americanum*; however, there are several possible reasons. First, in the current study, only right-half of salivary glands were removed from fed ticks and used for staining examination, whereas the entire salivary glands were examined for flat *A. americanum* adults. In the previous study with *T. parva*, the entire salivary glands were also examined for infection (Voigt et al. 1995). As *C. felis* DNA was detected from left-half salivary glands in 4 ticks by PCR, *C. felis* sporozoites may not be distributed equally in salivary glands of *A. americanum*. Secondly, since cat G (72-hour tick attachment) and all control cats became infected by *C. felis* and *C. felis* DNA was not detected on PCR from ticks that attached on cats for 72 hours or longer, most sporozoites likely were emitted from ticks after 48-hour attachment. Thirdly, unlike with life cycle of *T. parva* in *R. appendiculatus*, *C. felis* may locate in salivary glands for a short time or may take a longer time migrating to salivary glands. After *R. appendiculatus* ingested parasitized erythrocytes with *T. parva* while feeding on an infected animal host, piroplasms develop into dimorphic gametes in the lumen of the gut

in a tick (Dolan, 1989; Mehlhorn & Shein, 1984). These sexual forms fuse to form zygotes which enter the epithelial cells of the gut. Motile kinetes develop, pass through the haemolymph, and enter the salivary glands where they develop into sporoblasts and then sporozoites (Dolan, 1989). The complete life cycle of *C. felis* has not been determined yet, but *C. felis* may have a different, unique life cycle in *A. americanum* from that of *Theileria* spp. To increase sensitivity of staining examination, the entire salivary glands and other parts of *A. americanum*, such as gut and hemolymphs, may need to be tested for *C. felis* detection in fed ticks.

Additionally, the current study is the first report demonstrated *C. felis* can be transmitted from *A. americanum* to a domestic cat in ≤ 72 hours. Previous studies reported that transmission of *Theileria* spp. and *Babesia* spp. usually takes at least a few days of tick-attachment on animals (Shaw and Young, 1994; Piesman & Spielman, 1980). Transmission of *Babesia microti* (Piesman & Spielman, 1980) from an infected *Ixodes dammini* to human usually occurs in in $< 36\text{--}54$ hours of tick attachment (Piesman & Spielman, 1980). Ochanda et al. (1988) demonstrated a transmission of *Theileria parva parva* from an infected *R. appendiculatus* to a naïve cow in $< 24\text{--}72$ hours. A minimum length of tick attachment on a host to inoculate disease agents varies widely depending on tick species and type of pathogens (Fourie et al., 2013; Piesman et al., 1991; Piesman & Spielman, 1980). It remains unknown why cat H (96-hour tick attachment) and cat E in the group 3 (72-hour tick attachment) did not become infected by *C. felis*.

The engorgement indices in the current study showed statistically significant increases according to duration of attachment and differences between time intervals from 0 through 4 dpi (Table 1). However, it may be difficult and less practical to predict

the duration of tick attachment and probability of *C. felis* transmission by solely using tick morphometrics since there were overlaps of indices between time intervals in all indices. To increase the credibility of this technique, coxal index, a ratio of tick's ventral coxal gap and dorsal width of scutum, should have been also examined (Williams et al., 2015). However, it is important to note the limitations for estimating the risk of pathogen transmission based on tick morphometrics. It is less applicable to male ticks because *A. americanum* males do not engorge dramatically compared with females as other ixodid ticks and not all ticks will be infected with pathogens. However, it has been shown both female and male *A. americanum* can acquire disease agents and transmit them to animals (Anthony & Roby, 1966; Kocan & Stiller, 1992; Little et al., 2007; Steiner et al., 2008). Ratios of pathogen transmission between sexes of a given tick species seem variable depending on many factors, including tick species, disease agents, locations, and years of sampling (Steiner et al., 2008). Since ratios of *C. felis* transmission between male and female *A. americanum* are still unknown, when tick infestation is noticed on a cat patient, it is still important to follow the traditional recommendations: closely monitoring clinical signs of cytauxzoonosis and performing diagnostic examinations, such as clinicopathologic assessments, imaging, and PCR.

The current study is the first to report infection of *C. felis* in the salivary glands of *A. americanum* and transmission of *C. felis* from *A. americanum* to a domestic cat in ≤ 72 hours. Tick morphometric method was less applicable to predict duration of *A. americanum* attachment and probability of *C. felis* transmission to cats.

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Table 1. Engorgement indices for *Amblyomma americanum*

Index 1 = body length/scutal length; Index 2 = body length/scutal width; Index 3 = body width/scutal width

Duration of attachment (day)	Engorgement indices		
	Index 1	Index 2	Index 3
0	1.67–2.21	1.36–1.96	1.25–1.70
1	1.70–2.55	1.48–2.18	1.35–1.85
2	1.95–2.39	1.57–2.29	1.41–2.03
3	1.84–2.62	1.74–2.55	1.40–2.13
4	2.29–3.11	1.94–2.56	1.64–2.14

Engorgement indices 1–3 showed statistically significant differences at different intervals between 0, 1, 2, 3, and 4 days of attachment ($P < 0.0001$).

Figure 1: Partial feeding of *Amblyomma americanum* adults, acquisition fed as nymphs on a cytauxzoonosis survivor, on principal cats

A total of 30-paired *A. americanum* adults were infested on each principal cat in the group #1. All ticks were removed at 24 and 48 hours post-infestation from principal cat A and B, respectively. Ticks on principal cats C & D were allowed to feed till repletion, and replete ticks were collected. In similar fashion, 25-paired *A. americanum* adults were infested on each principal cat in the group #2 and 3. All ticks were removed at 24, 48, 72, 96 hours post-infestation from principal cat E, F, G, and H, respectively. All ticks were removed from cat E at 72 hours post-infestation. Ticks on cat I were allowed to feed till repletion, and replete ticks were collected.

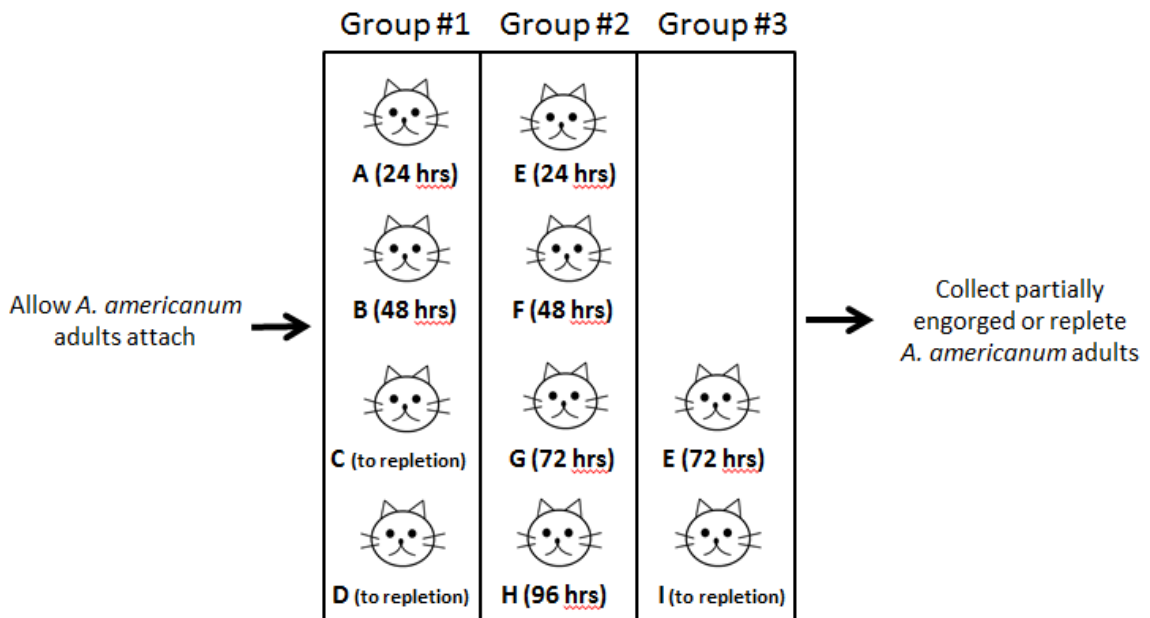


Figure 2. Methyl green pyronin staining of salivary gland acini of flat *Amblyomma americanum* infected with *Cytauxzoon felis* (magnification, x400). The infected acinar cells are indicated with arrows.

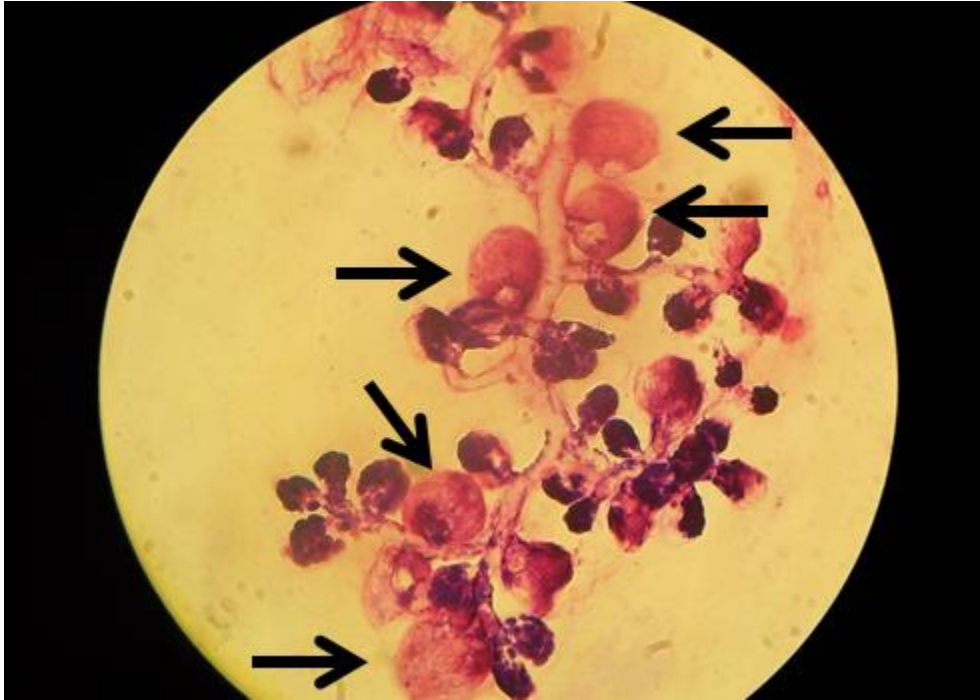
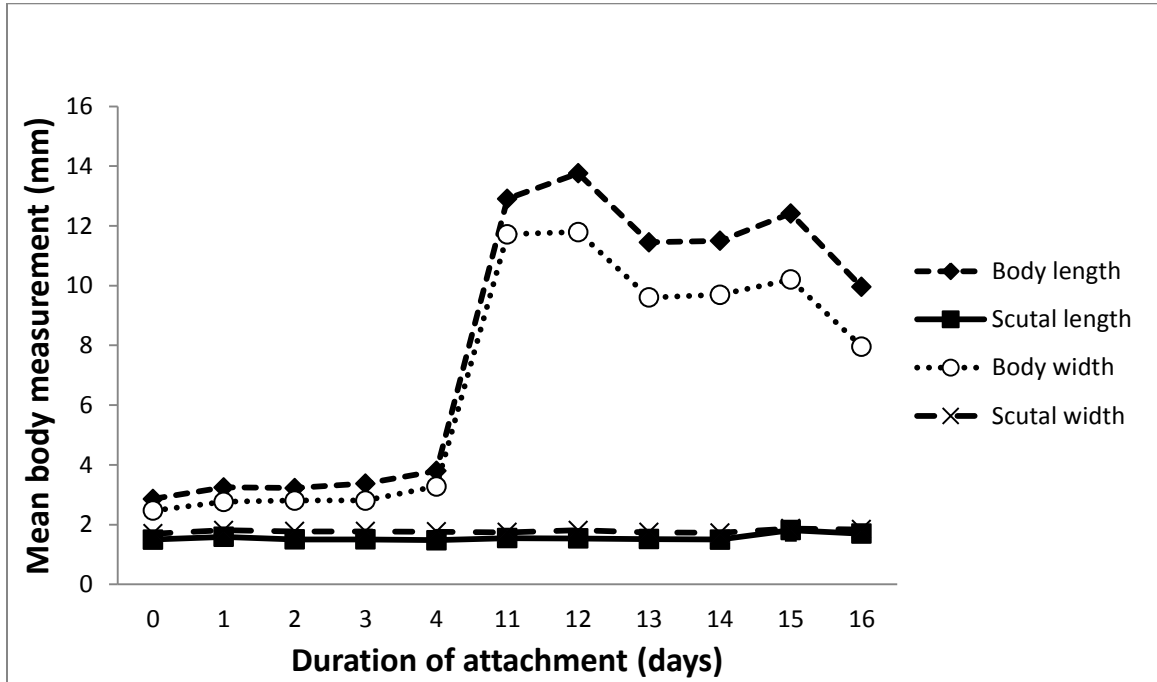


Figure 3. Comparisons of mean external body measurements of *Amblyomma americanum* female adults



CHAPTER V

NYMPHAL ENGORGEMENT WEIGHT PREDICTS SEX OF ADULT *AMBLYOMMA AMERICANUM*, *AMBLYOMMA MACULATUM*, *DERMACENTOR ANDERSONI*, *DERMACENTOR VARIABILIS*, AND *RHIPICEPHALUS SANGUINEUS*

ABSTRACT

Amblyomma americanum, *Amblyomma maculatum*, *Dermacentor andersoni*, *Dermacentor variabilis*, and *Rhipicephalus sanguineus* are common ticks in the United States. Sex of these ticks cannot be determined morphologically until they are adults. Our study evaluated if engorgement weight of replete nymphs differed between ticks that molted to males and females. Additionally we examined and compared length of feeding period of nymphs, molting success and durations, and sex ratios between these tick species. An adult sheep (*Ovis aries*) was used as a vertebrate host. Replete nymphs were weighed individually, allocated into different containers based on body weights, and allowed to molt to adults. Length of feeding duration was significantly different by species (F-value=1963.79; df=4; P<0.0001); *R. sanguineus* nymphs became replete fastest, followed by *A. americanum*, *D. variabilis*, *A. maculatum*, and *D. andersoni*. There was no significant difference in molting success between species. The mean body weight of engorged nymphs that became females was significantly greater than that of nymphs that molted to males in *A. americanum* (t=32.3; df=662; P<0.0001),

A. maculatum ($t=-9.70$; $df=255$; $P<0.0001$), *D. variabilis* ($t=15.7$; $df=751$; $P<0.0001$), and *R. sanguineus* ($t=5.17$; $df=560$; $P<0.0001$). In contrast, the mean body weight of *D. andersoni* engorged nymphs that became males was significantly greater ($t=8.71$; $df=480$; $P<0.0001$) than that of nymphs that molted to females. *Amblyomma maculatum* nymphs that molted to females fed to repletion faster ($t=3.33$; $df=265$; $P\leq 0.001$) than nymphs that molted to males. A significantly higher proportion ($X^2=48.4$; $df=1$, $P<0.0001$) of females molted than males of *A. maculatum* and *D. andersoni* ($X^2=8.19$; $df=1$, $P=0.004$). Our study demonstrated biological and behavioral differences between female-to-be and male-to-be nymphs and between the five tick species measured. These findings may help examining if there is a difference in prevalence of tick-borne pathogens between female and male ticks.

INTRODUCTION

Amblyomma americanum, *Amblyomma maculatum*, *Dermacentor andersoni*, *Dermacentor variabilis*, and *Rhipicephalus sanguineus* are common ixodid three-host ticks in the United States. Each of these ticks has significant medical importance as they can vector pathogens to animals and humans. Moreover, their bites can result in dermatitis, allergic responses, and paralysis, and feeding by large numbers of ticks can lead to anemia (Bowman, 2014).

Besides a difference in size of scutum, there are several biological and behavioral differences between female and male ixodid ticks. Female ticks have an ovary, paired oviducts, uterus, vagina, and seminal receptacle, whereas male ixodid ticks have testes, the tubular vasa deferentia, the seminal vesicle, and the ejaculatory duct (Sauer and Hair,

1986). Both female and male ticks in metastriate genera, such as *Amblyomma*, *Dermacentor*, and *Rhipicephalus*, need to feed first to become sexually mature (Sonenshine, 2005; Little et al., 2007). Females feed only once and swell enormously during a few days of attachment on a host, whereas males swell only slightly while feeding and may feed on several different hosts more than once (Sauer and Hair, 1986). Although both female and male metastriate ticks have been demonstrated capable of acquiring and transmitting pathogens (Anthony and Roby, 1966; Kocan and Stiller, 1992; Little et al., 2007; Zivkovic et al., 2007), there are limited reports showing differences in the prevalence, intensity, or abundance of tick-borne pathogens between females and males.

Sex of *A. americanum*, *A. maculatum*, *D. andersoni*, *D. variabilis*, and *R. sanguineus* cannot be determined morphologically until adult stages (Bowman, 2014). Previous studies demonstrated significant differences in body weight of engorged nymphs; heavier nymphs molted to females and lighter nymphs molted to males in *A. triguttatum* (Guglielmone, 1985), *A. neumannii* (Aguirre et al., 1999), *A. variegatum* (Centurion, 1979), *D. variabilis* (Hu and Rowley, 2000), *Hyalomma anatolicum* (Arthur and Snow, 1966), *Ixodes ricinus* (Kahl et al., 1990; Dusbabek et al., 1994; Dusbabek, 1996), *I. rubicundus* (Belozero et al., 1993), *I. scapularis* (Hu and Rowley, 2000), and *R. bursa* (Yeruham et al., 2000). In the current study, we determined if engorgement weight of replete nymphs differed between those ticks that molted to males and females in selected common ticks in the U.S.: *A. americanum*, *A. maculatum*, *D. andersoni*, *D. variabilis*, and *R. sanguineus*.

MATERIALS AND METHODS

An adult sheep (*Ovis aries*) was used as a vertebrate host for nymphs of *A. americanum*, *A. maculatum*, *D. andersoni*, *D. variabilis*, and *R. sanguineus*. To facilitate nymphal attachment, hairs were clipped in an approximately 12 cm diameter circle in six different locations: right and left sides of middle-lateral thorax and cranial- and caudal-lateral abdomen. Cylindrical white-colored 100% cotton interlock fabric (Hancock Fabrics) was secured to sheep using 3M Scotch-Weld Industrial Adhesive 4799 (St. Paul, MN) around each well. Nymphs were obtained from Oklahoma State University Entomology Tick Rearing Facility (Stillwater, OK). Batches of nymphs were released into different wells, one species per well, concurrently on the same sheep. The cloth for each cell was tied off with rubber bands to keep ticks from escaping. Nymphs were allowed to feed on the sheep until they were fully engorged. Sheep were maintained at room temperature and cared for daily. All replete nymphs were collected, and approximately 35–50% of replete nymphs of each species were randomly selected and weighed. Engorged nymphs were weighed individually to the nearest milligram with an analytical electric balance (Mettler Toledo; Switzerland) calibrated with 1-, 5-, and 10-gram certified weights (Troemner; NJ, USA). Nymphs were allocated into different containers in 1 mg increments based on their body weights. Ticks were maintained in humidity chambers (90–98% humidity at 25 °C with a 14-hour light-dark photophase) until they molted to adults. Sex of adult tick was identified morphologically. All animal procedures were approved by the Oklahoma State University Institutional Animal Care and Use Committee prior to initiation of the study.

A factorial experiment was used to examine and determine whether length of feeding duration was significantly different between species. Two-sample t tests were used to determine whether the body weight of engorged nymphs that molted to females was different from the body weight of engorged nymphs that molted to males. Fisher's exact tests were used to determine if there were significant differences in molting success rates between species. Two-sample t tests were also performed to examine whether nymphs that molted to female adults took longer to be replete than nymphs that molted to male adults. Chi-square goodness-of-fit tests were used to determine whether more female ticks molted successfully than males in each species. T tests and chi-square tests were performed by Microsoft Excel 2010 (Microsoft; Redmond, WA), and Fisher's exact tests were performed by SigmaPlot 13.0 (Systat Software, Inc.; San Jose, CA).

RESULTS

Feeding Period of Nymphs

Nymphs of 1,507 *A. americanum*, 1,105 *A. maculatum*, 1,385 *D. andersoni*, 2,110 *D. variabilis*, and 1,683 *R. sanguineus* were infested on the same sheep at the same time (Table 1). Fully engorged *A. americanum* nymphs were recovered on days 4–8 post-infestation with a mean of day 5.34 ($t=289.77$; $df=7785$; $P<0.0001$). Similarly, replete nymphs of *A. maculatum*, *D. andersoni*, *D. variabilis*, and *R. sanguineus* were collected on days 5–10 with a mean of day 6.51 ($t=302.73$; $df=7785$; $P<0.0001$), on days 6–10 with a mean of day 7.00 ($t\text{-value}=363.94$; $df=7785$; $P<0.0001$), on days 5–8 with a mean of day 6.10 ($t=391.96$; $df=7785$; $P<0.0001$), and on days 4–7 with a mean of day 4.99 post-infestation ($t=286.29$; $df=7785$; $P<0.0001$), respectively. Statistically, length of feeding

duration was significantly different by species; *R. sanguineus* nymphs became replete fastest, followed by *A. americanum*, *D. variabilis*, *A. maculatum*, and *D. andersoni* ($F=1963.79$, $df=4$, $P<0.0001$).

Molting success and durations

Randomly 682 of 1,507 (45.3%) *A. americanum* engorged nymphs were selected and weighed individually. In a similar fashion, 515 of 1,105 (46.6%) *A. maculatum*, 660 of 1,385 (47.7%) *D. andersoni*, 801 of 2,110 (38.0%) *D. variabilis*, and 618 of 1,683 (36.7%) *R. sanguineus* replete nymphs were weighed. A total of 681 (99.8%; $N=682$) *A. americanum*, 459 (89.2%; $N=515$) *A. maculatum*, 651 (98.1%; $N=660$) *D. andersoni*, 768 (95.8%; $N=801$) *D. variabilis*, and 613 (99.2%; $N=618$) *R. sanguineus* engorged nymphs successfully molted to the adult stage (Table 1). There were statistically significant differences in molting success between species ($P < 0.05$). Engorged nymphs of *A. americanum*, *R. sanguineus*, and *D. andersoni* molted more successfully than *D. variabilis* and *A. maculatum*. Nymphs of *A. americanum*, *A. maculatum*, *D. variabilis*, and *R. sanguineus* started to molt in 4–5 weeks and completed in 7–8 weeks after repletion. *D. andersoni* started to molt in 7–8 weeks and completed in 11-12 weeks.

Weights of engorged nymphs and resulting sexes of adults

Amblyomma americanum replete nymphs (Fig. 1) that molted to females ($N=354$) weighed between 6.0 and 21.0 mg with a mean body weight of 13.2 ± 2.2 mg (\pm SD) whereas males ($N=327$) ranged between 2.0 and 13.0 mg and had a mean of 8.4 ± 1.7 mg. In *A. maculatum*, the body weight of engorged nymphs (Fig. 2) that subsequently became females ($N=304$) ranged between 9.0 and 26.0 mg with a mean of 20.5 ± 2.4 mg whereas males ($N=155$) weighed between 6.0 and 25.0 mg and had a mean of 17.8 ± 3.0 mg. In *D.*

andersoni, the body weight of engorged nymphs (Fig. 3) that subsequently became females (N=362) ranged between 12.0 and 30.0 mg with a mean body weight of 20.1 ± 3.2 mg (N=362) whereas males (N=289) weighed between 8.0 and 34.0 mg and had a mean of 23.0 ± 4.8 mg. In *D. variabilis*, the body weight of replete nymphs (Fig. 4) that subsequently molted to females (N=386) ranged between 6.0 and 22.0 mg with a mean of 15.5 ± 2.3 mg whereas males (N=376) ranged between 4.0 and 19.0 mg and had a mean of 12.8 ± 2.5 mg. In *R. sanguineus*, replete nymphs (Fig. 5) that subsequently molted to female (N=306) weighed between 2.0 and 9.0 mg with a mean body weight of 5.7 ± 1.4 mg whereas males (N=307) ranged between 1.0 and 9.0 mg and had a mean body weight of 4.9 ± 2.0 mg.

The mean body weight of engorged nymphs that became females was significantly greater than that of nymphs that molted to males in *A. americanum* ($t=32.3$; $df=662$; $P<0.0001$), *A. maculatum* ($t=-9.70$; $df=255$; $P<0.0001$), *D. variabilis* ($t=15.7$; $df=751$; $P<0.0001$), and *R. sanguineus* ($t=5.17$; $df=560$; $P<0.0001$). In contrast, the mean body weight of *D. andersoni* engorged nymphs that became males was significantly greater ($t=8.71$; $df=480$; $P<0.0001$) than that of nymphs that molted to females (Table 1). However, in all tick species the body weights of engorged nymphs that molted to either males or females (Figs. 1–5) overlapped.

Length of Feeding Period Between Female-to-be and Male-to-be Nymphs

The length of feeding period between replete nymphs that molted to female and male is shown in Table 2. *Amblyomma maculatum* nymphs that molted to females fed to repletion faster ($t=3.33$; $df=265$; $P \leq 0.001$) than nymphs that molted to males. Significant

differences in the length of feeding period were not observed between females-to-be and males-to-be nymphs in any of the other tick species measured.

Sex Ratios

A significantly higher proportion ($X^2=48.4$; $df=1$, $P<0.0001$) of females molted than males of *A. maculatum* and *D. andersoni* ($X^2=8.19$; $df=1$, $P=0.004$). Statistically significant differences were not observed in sex ratios of *A. americanum*, *D. variabilis*, and *R. sanguineus*.

DISCUSSION

Engorgement weights of nymphs that molted to females were significantly greater than those of nymphs that molted to males in *A. americanum*, *A. maculatum*, *D. variabilis*, and *R. sanguineus*. Hu and Rowley (2000) discussed whether nymphal development to female or male is genetically controlled. Since XX-XO sex chromosome system has been reported in *A. americanum*, *A. maculatum*, *D. andersoni*, *D. variabilis*, and *R. sanguineus* (Guglielmone, 1985; Oliver, 1977), their hypothesis was that sex chromosomes could affect the feeding behavior; nymphs that become females may need to imbibe more blood than those that become males because females are ultimately larger and need more nutrition for reproduction. Although there were no previous studies that compared the length of feeding durations between female-to-be and male-to-be nymphs on sheep, the current study showed a significant difference in the feeding durations by sex in *A. maculatum*. *Amblyomma maculatum* nymphs that molted to females became engorged faster than those that molted to males.

Conversely, engorgement weight of *D. andersoni* nymphs that molted to males was significantly greater than those that molted to females. One striking difference between *D. andersoni* and other ticks we found was molting duration from nymph to adult. *Dermacentor andersoni* took approximately 12 weeks, while other species molted within 7–8 weeks. It is unclear why male-to-be nymphs of *D. andersoni* weighed more than female-nymphs-to-be, the opposite of the other tick species examined. Since *D. andersoni* has the same sex chromosomes, XO-XX, as the other tick species (Oliver, 1977), sex chromosomes are less likely related to cause the differences. *Dermacentor andersoni*, the Rocky Mountain wood tick, is distributed from western Nebraska and South Dakota, westward to the Cascades and Sierra Nevada Mountains, and from northern New Mexico and Arizona, northward into Canada (James et al., 2006). Compared with other tick species, the habitats of *D. andersoni* are generally higher in altitudes, cooler in temperature, and more dry in humidity. To successfully adapt to the less favorable environments, it might have been necessary for *D. andersoni* to evolve in a different way than others.

Observed differences in the length of feeding period between nymphs in the different species were most likely related to the body weights of replete nymphs. Since smaller ticks, such as *A. americanum* and *R. sanguineus*, required a smaller volume of blood, they engorged faster. In fact, size of adult *A. americanum* and *R. sanguineus* is generally smaller than others (Cooley, 1946; Cooley and Kohls, 1944; Cooley, 1938). The length of feeding duration is probably proportional to the body size and weight. We also observed more females successfully molted than males in *A. maculatum* and *D.*

andersoni. Since we did not examine the sex ratios in the entire batch of replete nymphs, we cannot determine if this difference was influenced by a sampling bias.

Belozerov et al. (1993) and Hu and Rowley (2000) examined *I. rubicundus* (n=33) and *I. scapularis* (n=121) respectively and demonstrated that all lighter nymphs molted into males and heavier nymphs molted into females. We increased the number of nymphs weighed; however, a weight threshold dividing female-to-be and male-to-be nymphs could not be established due to some level of overlap. Other studies (Arthur and Snow, 1966; Kahl et al., 1990; Dusbabek et al., 1994; Dusbabek, 1996; Aguirre et al., 1999; Hu and Rowley, 2000; Yeruham et al., 2000) also concluded no definite way to differentiate sex of engorged nymphs by body weights due to overlap between nymphs that became males and females. To predict sex of adult ticks by using body weight of engorged nymphs, it is important to note that outcomes may be affected by different tick species, tick populations, and animal hosts for the nymphs. Hu and Rowley (2000) examined body weight of engorged *D. variabilis* using laboratory mice as vertebrate host. Mean body weights of replete *D. variabilis* nymphs that molted to female and male were 11.8 ± 1.7 mg and 8.2 ± 1.7 mg, respectively, which are lighter than those of our results (16.0 ± 2.3 mg for female and 12.0 ± 2.5 mg for male). Dusbabek (1996) examined nymphal sexual dimorphism in laboratory-derived and field-collected *Ixodes ricinus* using BALB/c mice. Their results demonstrated engorged body weights of laboratory-derived *I. ricinus* nymphs (4.22 ± 0.50 mg for female 2.61 ± 0.32 mg for male) were significantly lighter than those of field-collected nymphs (4.94 ± 0.76 mg for female and 2.97 ± 0.44 mg for male). In nature active feeding seasons and preferred animal hosts for nymphs vary by different tick species (e.g. *R. sanguineus* nymphs stay active year round

and prefer to feed on dogs; while *D. andersoni* nymphs become active in early spring through late summer or early fall and prefer to feed on rodents) (Bowman, 2014). Even though engorgement weights of nymphs can be a good indicator to predict sex of adult ticks, there are limitations.

The body weight of engorged nymphs, which is presumably proportional to the blood volume ingested and concentrated, provides objective information on the amount of blood volume obtained from an animal host. Since most tick-borne disease agents are transmitted by tick bites from infected to naïve animals (Bowman, 2014), our findings can be helpful to determine if probability and prevalence of disease agents in ticks are proportionally related to body weight of engorged nymphs. Although ability of acquiring and transmitting tick-borne disease agents has been demonstrated in both female and male metastriate ticks (Kocan and Stiller, 1992; Little et al., 2007; Zivkovic et al., 2007), there is little information comparing prevalence of tick-borne pathogens between sexes of ticks. A survey conducted by Mixson et al. (2006) compared prevalence of *Ehrlichia chaffeensis* in female and male *A. americanum* and concluded its prevalence varied by locations; more females were infected in northeast, no female infected in Midwest, and approximately equal numbers of female and male were infected in South. Since our study demonstrated a significant difference in engorgement weights of nymphs that molted to females and males, there may be differences in prevalence, intensity, and abundance of tick-borne pathogens between female and male ticks. Further studies should be designed to test this hypothesis.

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Table 1. Comparisons of five different tick species.

	<i>A. americanum</i>	<i>A. maculatum</i>	<i>D. andersoni</i>	<i>D. variabilis</i>	<i>R. sanguineus</i>
Total number of replete nymphs	1,507	1,105	1,385	2,110	1,683
Number of weighed replete nymphs	682	515	660	801	618
Number of adults (molting success %)	681 (99.8%) ^a	459 (89.2%) ^d	651 (98.6%) ^b	768 (95.8%) ^c	613 (99.2%) ^{a,b}
Number of female : male	354 : 327	304 : 155*	362 : 289*	386 : 376	306 : 307
Mean body weight: Male (mg)	8.4±1.7	17.8±3.0	23.0±4.8	12.8±2.5	4.9±2.0
Mean body weight: Female (mg)	13.2±2.2	20.5±2.4	20.1±3.2	15.5±2.3	5.7±1.4
T-value	T=32.3**	T=-9.70**	T=8.71***	T=15.7**	T=5.17**
P-value	P<0.0001	P<0.0001	P<0.0001	P<0.0001	P<0.0001

^{a-d} Numbers not sharing the same letter are significantly different (p < 0.05)

* In *Amblyomma maculatum* and *Dermacentor andersoni*, a significantly higher proportion of female molted than males

** Engorgement weights of nymphs that molted to females were significantly greater than those of males in *Amblyomma americanum*, *Amblyomma maculatum*, *Dermacentor variabilis*, and *Rhipicephalus sanguineus*

*** Engorgement weight of *Dermacentor andersoni* nymphs that molted to males was significantly greater than those of females

Table 2. Length of day post-infestation (DPI) to repletion and number of replete nymphs that molted to male and female

	<i>A. americanum</i>		<i>A. maculatum</i> *		<i>D. andersoni</i>		<i>D. variabilis</i>		<i>R. sanguineus</i>	
	male	female	male	female	male	female	male	female	male	female
DPI 4	56	47	0	0	0	0	0	0	107	105
DPI 5	108	123	9	7	0	0	173	147	98	125
DPI 6	102	100	42	148	82	115	90	117	97	63
DPI 7	45	51	65	113	110	88	87	107	5	13
DPI 8	16	33	34	29	73	126	28	15	0	0
DPI 9	0	0	3	6	19	30	0	0	0	0
DPI 10	0	0	2	1	5	3	0	0	0	0

**Amblyomma maculatum* nymphs that molted to females reached to repletion significantly faster than nymphs that molted to males (U=18827.00; P=<0.001)

Figure 1. Sex of *Amblyomma americanum* as predicted by engorgement weight of 681 nymphs

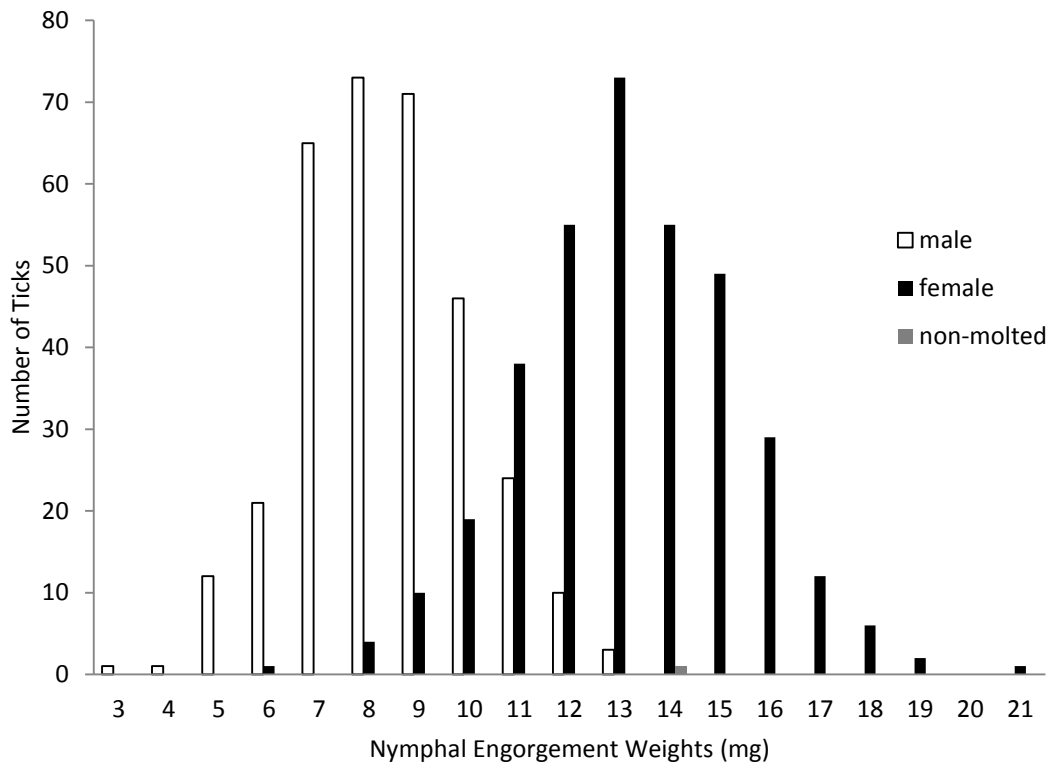


Figure 2. Sex of *Amblyomma maculatum* as predicted by engorgement weight of 459 nymphs

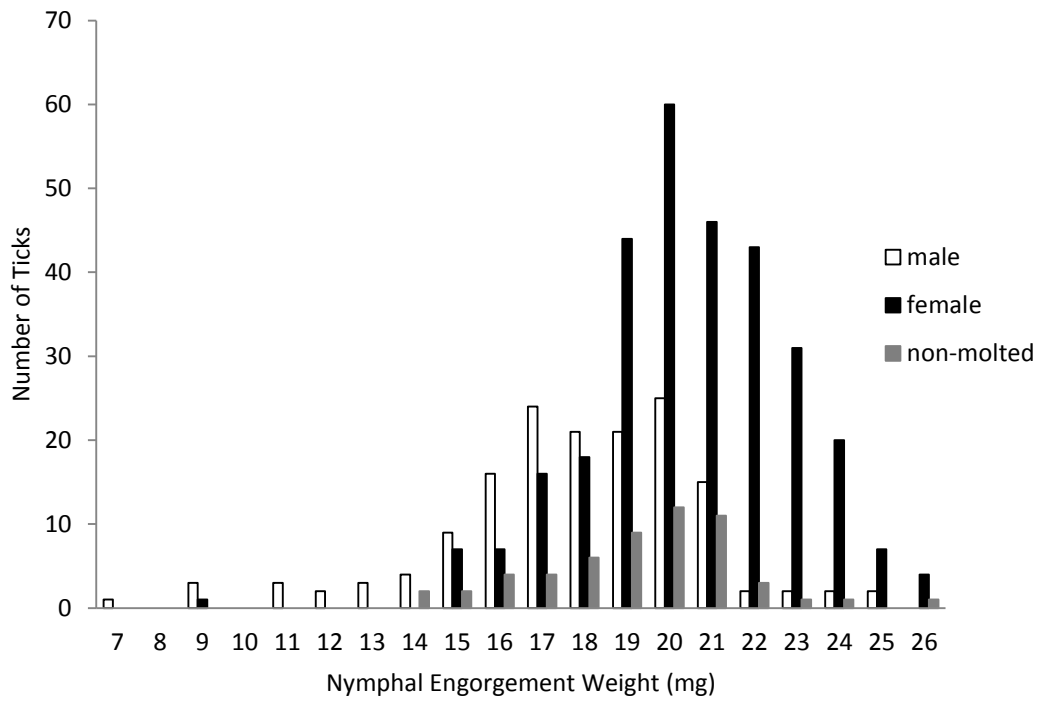


Figure 3. Sex of *Dermacentor andersoni* as predicted by engorgement weight of 651 nymphs

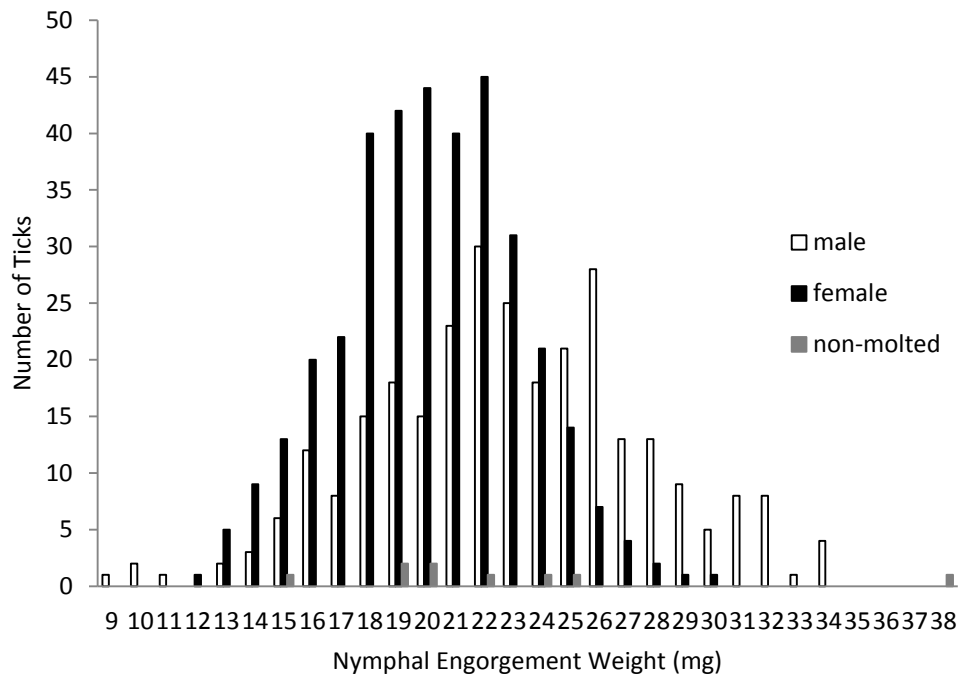


Figure 4. Sex of *Dermacentor variabilis* as predicted by engorgement weight of 768 nymphs

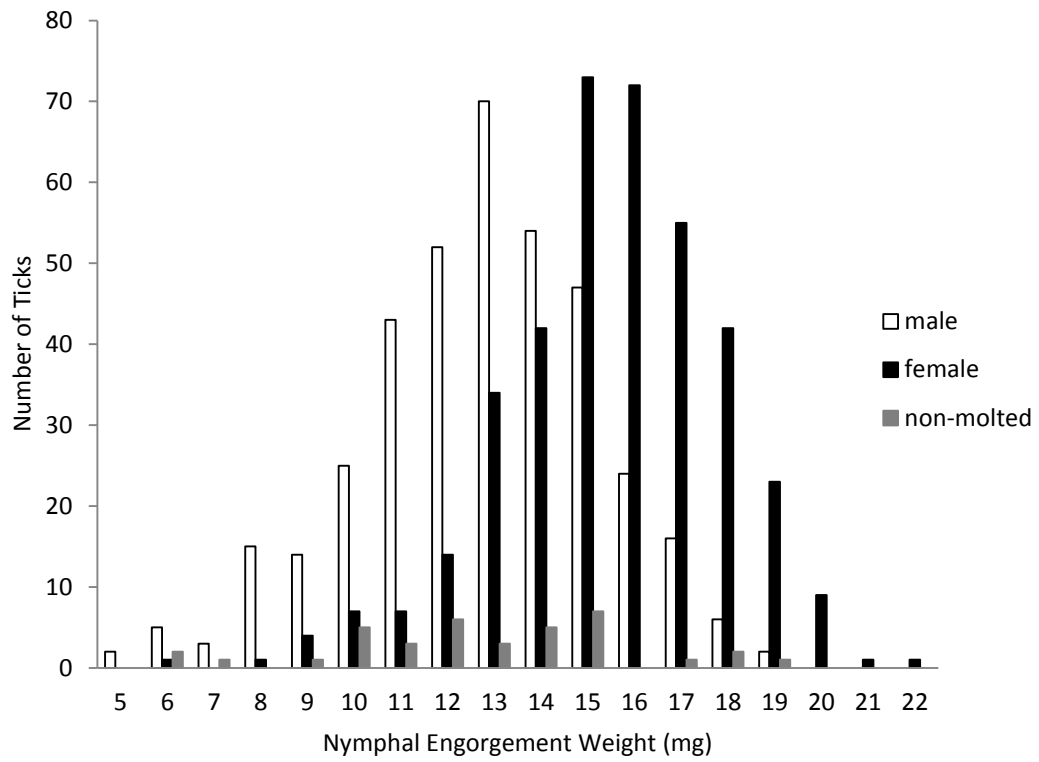
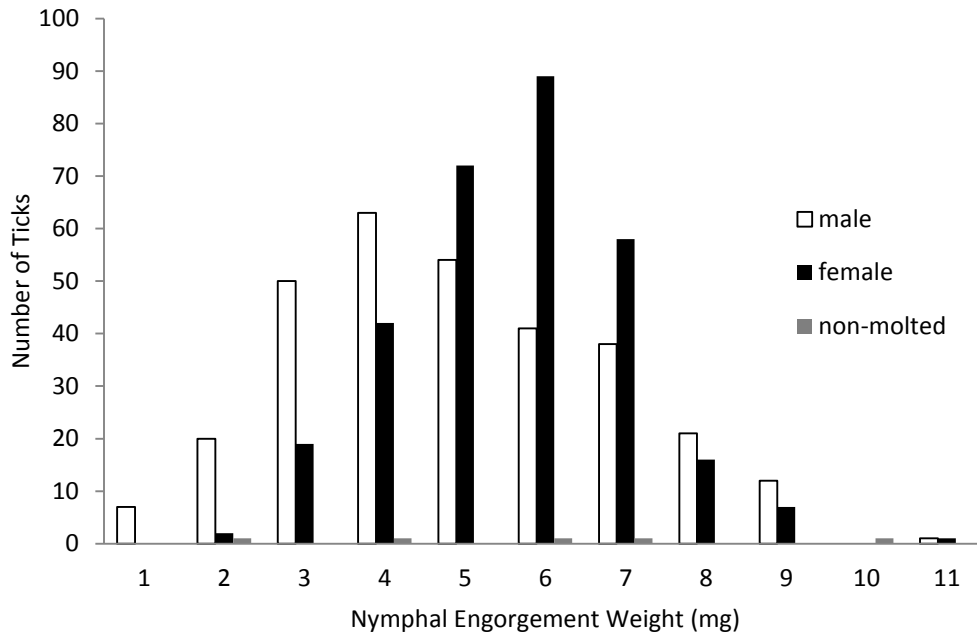


Figure 5. Sex of *Rhipicephalus sanguineus* as predicted by engorgement weight of 613 nymphs



CHAPTER VI

CONCLUSION

Ticks on domestic cats are often overlooked as parasites and vectors of disease agents compared with those on dogs. However, *Amblyomma americanum*, *Dermacentor andersoni*, *D. occidentalis*, *D. variabilis*, *Ixodes scapularis*, *I. pacificus*, and *Rhipicephalus sanguineus* have been commonly found and reported on domestic cats in the United States. Moreover, those tick species can transmit disease agents to cats, including *Anaplasma phagocytophilum*, *Cytauxzoon felis*, *Francisella tularensis*, *Borrelia burgdorferi*, and *Ehrlichia canis*-like agent. *Cytauxzoon felis* is a protozoan parasite that causes the most severe tick-borne disease of cats in the U.S. The geographic distribution and range of cytauxzoonosis seem to be increasing as the range of *Amblyomma americanum* expands. However, disease prevalence and life cycle have not been completely understood, and diagnosis and treatment remain challenging.

To contribute to work establishing the prevalence of *C. felis* infection, blood samples were collected from free-roaming cats since they live outdoors and are exposed to ticks more often. Although this study demonstrated a lower prevalence of *C. felis* infection than that in previous study, this result supported the previous report

demonstrating that the prevalence of *C. felis* in a given enzootic area can vary from location to location and from the population of cats sampled. Although DNA of *C. felis* was not detected in blood samples collected in Iowa, where *C. felis* is not considered as enzootic, it is important to keep monitoring for this parasite in non-enzootic areas since geographic distribution and range of *A. americanum* is expanding northward.

This study was the first report that visually demonstrated infection of *C. felis* in salivary glands of *A. americanum*. It was unexpected outcome that we detected infection of *C. felis* in the salivary glands only in flat *A. americanum*. It still remains unknown why we did not detect infected salivary glands in fed *A. americanum*. To increase sensitivity of staining examination, the entire salivary glands and other parts of *A. americanum*, such as gut and hemolymphs, may need to be tested for *C. felis* detection in fed ticks. In addition, this research was the first report demonstrated that *C. felis* can be transmitted from *A. americanum* to a domestic cat in ≤ 72 hours.

Although tick morphometric technique was demonstrated statistically significant, it is difficult to predict duration of *A. americanum* attachment and probability of *C. felis* transmission due to overlaps of indices between time intervals. In previous study, this technique was successfully applied as a supportive diagnostic method for female *I. scapularis* that transmits *B. burgdorferi*. It is important to note that this technique is less applicable to male *A. americanum* because they do not engorge dramatically compared with females and not all ticks will be infected with pathogens. Furthermore, it has been shown both female and male *A. americanum* can acquire disease agents and transmit them to animals. Since ratios of *C. felis* transmission between male and female *A. americanum* are still unknown, when tick infestation is noticed on a cat patient, it is still

important to follow the traditional recommendations: closely monitoring clinical signs of cytauxzoonosis and performing diagnostic examinations, such as clinicopathologic assessments, imaging, and PCR.

Nymphal sexual dimorphism in ticks was an incidental finding of this research. Although the phenomenon has been demonstrated in *D. variabilis*, this study was the first report showing the same phenomenon in *A. americanum*, *A. maculatum*, *D. andersoni*, and *R. sanguineus*. An unexpected result was the mean body weight of *D. andersoni* engorged nymphs that became males was significantly greater than that of nymphs that molted to females, whereas, the rest of tick species examined showed the opposite outcome. The body weight of an engorged nymph, which is presumably proportional to the blood volume ingested and concentrated, provides objective information on the amount of blood volume obtained from an animal host. Since most tick-borne disease agents are transmitted by tick bites from infected to naïve animals, our findings can be helpful information to determine if probability and prevalence of disease agents in ticks are proportionally related to body weight of engorged nymphs.

Overall, these studies have contributed to developing a better understanding of the prevalence of cytauxzoonosis and life cycle with timing of transmission from infected *A. americanum* to a domestic cat. In addition, the findings in significantly different engorgement weights of nymphs that molted to females and males may assist examining a hypothesis if there are differences in prevalence, intensity, and abundance of tick-borne pathogens between female and male ticks.

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