Vector-Host Prevalence Comparison of *Cytauxzoon felis* in south Georgia and north Florida

By

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Submitted to the Faculty of the Graduate School of

Valdosta State University

in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

in Biology

in the Department of Biology

of the College of Science and Mathematics

December 2022

Bachelor of Science in Exercise Physiology

Florida State University

Tallahassee, Florida

2016

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DocuSign Envelope ID: 038472D9-03EA-4F1F-9E17-E4DB3EC993F5

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ABSTRACT

Cytauxzoonosis is an infectious disease caused by the protozoan parasite *Cytauxzoon felis* that affects members of the family Felidae. *Cytauxzoon* belongs to the phylum Apicomplexa, order Piroplasmida, and family Theileriidae. Members of this family are tick-transmitted protozoans that exist in erythrocytic and leukocytic stages in their intended hosts. Historically, the disease was thought to be fatal to domestic cats, but recent cases of host survival have been documented. Studies on the genetic variation of the first and second internal transcribed spacer regions (ITS1 and ITS2) of the rRNA operon have identified five prominent genotypes (ITSA, ITSB, ITSC, ITSG, ITSI) associated with varying cytauxzoonosis severity in the bobcat and domestic cat. Little research has been done so far to uncover how these genotypes are expressed in the vectors of the pathogen, *Amblyomma americanum* (lone star tick) and *Dermacentor variabilis* (American dog tick). A number of studies have shown *A. americanum* to be a more competent vector in the wild. In this study PCR and gel electrophoresis were used to test for the presence of *C. felis* in blood samples from *Felis catus* (domestic cat), and the vector *A. americanum*. After testing for the presence of the parasite in each blood sample, positive samples were DNA sequenced in an attempt to identify distinct *C. felis* genotypes. Previously genotyped *Lynx rufus* (bobcat) DNA samples were used as positive controls and for comparison purposes. A total of 109 domestic feral cat samples were collected for this study from June 2019 to February 2020, and a total of 260 lone star tick samples were collected from March 2020 to May 2020. Of the collected samples eight samples from domestic cats and 17 from lone star ticks were sent to Florida State University for DNA sequencing. DNA sequence results showed that our samples were infected with more than one pathogen, the three genera within the order Piroplasmida were identified (*Babesia sp, Theileria sp,* and *Cytauxzoon sp)*.

Keywords: *Cytauxzoon felis,* domestic cat, *Amblyomma americanum* (lone star tick), Georgia, Florida, PCR

46 **INTRODUCTION**

47 The protozoan parasite *Cytauxzoon felis* is transmitted by *Amblyomma americanum* (lone star
48 tick) and *Dermacentor variabilis* (American dog tick) to members of the Felidae family (Jakob
49 and Wesemeier, 1996; Lewis et al., 2012; Peixoto et al., 2007). The pathogen was initially
50 discovered in Missouri in the late 1970s, and has since been documented in central, southeastern,
51 and south-central areas of the United States (Meiknoth and Kocan, 2005; Reichard et al., 2008)
52 (Fig. 1). *Cytauxzoon* belongs in the phylum Apicomplexa, order Piroplasmida, and family
53 Theileriidae. Related genera include *Babesia,* and *Theileria* (Wagner, J.E. 1976). Members of
54 this family are tick-transmitted protozoans that exist in erythrocytic and leukocytic stages in their
55 intended hosts (Wagner, J.E. 1976). Genera within the order have been grouped together based
56 on mechanisms of transmission within the vector, host cell infection type, parasite morphology,
57 and vertebrate host preference (Schreeg et al., 2016). Species within *Theileria* and *Cytauxzoon*
58 undergo transstadial transmission within the tick and infect nucleated cells within their vertebrate
59 host during initial infection (Schreeg et al., 2016). Species within *Babesia* exhibit transovarial
60 transmission within the tick and infect unnucleated cells within their vertebrate host (Schreeg et
61 al., 2016). Pathogens within this family infect both domestic and wild animals as well as
62 humans.

63 The *C. felis* life cycle exhibits sexual and asexual stages, both occur within the vector
64 while only asexual reproduction is documented within the mammalian host (Fig. 2). Within the
65 tick, sexual reproduction begins in the gut as macro- and micro- gametes, they produce a zygote
66 that penetrates the gut wall and then migrates to the salivary glands (Brown, 2010). The zygote
67 develops into a haploid kinete which invades the salivary glands. Within the salivary glands,
68 kinetes undergo asexual reproduction to produce the infective sporozoites that are inoculated into

69 the host during feeding (Greene et al., 2006). Once transmission into the intermediate host has
70 occurred, the pathogen undergoes two stages: schizogony and merogony. Initially in the
71 schizogony stage sporozoites transmitted from the tick infect white blood cells of the host and
72 undergo asexual reproduction (schizogony) to produce schizonts. Schizonts are commonly found
73 within lymph nodes, spleen, liver, lung, and bone marrow (Tarigo, 2022) (Fig. 3). These
74 schizont-infected white blood cells increase in size from 15µm up to 250µm leading to parasitic
75 thrombi with symptoms of ischemia and tissue necrosis (Tarigo, 2022). This schizogenous tissue
76 phase is when cats begin to show clinical illness, with symptoms appearing 10-14 days post
77 infection (Sherrill and Cohn, 2015). Schizonts within white blood cells can be visualized on the
78 edges of stained blood smears, as they are larger in size than the merozoites that infect red blood
79 cells, and can be visualized during acute cytauxzoonosis (Cohn, 2014; Brown, 2010). Once
80 schizont infected white blood cells rupture, they release merozoites (piroplasms) that either
81 infect host red blood cells or develop into gametocytes (Wikander et al., 2020). In the merogony
82 stage, symptoms include an increase in body temperature paired with a decrease in leukocyte
83 numbers (Tarigo, 2022). Visualization of piroplasms on stained blood smears can be used for
84 diagnosis of cytauxzoonosis, commonly Wright's stain or Diff Quik are used (Cohn, 2014).
85 Piroplasms are usually detected 1-3 days before the animal succumbs to infection, at which point
86 they are 1-1.5µm in size (Cohn, 2014; Brown, 2010) (Fig. 3). Fine needle aspiration of splenic
87 tissue, liver tissue, or lymph nodes can also be used to visualize mononuclear cells (Cohn, 2014).
88 If schizonts or piroplasms cannot be visualized microscopically but *C. felis* is suspected, a
89 polymerase chain reaction (PCR) test can be used to identify the pathogen (Tarigo, 2022).
90 *Amblyomma americanum* and *D. variabilis* have both been implicated as vectors of *C.*
91 *felis* (Schock et al., 2012) (Fig. 4). Both species are within the family of hard ticks, Ixodidae,

92 characterized by the hardened plate on their dorsal surface known as the scutum. They use their
93 chelicerae to pierce the skin of their host along with the pedipalps and hypostome that aid in
94 attachment (UMaine Cooperative Extension, n.d.). They make use of the chemosensory organ
95 (Haller's organ) that is found on their first pair of legs to detect carbon dioxide being emitted by
96 potential hosts (Carr et al., 2017). Members of this tick family exhibit one-host, two-host, or
97 three-host life cycles; both *C. felis* vectors exhibit three-host life cycles (Mangan et al., 2018).
98 Their life cycle spans two years and is dependent on host availability, humidity, ambient
99 temperature, and day length (Schulze T.L. and Jordan R.A., 2003; Reichard et al., 2010). The
100 preferred host for each species is dependent on life stage; *A. americanum* larvae and nymphs
101 feed on small avian or mammal hosts while adults feed on large mammals (Kollars, 1993).
102 *Dermacenter variabilis* larvae feed on small mammals, nymphs feed on medium-sized
103 mammals, and adults feed on large mammals (Matheson, 1950). Transstadial maintenance of the
104 parasite within the vector has been documented allowing nymphal and adult stages to infect hosts
105 (Wikander et al., 2020). Seasonal peaks in tick activity correspond to increased cases of
106 cytauxzoonosis in domestic cats, peaking in late spring and early fall (Wikander et al., 2020).
107 Initial discovery of the pathogen implicated *D. variabilis* as the natural vector, but more
108 recent studies have shown *A. americanum* to be a more capable vector (Reichard et al., 2010;
109 Reichard et al., 2009). Transmission from the American dog tick was confirmed in a study by
110 Blouin, et al. (1984) where they experimentally showed that transstadial transmission occurs in
111 this vector. A 2005 study in Missouri screened 352 ticks representing either *A. americanum* or *D.*
112 *variabilis* and found that only samples from *A. americanum* tested positive for the pathogen
113 (Bondy, et al., 2005). Reichard et al., performed a wild survey of both *A. americanum* and *D.*
114 *variabilis* that showed only *A. americanum* ticks were naturally infected with *C. felis* (2010).

115 Due to its role as primary vector and documented presence in south Georgia and north Florida,
116 this study will focus on the collection of *A. americanum* as the vector for *C. felis* (Bondy, et al.,
117 2005; Brown, et al., 2009) (Fig. 5).

118 Cytauxzoonosis in domestic cats historically was found to be a fatal disease (Reichard et
119 al., 2009). The bobcat experiences subclinical infection, followed by chronic parasitemia while
120 rarely experiencing fatal infections (Bondy, et al., 2005). Clinical symptoms within domestic cats
121 begin to appear 5-14 days post infection. Initial symptoms present as lethargy and anorexia,
122 followed by elevated body temperature, jaundice, inflammation of lymph nodes, or dyspnea.
123 Without treatment death usually occurs 2-3 days after an elevated body temperature (Tarigo,
124 2022). Recent studies have shown that the domestic cat is capable of surviving cytauxzonoosis,
125 leading to their potential as reservoir hosts for the parasite (Haber, et al., 2007; Birkenheur, et al.,
126 2006).

127 Studies on the genetic variation of the first and second internal transcribed spacer regions
128 (ITS1-ITS2) of the rRNA operon of *C. felis* have found that there are 11 different sequences
129 resulting in three genotypes within domestic cats (Brown et al., 2009). This region is a
130 noncoding region on the rRNA operon, it does not have structure-function constraints, and is
131 likely to be replaced quickly. Due to its high variability, this region is useful in studying
132 presumed strains of *C. felis*. Genotypes ITSA, ITSB, ITSC were associated with varying
133 survival rates of domestic cats in a previous study, it was hypothesized that varying
134 pathogenicity is due to these different strains of the disease (Brown, 2010). Domestic cats with
135 genotype ITSA were identified as having the highest survival rate of the three genotypes, ITSB
136 had an intermediate survival rate and domestic cats with ITSC all experienced death (Brown et
137 al., 2010). In the same study, *C. felis* ITS1 and ITS2 sequence data was collected from 25/34

138 bobcat tissue samples, some of which will be used for this study (Brown et al., 2010). Sequence
139 data from the 2010 study uncovered 11 genotypes in bobcats, of these ITSA and ITSB were
140 shared between both domestic cats and bobcats. (Brown et al., 2010). Another study of the *C.*
141 *felis* ITS1-ITS2 region found ITS1 and/or ITS2 sequences for 145/161 *C. felis* infected DNA
142 bobcat samples (Schock et al., 2012). Of the 145 sequences screened, only 111 gave combined
143 ITS1-ITS2 sequences, for a total of 25 described genotypes (Shock et al., 2012).

144 Pathogen virulence was associated with ITSA, ITSB, and ITSC genotypes in the Brown
145 (2009) study, subsequent research did not find clinical outcome to be associated with *C. felis*
146 genotypes (Brown et al., 2010; Cohn et al., 2011; Shock et al., 2012). Published data associated
147 with the vectors of the pathogen have focused on efficacy for transmission, transmission time,
148 presence of the pathogen, or distribution of vectors (Blouin et al., 1984; Reichard et al., 2009;
149 Reichard et al., 2010, Nagamori, 2016). The following is a comprehensive study on the
150 prevalence of *C. felis* in *A. americanum* and *F. catus* in north Florida and South Georgia. We
151 hoped to characterize strains of the parasite in the vector. *L. Rufus* tissue samples from a previous
152 predator control study were used to compare prevalence between all three hosts of the parasite.
153 We aimed to compare genotypes to previously published sequences in Genbank to assess
154 sequence variability. Previous studies have shown that the bobcat is the natural reservoir host,
155 capable of surviving infection and remaining subclinically infected (Blouin et al., 1984; Shock et
156 al., 2012). In Florida *C. felis* has also been documented in free ranging Florida panthers as well
157 as a captive white tiger (Butt et al., 1991; Garner et al., 1996). Unlike large felids, domestic cats
158 usually experience rapid disease progression that leads to death after the onset of disease (Pollard
159 et al., 2017). Feral domestic cats tend to live solely outdoors with little human contact, they have
160 greater exposure to ectoparasites and other mammals making them ideal subjects for studying

161 prevalence and distribution of *C. felis* (Nagamori, 2016). We suspected higher genotypic
162 diversity within the tick and bobcat as they are exposed to a variety of pathogen strains and host-
163 parasite co-evolution (Maizels and Kurniaqan-Atmadja, 2002; Matrajt, 2010). In domestic cats
164 we expected that ITSA would be the most common genotype (Brown et al., 2010).
165

166 **MATERIALS AND METHODS**

167 **Ethical Approval**

168 All study activities involving animals and management of animal data were performed in
169 accordance with an approved Valdosta State University Institutional Animal Use Protocol
170 (Appendix I).

171 **Blood collections from domestic cats:**

172 Blood from domestic cats was collected from three animal welfare groups that participate
173 in Trap Neuter and Release programs: PAWS of Wakulla Inc., Wakulla, FL, Humane Society of
174 Valdosta, Valdosta, GA and South Georgia Low Cost Spay and Neuter Clinic, Thomasville, GA.
175 Collections occurred on days where feral cats from Tallahassee, FL, Crawfordville, FL, or
176 Valdosta, GA were undergoing hysterectomy or neuter procedures. Permission was granted by
177 the manager of each clinic for phlebotomy to be performed post-surgery on subjects. All clinics
178 required cats to be brought in a humane animal trap that allowed for anesthesia induction by a
179 syringe pole. Post hysterectomy or neuter, 1-2 mL of whole blood was collected via venipuncture
180 of the femoral artery of each cat and placed into blood collection tubes with EDTA. Cats were
181 then placed back into their humane traps under heat lamps to regulate their body temperature,and
182 they were monitored for several hours post-surgery (Fig. 6). Whole blood samples were used to
183 test *for C. felis* since the parasite reproduces in both white and red blood cells. Blood samples

184 were placed in coolers for transport to Valdosta State University and were held at -20°C until
185 they underwent DNA extraction. A total of 109 domestic feral cat samples were collected for this
186 study from June 2019 to February 2020.

187 *Thomasville Low Cost Spay and Neuter clinic*

188 After intake, cats were anesthetized with Telazol powder mixed with 2.5mL of
189 Butorphanol and 2.5mL of DexDomitor (Jones C, personal communication. November 20,
190 2018). The cats remained in their humane traps for induction of anesthesia via a syringe pole
191 (Clinic Procedures, 2016.). This clinic provided 12 samples for the study.

192 *PAWS of Wakulla*

193 TNR clinics were organized to occur on Sundays allowing participants to drop cats off early
194 Sunday mornings for intake. Drugs for induction at this clinic consisted of a mixture of Telazol,
195 DexDomitor, and Butorphanol with Meloxicam as an NSAID. A total of 29 samples were
196 collected from this clinic.

197 *Humane Society of Valdosta*

198 TNR clinics were organized to occur on Sundays allowing participants to drop cats off
199 early Sunday mornings for intake. Drugs for induction at this clinic consisted of a mixture of
200 Telazol, Ketamine, and Xylazine. This clinic provided the largest number of samples at 68.

201 **Domestic cat DNA Extractions**

202 Following the methods performed in a similar study by Brown (2010), DNA extraction
203 from EDTA whole blood samples followed the manufacturer's instructions for the Illustra blood
204 genomic Prep Mini Spin Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). 200 µL of
205 whole blood from 3mL EDTA tubes were used for each extraction. The extracted DNA was
206 stored at -20 °C for PCR.

207 **Bobcat DNA samples:**

208 *Lynx rufus* (bobcat) frozen tissue samples from a previous study by one of the committee
209 members were used as positive controls in this study. These samples provided a comparison for
210 genotypes between hosts and vectors (Brown et al., 2010). Samples from the study were obtained
211 at Tall Timbers Research Station in Tallahassee, FL, Pebble Hill Plantation in Thomasville, GA
212 and Pinebloom Plantation in Newton, GA from 2003-2006 (Brown et al., 2010). Samples were
213 maintained at Valdosta State University at -20 °C. Only samples collected at Tall Timbers
214 Research station were utilized; genotypes for these samples were provided by the Peterson lab at
215 the University of Georgia.

216 **Bobcat Tissue DNA Extraction**

217 The Qiagen DNeasy Blood & Tissue kit (QIAGEN Inc, Germantown, Maryland, USA)
218 was used for DNA extraction from bobcat tissue samples. Spleen, liver, or lung samples were
219 used for DNA extraction, as it has been demonstrated that *C. felis* disseminates into those organs
220 (Tarigo, 2022). A section of tissue, less than 25mg in size, was cut from each sample and placed
221 into a labeled 1.5mL microcentrifuge tube with 180 µL of Buffer ATL and 20 µL of Proteinase
222 K. Samples were vortexed for 15s and incubated until tissue was completely lysed. After
223 incubation they were vortexed for 15s, then 200 µL of Buffer AL was added followed by
224 vortexing for 15s. 200 µL of pure ethanol was added to each sample followed by vortexing for
225 15s. Mixtures were pipetted into prepared DNeasy spin columns and centrifuged at 8,000 rpm for
226 1 min. Flow through was discarded and the spin column was placed into a new collection tube.
227 500 µL of Buffer AW1 was added into each spin column and centrifuged at 8,000 rpm for 1
228 minute. Flow through was discarded and spin columns were added into a new collection tube.
229 500 µL of Buffer AW2 was added into each spin column and centrifuged at 14,000 rpm for 3

230 mins. Flow through and collection tubes were discarded, spin columns were placed into new

231 1.5mL microcentrifuge tubes. Samples were eluted with 200 μL of Buffer AE and allowed to

232 incubate at room temperature for 1 min. Samples were microcentrifuged at 8,000 rpm for 1 min

233 and spin columns were then discarded. DNA extraction products were stored at -20 °C for PCR.

234 These steps were performed on two bobcat tissue samples from the 2003-2006 predator study

235 that were collected at Tall Timbers Research Station: PRS05-354 and PRS05-466. Confirmation

236 of parasite presence was conducted by PCR.

237 ***Amblyomma americanum* collection:**

238 Permission to collect lone star ticks was granted by Tall Timbers Research Station in

239 Tallahassee, FL (Fig. 7). Tall Timbers is a prescribed fire research station that is home to a

240 longleaf pine ecosystem. *A. americanum* are hunting ticks that travel to follow carbon dioxide

241 gradients, with peak activity levels during periods of high temperature and low humidity

242 (Schulze et al., 2001; Schulze and Jordan, 2003). Previous studies on the efficacy of dry-ice

243 baited traps versus field dragging have shown that CO_2 traps are more effective in collection of

244 adult *A. americanum* (Keisenger and Allan, 2011; Schulze et al., 1997;). This study focused on

245 adult *A. americanum* for ease of identification and to increase the likelihood of *C. felis* presence

246 associated with the life stage. Traps consisted of a cardboard box outlined by clear duct tape, the

247 center of each trap had about 0.5 kg of dry ice (Fig. 8). Collections occurred from March to May

248 2020, during which 260 lone star ticks were trapped. Traps were set mid-morning and allowed to

249 sit for evaporation of dry-ice for 4-5 hours into the warmest parts of the day (Schulze and Jordan,

250 2003). A total of 8-11 traps were set each collection day, for five collection dates. Tick

251 collections were labeled per day and trap, ticks were placed in glass collection vials with 70%

252 isopropyl alcohol until DNA extractions were performed.

253 ***Amblyomma americanum* DNA Extraction:**

254 *Amblyomma americanum* ticks were identified when brought back to the lab (Fig. 9). A
255 dissection microscope was used to visualize the capitulum and scutum of ticks, *A. americanum*
256 was identified using the identification guide provided by the University of Missouri Extension
257 program (Houseman, 2013). The Qiagen DNA Easy Blood and Tissue kit (QIAGEN Inc,
258 Germantown, Maryland, USA) with the following protocol was used to extract DNA from the
259 whole body of ticks (Bondy et al., 2005). Ticks were not separated into prosoma and
260 opisthosoma as the objective of this study was uncovering the presence of *C. felis* in samples
261 followed by genotyping positive samples. DNA from ticks was extracted individually to allow
262 for determination of the specific genotype present in our sample.

263 Ticks were individually removed from 70% isopropyl alcohol vials and placed into 1.5
264 mL microcentrifuge tubes labeled with their sex and date of collection. 180 µL of Buffer ATL
265 was added to microcentrifuge tube followed by 15s of samples being vortexed. The moistened
266 body of the tick was removed from its microcentrifuge tubes and placed on a clean petri dish.
267 The body of the tick was cut longitudinally and diagonally with a scalpel, placed back into
268 microcentrifuge tubes and vortexed for 10 mins. 20 µL of Proteinase K was added, samples were
269 vortexed for 15s and incubated at 56 °C in a water bath for one hour and forty-five mins.
270 Samples were vortexed for 15s after being removed from the water bath. 200µL of Buffer AL
271 was added to each sample, followed by vortexing for 15s. Samples were incubated at 70 °C for
272 10 mins and then pipetted into prepared 2mL spin columns and collection tubes. Spin columns
273 were centrifuged at 8,000 rpm for 1 min. Flow through and collection tubes were discarded. Spin
274 columns were placed into new collection tubes, 500 µL of Buffer AW1 was added to each. Spin
275 columns were centrifuged at 8,000 rpm for 1 min. Flow through and collection tubes were

276 discarded, spin columns were placed into new collection tubes. 500 μL of Buffer AW2 was
277 added to each followed by centrifugation at 14,000 rpm for 3 mins. The flow through and
278 collection tube were discarded, and the spin column was placed into a new 1.5 mL centrifuge
279 tube. 35 μL of Buffer AE was added to each sample and samples were incubated at room
280 temperature for 1 min, then centrifuged at 8,000 rpm for 1 min. 30 μL of Buffer AE was added
281 to each column a second time followed by incubation at room temperature for 1 min, and
282 centrifugation at 8,000 rpm for 1 min. Extracted DNA was stored at -20 °C prior to PCR.

283 **Polymerase Chain Reaction (PCR) amplification for detection of ITS1-ITS2 region**

284 Information from previously sequenced ITS1-ITS2 regions of *C. felis* found in GenBank
285 was used to prepare primers to detect the presence of the pathogen in DNA extracted from
286 collected lone star ticks and blood samples from cats. Both the ITS1 and ITS2 region were
287 targeted by specific forward and reverse primers in order to identify genetic variability in
288 positive samples. Primer information was retrieved from Brown et al. genetic variability study
289 (2009). Primers were purchased from Integrated DNA Technologies (Integrated DNA
290 Technologies, Coralville, IA, USA). The sequences for ITS1 forward and reverse primers were
291 5'-CGATCGAGTGATCCGGTGAATTA-3' and 5'-GCTGCGTCCTTCATCGATGTG-3'
292 respectively. Primers were expected to produce a 651bp amplicon that includes the 458bp ITS1
293 region plus 18S and 5.8S partial flanking regions. The sequences for ITS2 forward and reverse
294 primers were 5'-TGAACGTATTAGACACACCACCT-3' and 5'-
295 TCCTCCCGCTTCACTCGCCG-3' respectively. ITS2 primers were expected to produce a
296 431bp amplicon that encompasses the 265bp ITS2 region plus 18S and 5.8S partial flanking
297 regions. Bobcat DNA samples from a previous study were used as positive controls, negative
298 controls consisted of molecular-biology grade water (Brown et al., 2010). Each PCR reaction

299 consisted of 1X Green GoTaq Flexi Buffer (Promega, Madison, WI), 3mM $MgCl_2$ (Promega,
300 Madison, WI), 400 μM each of dATP, dCTP, dGTP, dTTP (Fischer Scientific, Pittsburgh, PA),
301 0.4 μM each of either ITS1 or ITS2 *C. felis* forward and reverse primers (IDT, Coralville, IA),
302 and 1 unit of GoTaq DNA Polymerase (Promega, Madison, WI). Each reaction was brought up
303 to 25 μL using 10.8 μL of molecular grade water. Cycling parameters were as follows:
304 denaturation at 95°C for 5 min, followed by 50 amplification cycles each consisting of 95°C for
305 60 s, 59.2°C for 60 s, 72°C for a 90s, with a final extension step at 72°C for 5 min. PCR products
306 were size fractionated on 3% agarose gels stained with ethidium bromide and visualized under
307 ultraviolet light.

308 **Purification of presumed positive PCR products**

309 PCR products from ticks and domestic cats were purified using the Qiagen QIAquick
310 PCR Purification Kit (QIAGEN Inc, Germantown, Maryland, USA). Purification followed the
311 protocol outlined by Qiagen. Sodium acetate was added to the mixture of PCR product and
312 Buffer PB. DNA was eluted with water for the final two steps. Purified DNA was packaged on
313 ice for overnight shipment to Florida State University's Sequencing Facility.

314 **DNA Sequencing**

315 Two different regions were targeted for sequencing, the ITS1 and ITS2 regions of the
316 rRNA operon. Automated bi-directional DNA sequencing was performed at Florida State
317 University's laboratory (FSU Biological Core Facility, Florida State University, Tallahassee, FL,
318 USA). Samples were sent overnight to the facility along with ITS1 and ITS2 forward and
319 reverse primers. Bobcat samples had been previously sequenced at a university laboratory or a
320 commercial laboratory per Brown's 2010 study (Georgia Genomics Facility, University of
321 Georgia, Athens, GA; MACROGEN, Rockville, MD, USA).

322 **Chromatogram Analysis**

323 Chromatograms were analyzed using 4Peaks (Nucleobytes BV, Aalsmeer, The
324 Netherlands) sequence viewer and editor. Sequences received from FSU's Biological Core
325 Facility were trimmed based on sequencing quality scoring. According to the sequencing quality
326 score of a given base, Q, where $Q = -10log_{10}e$ the higher the base score the lower probability
327 of error (Illumina Inc., San Diego, CA, USA). Sequences were trimmed based on quality base
328 scores; the beginning of the sequence had a minimum of five bases with a quality score higher
329 than 20. Sequences were trimmed when quality base scores fell below 20 for five bases in a row.
330 According to the sequencing quality score equation a base score of 20 represents an error of 1 in
331 100, or a base accuracy of 99% (Illumina Inc., San Diego, CA, USA).

332 **Sequence Identification**

333 Trimmed sequences were entered into the National Center for Biotechnology Information
334 Nucleotide BLAST program (U.S. National Library of Medicine, Rockville, MD, USA). Search
335 parameters for sequences were set for "standard databases," and "somewhat similar sequences."
336 BLAST searches were conducted twice per sequence, the initial search did not specify a
337 particular organism. The secondary BLAST search specified "*Cytauxzoon felis*" as the organism.
338 Sequence description results, total score, query cover, e-value, and percentage identification
339 were compiled for each.

340 **RESULTS**

341 A total of 109 samples were collected from feral domestic cats from three clinics. Eight
342 samples appeared presumptively positive for *C. felis* amplicons following gel electrophoresis,
343 yielding an initial infection rate of 7.3% from the collected feral cats. Six of these samples were
344 collected at Valdosta Humane Society's TNR clinic and two were collected at PAWS of

345 Wakulla. Seven samples were from spayed female domestic cats and one from a neutered male
346 (Table 1). The putative *C. felis* amplicons were sent to Florida State University for DNA
347 sequencing.

348 DNA from 260 lone star ticks was extracted and amplified. From these 260 individuals,
349 17 appeared presumptively positive for *C. felis* amplicons following gel electrophoresis (Fig.10),
350 yielding a 6.5% infection rate among the ticks collected. Fourteen of these samples were
351 extracted from female lone star ticks and three from male lone star ticks (Table 2). These 17
352 positive samples were sequenced at Florida State University.

353 The sequencing of putative *C. felis* amplicons from lone star ticks and domestic cats
354 yielded ITS1 and/or ITS2 sequences from 18 of 27 submitted samples (66.7%). ITS1 sequence
355 data was obtained from 8 lone star tick samples and none of the domestic cat samples, while
356 ITS2 sequence data was obtained from 7 tick samples and 3 cat samples. Combined ITS1/ITS2
357 data was collected from four tick samples while none of the cat samples produced sequence data
358 from both regions. BLAST result descriptions, GENBANK accession numbers, query cover, e-
359 values, total score, and percentage identification were documented for the 18 samples that gave
360 ITS1 and/or ITS2 sequences. Two BLAST searches were performed per sample, the initial
361 BLAST search was to identify the organism present in the sample, the second BLAST search
362 was specific for *C. felis.*

363 Initial BLAST inquiry results for ITS1/ITS2 data showed that several samples were
364 infected with pathogens other than *C. felis* (Tables 3a, 3b, 3c). ITS1 *A. americanum* samples
365 yielded three sequences that matched *Babesia* species isolates, four that matched *Theileria*
366 species isolates, and one of the samples did not match any members within Theileriidae (Table
367 3a). Sample F12 (04/09) matched *Hepatozoon canis,* a protozoan parasite whose main vector is

368 *Rhipicephalus sanguineus;* the lone star tick is not a known vector (Otranto, et. al, 2011).

369 Samples F47 (04/09), and F8 (04/09) resulted in *Babesia* species isolates, sample F21 (04/09)

370 only yielded a forward sequence that also resulted in *Babesia* species isolates. Samples F2

371 (04/30), F1 (05/14), F5 (05/14), and F10 (05/14) only gave a reverse sequence, resulted in

372 *Theileria* species isolates for the ITS1 region. We did not receive any ITS1 sequences for

373 domestic cat samples. BLAST results for ITS2 were performed for *A. americanum* and *F. catus*

374 samples. *Amblyomma americanum* ITS2 sequences yielded *C. felis, Theileria* species, and one

375 sample gave results outside of the Theileriidae family (Table 3b). Samples F11 (04/09) and F47

376 (04/09) only gave a reverse sequence, and M4 (04/02) resulted in *C. felis* isolates, F1 (05/14) led

377 to *Theileria* species organisms. All *F. catus* samples yielded "*Felis catus* Senzu DNA,

378 chromosome: E1, American Shorthair breed," with a percentage identification higher than 90%

379 (Table 3c).

380 Secondary BLAST searches specific to *C. felis* generated more results for *A. americanum*

381 than the initial general BLAST search. Seven samples of *A. americanum* yielded *C. felis* for ITS1

382 upon the secondary BLAST search, and seven for ITS2 (Tables 4a and 4b). F47 (04/09), F10

383 (05/14), F8 (05/14), and F1 (05/14) ITS1 reverse sequences all yielded *C. felis* under the

384 accession number AY531524.1. Samples F21 (04/09), F8 (05/14), and F5 (05/14) ITS1 forward

385 sequences all yielded *C. felis* accession number KC122660.1, while sample F12 (04/09) yielded

386 accession number DQ458797.1. All samples had a greater than 80% identification. In terms of

387 ITS2 *C. felis* results F11 (04/09), F12 (04/09), F47 (04/09), F10 (05/14), and M4 (04/02) reverse

388 sequences all yielded accession number HQ383911.1. Samples F12 (04/09), F47 (04/09), F10

389 (05/14), M4 (04/02), and M7 (05/14) ITS2 forward sequences resulted in *C. felis* accession

390 number JF330260.1 with 100% identification. The forward sequence of F1(05/14) shared 88.8%

391 identity with GENBANK accession number AY531524, while the reverse shared 96.3%
392 homology with *C. felis* GENBANK accession number MG584567. Four samples gave *C. felis*
393 for both ITS1 and ITS2, sample F12 (04/09), F47 (04/09), F10 (05/14), and F1 (05/14). There
394 were no sequences received for ITS1 with regards to the domestic cat samples, all ITS2 samples
395 resulted in *C. felis* GENBANK accession number JF330260.1 (Table 4c).

396 **DISCUSSION**

397 A survey of field-collected adult lone star ticks and feral-trapped domestic cat blood
398 samples was performed for *C. felis* exposure. The goal of this study was to test for the presence
399 of *C. felis* in its natural vector and one of its hosts, after which genotyping of the ITS1-ITS2 was
400 performed. Feral cats were selected for the survey since they are at an increased risk for parasite
401 exposure. The area for lone star tick collection was selected based on a previous study conducted
402 by Brown et al. in which surveyed bobcats in the area were positive for *C. felis* (Brown et al.,
403 2010). A total of 260 lone star ticks were collected, along with 109 whole blood samples from
404 feral domestic cats. Sequencing of presumed positive samples of both subjects revealed the
405 presence of parasitic members of the family Theileriidae, including *Babesia, Theileiria,* and
406 *Cytauxzoon.* The aim of this study was to compare host and vector genotypes against published
407 virulence data on *C. felis.* We successfully performed positive amplification from our PCR
408 protocol, however our results could not be used for genotypic comparison due to the presence of
409 parasitic co-infection.

410 This study focused on *A. americanum* as the vector for transmission of *Cytauxzoon felis*
411 and was aimed at finding distinct genotypes in the vectors to compare to hosts in the same
412 region. DNA sequencing of samples occurred in two phases: initial BLAST for amplicons were
413 non-specific; secondary BLAST inquiries were specific for *C. felis*. Of 17 samples that resulted

414 in positive PCR amplification, no ITS1 amplicons and three ITS2 amplicons matched published
415 *C. felis* sequences in GENBANK for both initial and specific BLAST searches (17.7%). In total
416 3/109 (2.8%) domestic cat samples resulted in *C. felis* amplifications. All three of these samples
417 were collected at the Valdosta Humane Society that serves Lowndes Co. and surrounding
418 counties. The secondary BLAST search of all *A. americanum* sequences showed sequence
419 homology with *C. felis* resulting in 5.4% prevalence of infection within this species (14/206).

420 Prevalence of the pathogen in domestic cats has been studied using data from trap-neuter-
421 release clinics among midwestern, mid-Atlantic, and southcentral states. Studies within Florida
422 and Georgia are limited. In Kansas 1104 cats were evaluated for *C. felis* by qPCR, and 270/1104
423 (25.8%) tested positive (Wikander et al., 2020). A study by Haber et al. of cats brought to a trap-
424 neuter-release program found an overall prevalence of 0.3% from cats in North Carolina (0/392),
425 Tennessee (1/75), and Florida (2/494) (Haber et al., 2007). A study that utilized private
426 veterinary clinics examined a total of 902 samples from clinics in Missouri, Arkansas, and
427 Oklahoma and found *C. felis* in 56/902 cats (6.2%) (Rizzi et al., 2015). The study by Rizzi was
428 conducted over a four-year period, while Haber collected samples from June 1999 to February
429 2000. In our study, samples were collected from June 2019 to February 2000, yielding a
430 prevalence rate of 2.8%. Time permitting, blood collections would have continued through the
431 end of the summer to coincide with peaks in adult lone star ticks in the area.

432 Numerous *in vivo* studies have focused on the transmission ability of *A. americanum* to
433 transmit *C. felis* at different life stages confirming that *A. americanum* is the more competent
434 vector for the pathogen (Reichard et al., 2010; Reichard et al., 2009). This study used a
435 molecular approach to detect *C. felis* within *A. americanum,* resulting in a 5.4% (14/206) parasite
436 presence within the vector. Bondy et al., (2005) analyzed ITS1 of the rRNA region from ticks

437 removed from cats and dogs in Missouri and found 3/1,362 individual or pooled samples to be
438 positive. This study is one of the first to focus specifically on the genetic variability of *C. felis*
439 within *A. americanum.* Although the possibility of having multiple parasites present within our
440 samples was minimized by individually testing the ticks, genotypic analysis still found members
441 of the *Babesia,* and *Theileria,* genera. To more specifically isolate samples with only *C. felis,* a
442 qPCR procedure could have been used. Alternatively, using two primers might have increased
443 the sensitivity of the PCR (Bondy et al., 2005).

444 The prevalence of parasitemia with *C. felis* found in this study cannot be compared
445 directly with other published findings because different assay methods and target genes were
446 used to detect infection. This study is one of the first to directly test for infection in domestic cats
447 and ticks from Florida and Georgia. Previous studies focused on the presence of infection or
448 transmission ability within host or vector. Genotypes published by Brown et al., identified 11
449 sequences that resulted in three genotypes for the ITS1-ITS2 region of the rRNA operon (2009).
450 Although this study was intended to compare genotypes found within the samples, the presence
451 of coinfection with multiple parasites made this impossible. The presence of *C. felis* in domestic
452 cats as well as within the vector supports other studies that have detected the parasite in large
453 felids from Florida (Brown et al., 2010; Butt et al., 1991; Shock et al., 2012). Further work with
454 these blood samples can focus on other tick-borne pathogens such as *Francisella tularensis,* or
455 members of the family Theileriidae that can be transmitted to felids. Additionally, the domestic
456 cat samples can also be tested for *Bartonella henselae, Dirofilaria immitis,* or *Toxoplasma*
457 *gondii.*
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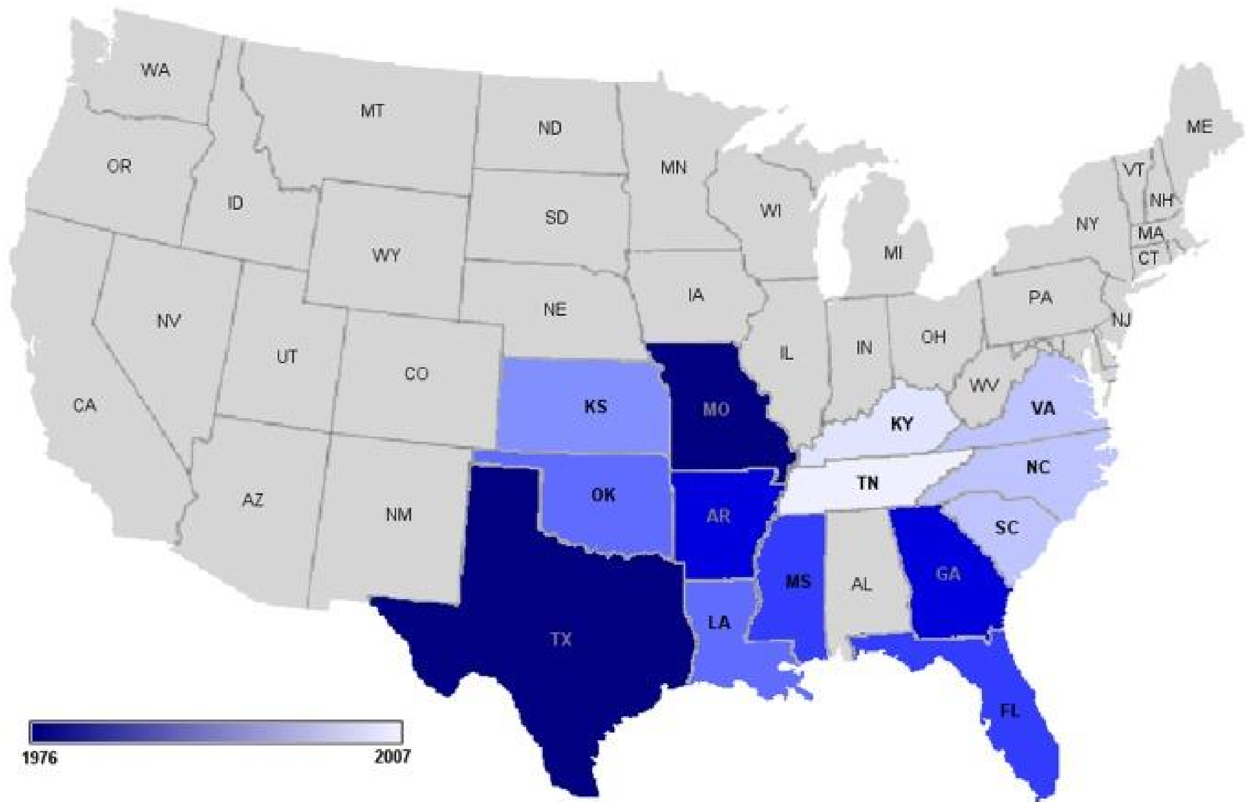
483 **Figures**

484 **Figure 1:** Distribution of *Cytauxzoon felis* as of 2013 in the United States (Tarigo et al., 2013)



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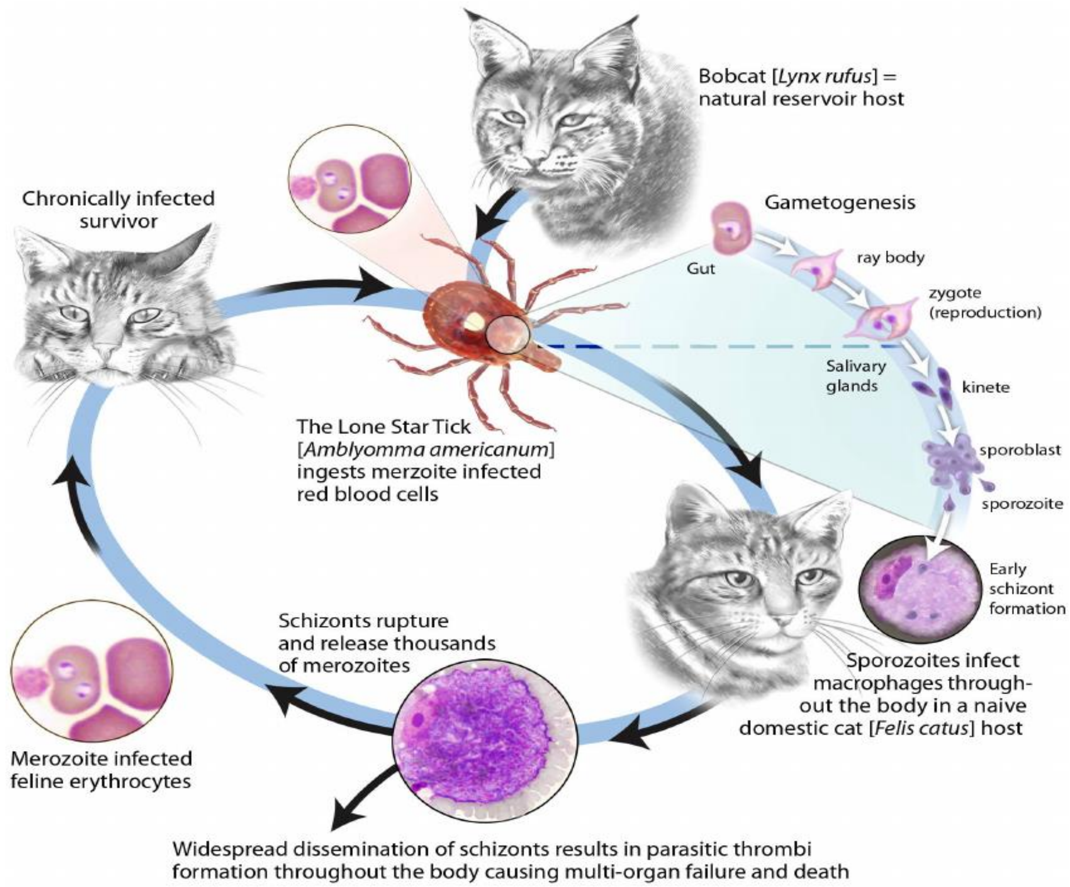
497 **Figure 2:** Life cycle of *Cytauxzoon felis* with *Amblyomma americanum* vector. Sexual

498 reproduction is depicted within the tick, infection begins when sporozoites are inoculated into the

499 host during tick feeding. Asexual reproduction occurs within the white blood cells of the host.

500 (Sherrill and Cohn, 2015)



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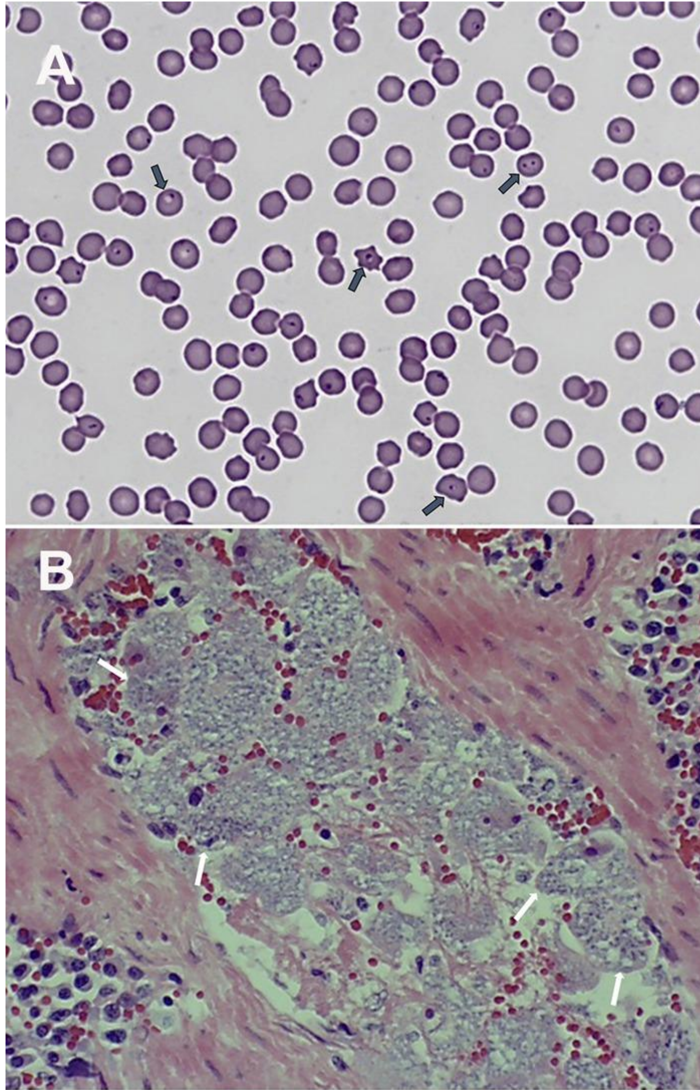
508 **Figure 3:** The dark arrows in Figure A shows intraerythrocytic piroplasms in a blood smear from

509 an infected cat. The white arrows in Figure B show schizont-laden macrophages in a splenic

510 blood vessel (Wang et al., 2017).



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523 **Figure 4:** Proposed transmission cycles with both vectors, *Dermacentor variabilis* and
524 *Amblyomma americanum,* to domestic cats and bobcats (Allen et al., 2019).
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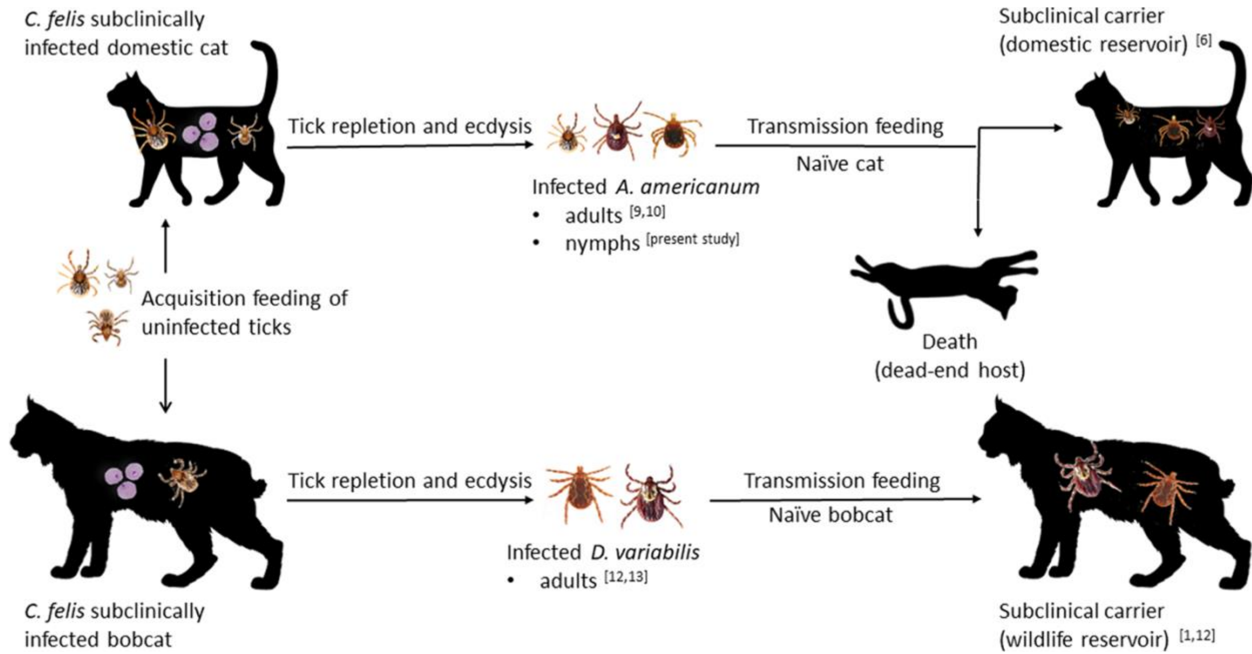

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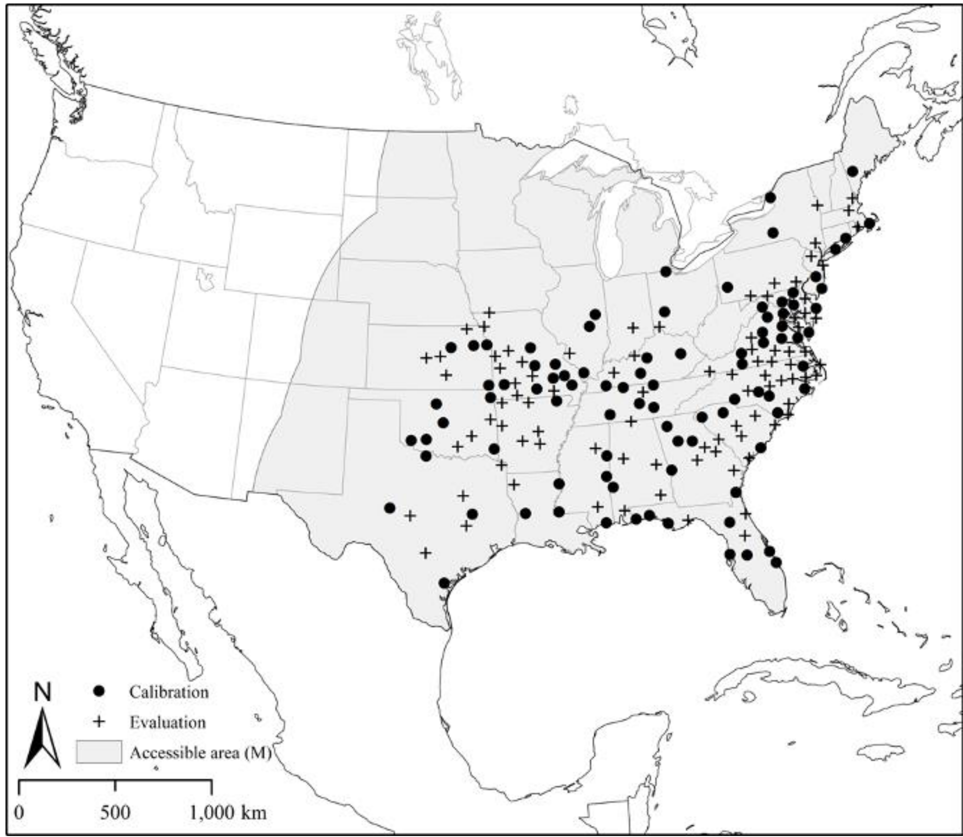
539 **Figure 5:** Map of known collection sites of *A. americanum* based on a records held at the Walter

540 Reed Biosystematics Unit. This study evaluated current and future range of *A. americanum*

541 based on ecological niche modeling. (Raghavan, et al., 2019)



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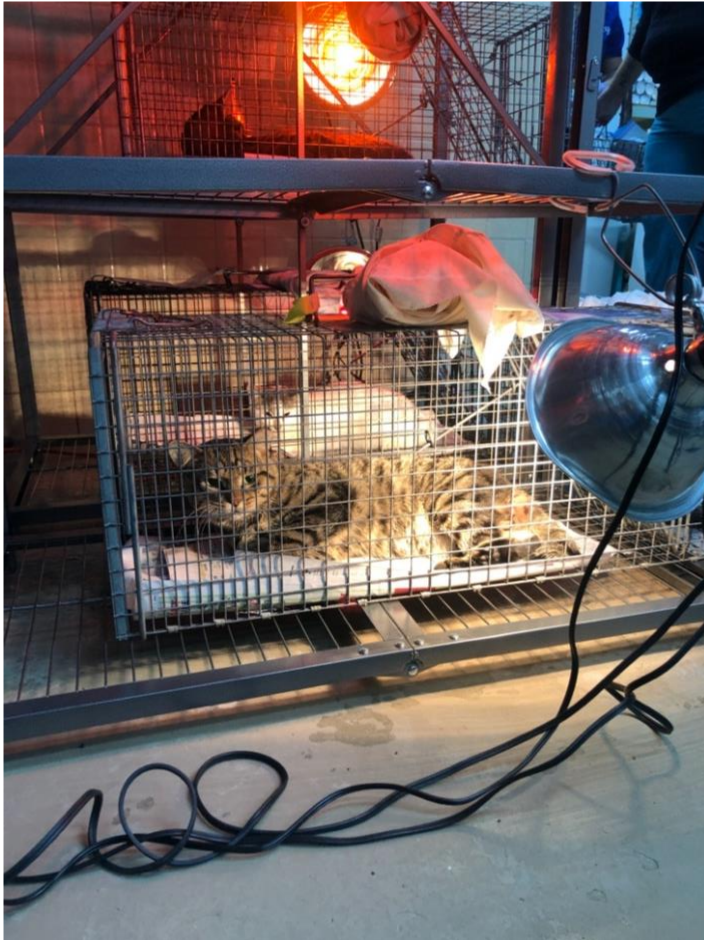
551 **Figure 6:** Male subject recovering from surgery and post blood draw. Cats were placed back in
552 their traps to ensure safety of volunteers. They were monitored and placed under heat lamps until
553 full recovery from anesthesia.



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560 **Figure 7:** Map of Tall Timbers Research Station, a long leaf pine ecosystem that studies

561 prescribed fire management. A digital version of this map was used to maintain trap locations,

562 the red circle indicates the area of collection.

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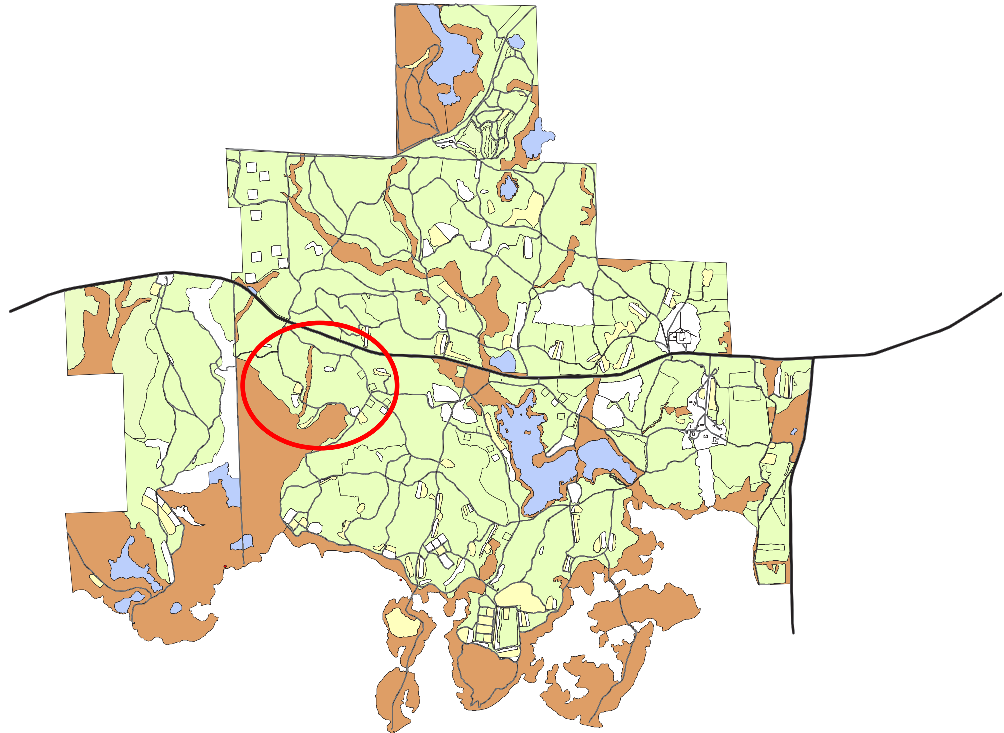

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Figure 8: Carbon dioxide traps at Tall Timbers were constructed from cardboard boxes outlined with clear duct tape and carbon dioxide placed on top. Each trap received about 0.5 kg of dry ice. Traps were allowed to evaporate for 4-5 hours.

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598 **Figure 9:** Identification markers of female and male *Amblyomma americanum* viewed under a
599 dissection microscope (captured by author).
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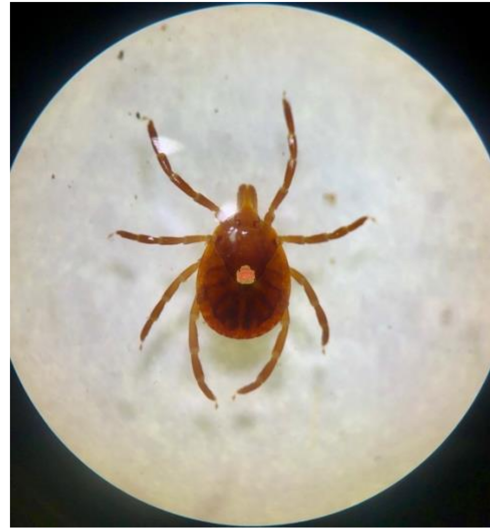

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603 Male *A. americanum* Female *A. americanum*
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615 **Figure 10.** Representative agarose gel of individual A. americanum ticks after PCR
616 amplification of *C. felis* 18s rRNA ITS1 primer. Lane 1 is 100bp molecular weight marker, lane
617 2 is the positive control *F. rufus* lung tissue sample. Lanes 3-9 are *A. americanum* samples: lane
618 3 sample F9 (05/14), lane 4 sample F7 (05/14), lane 5 F1 (05/14), lane 6 F2 (05/14), lane 7 F3
619 (05/14), lane 8 F4 (05/14), lane 9 F5 (05/14). Lane 10 is molecular grade water which acted as a
620 negative control.
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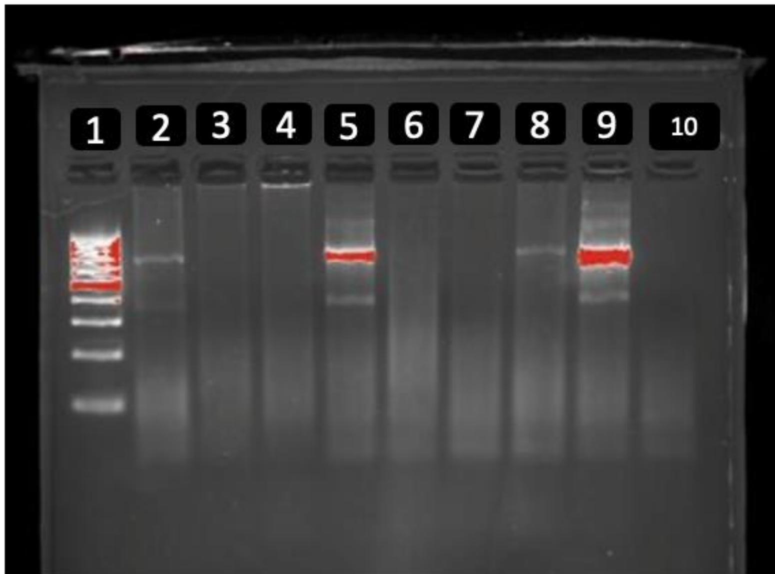

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630 **Tables**

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632 **Table 1:** Presumed positive domestic cat sequencing results.

Identification Label	Date of Collection	Sex	Clinic
HBRO	7/26/19	Neutered Male	Thomasville Low Cost Spay & Neuter Clinic
SAB	7/26/19	Spayed Female	Thomasville Low Cost Spay & Neuter Clinic
42	2/16/20	Spayed Female	Valdosta Humane Society
43	2/16/20	Spayed Female	Valdosta Humane Society
24	2/16/20	Spayed Female	Valdosta Humane Society
40	2/16/20	Spayed Female	Valdosta Humane Society
11	2/16/20	Spayed Female	Valdosta Humane Society
36	2/16/20	Spayed Female	Valdosta Humane Society

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650 **Table 2:** Presumed positive PCR lone star tick sequencing results.
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Date of Collection	Sex	Identification Label
4/2/2020	Female	F2 04/02
4/2/2020	Male	M4 04/02
4/9/2020	Female	F5 04/09
4/9/2020	Female	F11 04/09
4/9/2020	Female	F12 04/09
4/9/2020	Female	F13 04/09
4/9/2020	Female	F21 04/09
4/9/2020	Female	F35 04/09
4/9/2020	Female	F47 04/09
4/30/2020	Female	F2 04/30
5/14/2020	Female	F1 05/14
5/14/2020	Female	F4 05/14
5/14/2020	Female	F5 05/14
5/14/2020	Female	F8 05/14
5/14/2020	Female	F10 05/14
5/14/2020	Male	M7 05/14
5/14/2020	Male	M8 05/14

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657 **Table 3a:** *Amblyomma americanum* ITS1 BLAST Results
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Identification Label	*A. americanum* Sample Number	Forward/ Reverse Sequence	Male or Female (M/F)	Organism	Accession Number	Percent Identification	E-Value
F12 04/09	Cytaux 2	Forward	F	*Hepatozoon canis*	MH615006.1	93.62%	1.00E-29
F12 04/09	Cytaux 2	Reverse	F	*Gossypioides kirkii*	CP032249.1	76.60%	3.00E-05
F47 04/09	Cytaux 5	Forward	F	*Babesia*	KC162889.1	94.02%	0.00E+00
F47 04/09	Cytaux 5	Reverse	F	*Babesia*	KC119624.1	94.02%	1.00E-155
F21 04/09	Cytaux 6	Forward	F	*Babesia*	JX021526.1	99.27%	0.00E+00
F2 04/30	Cytaux 7	Forward	F	*Theileria*	KC119627.1	99.38%	0.00E+00
F2 04/30	Cytaux 7	Reverse	F	*Theileria*	KC122621.1	99.55%	0.00E+00
F10 05/14	Cytaux 8	Reverse	F	*Theileria*	KC122615.1	97.27%	0.00E+00
F8 05/14	Cytaux 9	Forward	F	*Babesia*	JX021526.1	99.42%	2.00E-170
F8 05/14	Cytaux 9	Reverse	F	*Babesia*	KC119621.1	76.04%	5.00E-93
F1 05/14	Cytaux 10	Forward	F	*Theileria*	KC122615.1	94.14%	9.00E-98
F1 05/14	Cytaux 10	Reverse	F	*Theileria*	KC122669.1	72%	0
F5 05/14	Cytaux 11	Forward	F	*Theileria*	KC122615.1	95.33%	3.00E-110
F5 05/14	Cytaux 11	Reverse	F	*Theileria*	KC122669.1	100%	3.00E-129

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662 **Table 3b:** *Amblyomma americanum* ITS2 BLAST Results
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Identification Label	*A. americanum* Sample Number	Forward/ Reverse Sequence	Male or Female (M/F)	Organism	Accession Number	Percent Identification	E-value
F11 04/09	Cytaux 1	Forward	F				
F11 04/09	Cytaux 1	Reverse	F	*Cytauxzoon*	MF966372.1	100%	0.003
F12 04/09	Cytaux 2	Forward	F				
F12 04/09	Cytaux 2	Reverse	F				
F47 04/09	Cytaux 5	Forward	F				
F47 04/09	Cytaux 5	Reverse	F	*Cytauxzoon*	MF966372.1	100%	
F10 05/14	Cytaux 8	Forward	F				1.90E-01
F10 05/14	Cytaux 8	Reverse	F	*Gadus morhua*	LR633947.1	100%	1.00E-04
F1 05/14	Cytaux 10	Forward	F	*Theileria*	MH491841.1	96.97%	1.00E-38
F1 05/14	Cytaux 10	Reverse	F	*Theileria*	KT124535.1	96.72%	2.00E-18
M4 04/02	Cytaux 14	Forward	M	*Cytauxzoon felis*	JF330260.1	100%	2
M4 04/02	Cytaux 14	Reverse	M	*Cytauxzoon felis*	HQ383911.1	100%	0.11

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674 **Table 3c:** *Felis catus* ITS2 BLAST Results
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Identification Label	*Felis catus* Sample Number	Forward/ Reverse Sequence	Male or Female (M/F)	Organism	Accession Number	Percent Identification	E-Value
42 02/16/20	Cytaux 20	Forward	Spayed Female	*Felis catus*	AP023165.1	94.23%	0.00E+00
42 02/16/20	Cytaux 20	Reverse	Spayed Female	*Felis catus*	AP023165.1	95.36%	0.00E+00
36 02/16/20	Cytaux 21	Forward	Spayed Female	*Felis catus*	AP023165.1	98.70%	0.00E+00
36 02/16/20	Cytaux 21	Reverse	Spayed Female	*Felis catus*	AP023165.1	99%	0.00E+00
40 02/16/20	Cytaux 24	Forward	Spayed Female	*Felis catus*	AP023165.1	93.20%	4.00E-118
40 02/16/20	Cytaux 24	Reverse	Spayed Female	*Felis catus*	AP023165.1	96.11%	2.00E-178

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690 **Table 4a.** *Amblyomma americanum Cytauxzoon felis* ITS1 BLAST results
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Identification Label	*A. americanum* Sample Number	ITS1 Forward/ Reverse Sequence	Male or Female (M/F)	Organism	Accession Number	Percent Identification	E-value
F12 04/09	Cytaux 2 Forward	Forward	F	*Cytauxzoon felis*	DQ458797.1	98.57%	9.00 E-30
F47 04/09	Cytaux 5 Forward	Forward	F	*Cytauxzoon felis*	KC122661.1	100%	3.00 E-16
F47 04/09	Cytaux 5 Reverse	Reverse	F	*Cytauxzoon felis*	AY531524.1	89.05%	1.00 E-46
F21 04/09	Cytaux 6 Forward	Forward	F	*Cytauxzoon felis*	KC122660.1	100%	9.00 E-22
F2 04/30	Cytaux 7 Forward	Forward	F	*Cytauxzoon felis*	KT783524.1	96.88%	1.00 E-08
F10 05/14	Cytaux 8 Reverse	Reverse	F	*Cytauxzoon felis*	AY531524.1	87.25%	2.00 E-46
F8 05/14	Cytaux 9 Forward	Forward	F	*Cytauxzoon felis*	KC122660.1	100%	5.00 E-24
F8 05/14	Cytaux 9 Reverse	Reverse	F	*Cytauxzoon felis*	AY531524.1	96.97%	5.00 E-09
F1 05/14	Cytaux 10 Forward	Forward	F	*Cytauxzoon felis*	DQ458797.1	100%	6.00 E-35
F1 05/14	Cytaux 10 Reverse	Reverse	F	*Cytauxzoon felis*	AY531524.1	83.45%	8.00 E-38
F5 05/14	Cytaux 11 Forward	Forward	F	*Cytauxzoon felis*	KC122660.1	100%	6.00 E-22

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703 **Table 4b.** *Amblyomma americanum Cytauxzoon felis* ITS2 BLAST results
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Identification Label	*A. americanum* Sample Number	ITS 2 Forward/ Reverse Sequence	Male or Female (M/F)	Organism	Accession Number	Percent Identification	E-value
F11 04/09	Cytaux 1	Forward	F	*Cytauxzoon felis*	HQ383877.1	100%	6.00 E-06
F11 04/09	Cytaux 1	Reverse	F	*Cytauxzoon felis*	HQ383911.1	100%	5.00 E-09
F12 04/09	Cytaux 2	Forward	F	*Cytauxzoon felis*	JF330260.1	100%	2.00 E-05
F12 04/09	Cytaux 2	Reverse	F	*Cytauxzoon felis*	HQ383911.1	100%	8.00 E-07
F47 04/09	Cytaux 5	Forward	F	*Cytauxzoon felis*	JF330260.1	100%	4.00 E-06
F47 04/09	Cytaux 5	Reverse	F	*Cytauxzoon felis*	HQ383911.1	100%	8.00 E-08
F10 05/14	Cytaux 8	Forward	F	*Cytauxzoon felis*	JF330260.1	100%	4.00 E-06
F10 05/14	Cytaux 8	Reverse	F	*Cytauxzoon felis*	HQ383911.1	100%	6.00 E-07
F1 05/14	Cytaux 10	Forward	F	*Cytauxzoon felis*	AY531524.1	88.78%	3.00 E-25
F1 05/14	Cytaux 10	Reverse	F	*Cytauxzoon felis*	MG584567.1;	96.30%	2.00 E-20
M4 04/02	Cytaux 14	Forward	M	*Cytauxzoon felis*	JF330260.1	100%	1.00 E-06
M4 04/02	Cytaux 14	Reverse	M	*Cytauxzoon felis*	HQ383911.1	100%	3.00 E-07
M7 05/14	Cytaux 15	Forward	M	*Cytauxzoon felis*	JF330260.1	100%	1.00 E-05

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709 **Table 4c.** *Felis catus Cytauxzoon felis* ITS2 BLAST results
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Identification Label	***Felis catus* Sample Number**	**ITS2 Forward/ Reverse Sequence**	**Male or Female (M/F)**	**Organism**	**Accession Number**	**Percent Identification**	**E-value**
42 02/16/20	Cytaux 20	Forward	Spayed Female	*Cytauxzoon felis*	JF330260.1	71.43%	1.00 E-09
42 02/16/20	Cytaux 20	Reverse	Spayed Female	*Cytauxzoon felis*	JF330260.1	86.17%	8.00 E-25
36 02/16/20	Cytaux 21	Forward	Spayed Female	*Cytauxzoon felis*	JF330260.1	93.55%	5.00 E-07
36 02/16/20	Cytaux 21	Reverse	Spayed Female	*Cytauxzoon felis*	JF330260.1	90.30%	1.00 E-59
40 02/16/20	Cytaux 24	Forward	Spayed Female	*Cytauxzoon felis*	JF330260.1	93.94%	2.00 E-07
40 02/16/20	Cytaux 24	Reverse	Spayed Female	*Cytauxzoon felis*	JF330260.1	100%	6.00 E-08

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Appendix I- Animal Use Protocol

Institutional Animal Care and Use Committee (IACUC)

ANIMAL USE PROTOCOL APPROVAL

January 30, 2020

Dr. Eric Chambers
Department of Biology
Valdosta State University

Dear Dr. Chambers;

Animal Use Protocol (AUP) *"A determination of the prevalence and geographic distribution of ITS1/ITS2 genotypes of the pathogen Cytauxzoon felis in ticks, domestic cats, and wild cats in South Georgia and North Florida.*" (AUP-00077-2020) has been approved by the Institutional Animal Care and Use Committee (IACUC). This approval is from 01.30.2020 – 01.30.2023. In order to keep your protocol active, an Annual Review & Protocol Continuation Request form must be submitted to the IACUC each year. The Office of Sponsored Programs and Research Administration (OSPRA) will email you the Annual Review & Protocol Continuation Request form approximately one month before the annual report is due.

Please remember that you must obtain IACUC approval before amending, or altering the scope, or procedures of the protocol. You are also required to report to attending Veterinarian, the IACUC Chair, and the IACUC Administrator any unanticipated problems with the animals that become apparent during the course, or as a result of the research, or teaching activity.

Should you have questions concerning your approved research, please contact Tina Wright, Research Officer, at 229.253.2947, or email IACUC at iacuc@valdosta.edu.

Sincerely,
Ann
Elizabeth "Ann" Olphie
IACUC Administrator

cc: Dr. Becky da Cruz, Associate Provost for Graduate Studies and Research
Dr. Teresa Doscher, Attending Veterinarian
Dr. Robert L. Gannon, Department Head

Sources

1. Allen, K. E., Reichard, M. V., Thomas, J. E., and Wohltjen, M. L.(2019). Transmission of *Cytauxzoon felis* to domestic cats by *Amblyomma americanum* nymphs. Parasites and Vectors, 12-28.

2. Birkenheur A. J., Le J. A., Valenzisi A. M., et al. (2006). *Cytauxzoon felis* infection in cats in the mid Atlantic states: 34 cases (1998-2004). Journal of American Veterinary Medicine Association. Vol 228, 568-571.

3. Blouin E. F., Glenn B. L, Kocan A. A., et al. (1984) Transmission of *Cytauxzoon felis* Kier, 1979 from Bobcats, *Felis rufus* (Schreber), to Domestic Cats by *Dermacentor variabilis* (Say). Journal of Wildlife Diseases. Vol 20 (3), 241-242.

4. Bondy P. J., Cohn L. A., Marsh A. E., and Tyler J. W. (2005). Polymerase Chain Reaction Detection of *Cytauxzoon felis* From Field Collected Ticks and Sequence Analysis of the Small Subunit and Internal Transcribed Spacer 1 Region of the Ribosomal RNA Gene. Journal of Parasitology. Vol 91 (2), 458-461.

5. Brown M. H. (2010). *Cytauxzoon felis:* Assessing genetic variability in an emerging feline pathogen. (Doctoral Dissertation) Retrieved from the library of The University of Georgia.

6. Brown M. H, Berghaus D. R., Latimer S. K., et al. (2009). Genetic Variability of *Cytauxzoon felis* from 88 infected domestic cats in Arkansas and Georgia. Journal of Veterinary Diagnostic Investigation, 21 (1), 59-63.

7. Brown M. H., Latimer S. K., Lockhart J. M., et al. (2010). Identification and genetic characterization of *Cytauxzoon felis* in asymptomatic domestic cats and bobcats. Journal of Veterinary Parasitology, 172, 311-316.

8. Butt, M. T., Barr, M. C., Bowman, D., and Roelke, M. E. (1991). Iatrogenic transmission of *Cytauxzoon felis* from a Florida panther (*Felix concolor coryi)* to a domestic cat. Journal of Wildlife Diseases, 27, 342-347.

9. Carr, A. L., Bissinger, B. W., Dhammi, A., et al. (2017). Tick Haller's Organ, a New Paradigm for Arthropod Olfaction: How Ticks Differ from Insects. *International journal of molecular sciences*, *18*(7), 1563.

10. Clinic Procedures. (2016). Feral Cat Spay and Neuter Project. Retrieved from http://www.feralcatproject.org/instructions-for-specific-procedures/

11. Cohn, L. A., Birkenheur, A. J., Brunker, J. D., et al. (2011). Efficacy of atovaquone and azithromycin or imidiocarb dipropionate in cats with acute cytauxzoonosis. Journal of Veterinary Entomology, 25, 55-60.

822 **12.** Cohn, L. A. (2014). Canine and Feline Infectious Diseases, 1st ed. Saunders
823 Publishing, Philadelphia, Pennsylvania, 739-746.
824
825 **13.** Garner, M. M., Citino, S., Greiner, E. C., et al. (1996). Fatal cytauxzoonosis in a
826 captive-reared white tiger (*Panthera tigris).* Veterinary Pathology, 33 (1), 82-86.
827
828 **14.** Greene, C. E., Kocan, A. A., and Meinkoth, J. (2006). Infectious Diseases of the Dog
829 and Cat, 3rd ed. Saunders Elsevier, St. Louis, Missouri, 722-733.
830
831 **15.** Haber M. D., Marr, H. S., Tucker M. D., et al. (2007). The detection of *Cytauxzoon*
832 *felis* in apparently healthy free-roaming cats in the USA. Journal of Veterinary
833 Parasitology. Vol 146, 316-320.
834
835 **16.** Houseman, R. M. (2013). Integrated Pest Management Guide to Ticks and Tick
836 Management. University of Missouri Extension Publications. 1-34.
837
838 **17.** Jakob, W., and Wesemeier, H. (1996). A fatal infection in a Bengal tiger resembling
839 cytauxzoonosis in domestic cats. Journal of Comparative Pathology, 114(4), 439-444
840
841 **18.** Kensinger, B. J., and Allan, B. F. (2011). Efficacy of dry ice-baited traps for sampling
842 *Amblyomma americanum* (Acari: Ixodidae) varies with life stage but not habitat.
843 Journal of Medical Entomology, 48, 708-711.
844
845 **19.** Kollars, T. M. (1993). Ticks (Acari: Ixodidae) infesting medium-sized wild mammals
846 in southwestern Tennessee. Journal of Medical Entomology, 30 (5), 896-900.
847
848 **20.** Lewis, K. M., Birkenheuer, A. J., Cohn, L.A., et al. (2012). Evaluation of *Cytauxzoon*
849 *felis* infection status in captive-born wild felis housed in an area endemic for the
850 pathogen. Journal of American Veterinary Medicine Association, 241(8), 1088-1092.
851
852 **21.** Maizels, R. M., and Kurniaqan-Atmadja, A. (2002). Variation and polymorphism in
853 helminth parasites. Parasitology, 125, S25-S37.
854
855 **22.** Mangan, M. J., Foré, S. A., and Kim, H. J. (2018). Ecological modeling over seven
856 years to describe the number of *Amblyomma americanum* in each life stage in
857 northeast Missouri. Journal of Vector Ecology, 43 (2) 271-284.
858
859 **23.** Matheson, R. (1950). Medical Entomology, 2nd Edition. Comstock Publishing
860 Company, Inc. Ithica, NY.
861
862 **24.** Matrajt, M. (2010). Non-coding RNA in apicomplexan parasites. Molecular
863 Biochemistry Parasitology, 174, 1-7.
864
865 **25.** Meinkoth, J. H. and Kocan, A. A. (2005). Feline cytauxzoonosis. *Veterinary Clinics of*
866 *North America Small Animal Practice,* 35(1), 89-101,

867 vvi.doi:10.1016/j.cvsm.2004.08.003
868
869 **26.** Nagamori, Y. (2016). Transmission of *Cytauxzoon felis* by *Amblyomma americanum:*
870 Engorgement Weight of Nymphs and Attachment Time of Adults for Transmission to
871 Domestic Cats. M.S. Thesis. Oklahoma State University, Stillwater, Oklahoma, 90 p.
872
873 **27.** Otranto, D., Baneth, G., Capeli, G., et al. 2011). Diagnosis of *Hepatozoon canis* in
874 young dogs by cytology and PCR. Parasites and Vectors, Vol 4 (5).
875
876 **28.** Peixoto, P. V., Barros, S. S., Franca, T. N., et al. (2007). Fatal cytauxzoonosis in
877 captive-reared lions in Brazil. Veterinary Parasitology, 145(3-4), 383-387.
878
879 **29.** Pollard, D. A., Cohn, L. A., Holman, P. J., et al. (2017). Genetic variability of cloned
880 *Cytauxzoon felis* ribosomal RNA ITS1 and ITS2 genomic regions from domestic cats
881 with varied clinical outcomes from five states. Journal of Veterinary Parasitology, 244,
882 136-143.
883
884 **30.** Raghavan, R. K., Cobos, M. E., Foley, D., et al. (2019). Current and Future
885 Distribution of the Lone Star Tick, *Amblyomma americanum* (L.) (Acari: Ixodidae) in
886 North America. PLOS ONE 14(1): e0209082.
887
888 **31.** Reichard, M. V., Baum, K. A., Cadenhead, S. C., and Snider, T. A. (2008). Temporal
889 occurrence and environmental risk factors associated with cytauxzoonosis in domestic
890 cats. Veterinary Parasitology, 152(3-4), 314-320.
891
892 **32.** Reichard, M.V., Blouin E. F., Edwards A. C., et al. (2009). Transmission of
893 *Cytauxzoon felis* to a domestic cat by *Amblyomma americanum.* Journal of Veterinary
894 Parasitology, 161, 110-115.
895
896 **33.** Reichard, M. V., Edwards, A. C., Heinz, R. E., et al. 2010). Confirmation of
897 *Amblyomma americanum* (Acari: Ixodidae) as a Vector for *Cytauxzoon felis*
898 (Piroplasmorida: Theileriidae) to Domestic Cats. Journal of Medical Veterinary
899 Entomology, 47 (5), 890-896.
900
901 **34.** Rizzi, T. E., Birkenheuer, A. J., Cohn, L. A., et al. (2015) Parasites and Vectors, Vol 8
902 (13).
903
904 **35.** Schreeg, M. E., Bird, D. M., Cohn, L. A., et al. (2016). Mitochondrial Genome
905 Sequences and Structures Aid in the Resolution of *Piroplasmida* phylogeny. PLoS
906 ONE 11(11): e0165702.
907
908 **36.** Schulze, T. L., Hung, R. W., and Jordan R. A. (1997). Biases associated with several
909 sampling methods used to estimate abundance of Ixodes scapularis and Amblyomma
910 americanum (Acari: Ixodidae). Journal of Medical Entomology, 34, 615–623.
911

912 **37.** Schulze, T. L., Hung, R. W., and Jordan, R. A. (2001). Potential effects of animal
913 activity on the spatial distribution *Ixodes scapularis* and *Amblyomma americanum*
914 (Acari: Ixodidae). Environmental Entomology, 30, 318-324.
915
916 **38.** Schulze, T. L. and Jordan, R. A. (2003). Meteriologically Mediated Diurnal Questing
917 of *Ixodes scapularis* and *Amblyomma American* (Acari: Ixodidae) Nymphs. Journal of
918 Medical Entomology, 40 (4), 395-402.
919
920 **39.** Sherrill M. K., and Cohn, L. A. (2015). Cytauxzoonosis Diagnosis and Treatment of
921 an Emerging Disease. Journal of Feline Medicine and Surgery 17, 940-948
922
923 **40.** Shock, C. B., Birkenheuer, J. A., Patton, L. L., et al. (2012). Variation in the ITS1-
924 ITS2 rRNA genomic regions of *Cytauxzoon felis* from bobcats and pumas in the
925 eastern United States and comparison with sequences from domestic cats. Journal of
926 Veterinary Parasitology, 190, 29-35.
927
928 **41.** Simo, L., Kazimirova, M., Richardson, J., et al. (2017). The Essential Role of Tick
929 Salivary Glands and Saliva in Tick Feeding and Pathogen Transmission. Frontiers in
930 Cellular and Infection Microbiology, Vol 7, (281).
931
932 **42.** Tarigo, L. J. (2022, March) *Cytauxzoonosis in cats*.
933 https://www.merckvetmanual.com/circulatory-system/blood-parasites/cytauxzoonosis-
934 in-cats
935
936 **43.** Tarigo, J. L., Birkenheuer, A. J., Brown, C. C., et al. (2013). A novel candidate
937 vaccine for cytauxzoonosis inferred from comparative apicomplexan genomics. *PloS*
938 *one*, *8*(8), e71233. https://doi.org/10.1371/journal.pone.0071233
939
940 **44.** U Maine Cooperative Extension: Insect Pests, Ticks and Plant Diseases. (n.d.)
941 Retrieved from https://extension.umaine.edu/ipm/tickid/tick-biology/
942
943 **45.** Wagner, J. E. (1976). A fatal cytauxzoonosis-like disease in cats. *Journal of the*
944 *American Veterinary Medical Association,* 168, 585-588.
945
946 **46.** Wang, J. L., Li, T. T., Liu, G. H., et al. (2017). Two Tales of *Cytauxzoon felis*
947 Infections in Domestic Cats. Clinical Microbiology Reviews, 30(4), 861–885.
948
949 **47.** Wikander, Y. M., Kang, Q., and Reif, K. E. (2020) Acute *Cytauxzoon felis* Cases in
950 Domestic Cats from Eastern Kansas, a Retrospective Case-Control Study (2006-2019).
951 Journal of Veterinary Sciences, 7 (4), 205.
952
953