Investigations into *Cytauxzoon felis* among the domestic cat population of eastern Kansas.

by

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Abstract

Cytauxzoon felis is a tick-transmitted, obligate, hemoprotozoal, piroplasmid pathogen of felids and the causative agent of cytauxzoonosis. It has a complex life cycle which includes a tick as its definitive host and a felid as its intermediate host. Since its first description in 1976, *C. felis* infections of felids have been reported in several southeastern and south-central U.S. states, overlapping with the ranges of its two known biological vectors, *Amblyomma americanum* (Lone Star tick) and *Dermacentor variabilis* (American Dog tick). Infected felids demonstrate disease as either an acute, often-fatal, infection or a subclinical carrier infection. While statewide awareness and concern of *C. felis* infections have increased, few studies have evaluated the incidence of acute cytauxzoonosis, prevalence of carriers, and associated disease risk factors among domestic cats in endemic areas such as eastern Kansas. As such, the objective of this thesis was to investigate *C. felis* infections among the domestic cat population of eastern Kansas. Our first objective was to perform a retrospective review of Kansas State Veterinary Diagnostic Laboratory (KSVDL) feline records to identify: i) the incidence of acute cytauxzoonosis in Kansas over a 14-year period (2006-2019), and ii) risk factors associated with a diagnosis of acute cytauxzoonosis. The overall incidence trend was largely unchanged for the case review period and the feline risk factors most commonly associated with acute disease was ≥1-year old male owned cats' samples submitted during the spring and summer. Felids that survive acute disease often remain infected and serve as reservoirs for subsequent tick transmission to other susceptible felines. Several states near Kansas (Arkansas, Oklahoma, Missouri) have identified *C. felis*-carrier domestic cat populations. Thus, the second objective of our study was to determine: i) the prevalence of *C. felis*-carriers in the domestic cat population in eastern Kansas using a quantitative PCR assay targeting the *C. felis* Cox3 mitochondrial gene, and ii) risk factors associated with cats that develop into *C. felis* carriers.

An overall asymptomatic feline *C. felis* infection prevalence of 25.8% was determined in eastern Kansas with a seasonal fluctuation of more *C. felis*-survivors identified in spring and fall. Our study demonstrates that *C. felis*-domestic cat carriers are common among cats in eastern Kansas and suggests that more cats likely survive cytauxzoonosis than previously expected. Collectively, our studies present new information on the state of acute and carrier cytauxzoonosis cases among domestic cats in eastern Kansas. Investigating the incidence of acute cytauxzoonosis, patient risk factors, roles of domestic cat carriers, and ecological variables that influence disease transmission are important towards developing and communicating the need for effective cytauxzoonosis control strategies for high-risk cat populations, including recommending year-round use of acaricide products for all cats that spend any time outdoors. More studies are needed to further identify factors affecting *C. felis* and other *Cytauxzoon* spp. infections transmission, progression, and treatment options and outcomes within the U.S. and globally.

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Dedication

I would like to dedicate this manuscript to my family for their unfailing support of my seemingly never-ending endeavors. Especially, to my mother and father, **Truus and Jim Wikander**, for their good counsel, and to my son, **Tapweski Sandaine**, for always having my back.

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Chapter 1 - *Cytauxzoon felis*, an overview

Apicomplexa.

Cytauxzoon felis is an obligate, hemoprotozoal, piroplasmid pathogen of felids and the agent of cytauxzoonosis, an often-fatal disease of domestic cats residing in the southeastern and south-central United States (U.S.). Cytauxzoon organisms are apicomplexan protozoa in the subclass Hematozoa, order Piroplasmida, family Theileriidae (Figure 1.1). The microscopic intra-erythrocytic pathogens of the Babesia and Theileria families are often called piroplasms due to their 1-2-micron diameter pear-shaped or circular (signet ring) appearance. The history of *C. felis*, its phylogenetic placement, anatomy, and complex life cycle has been studied for over four decades with many questions still unanswered.

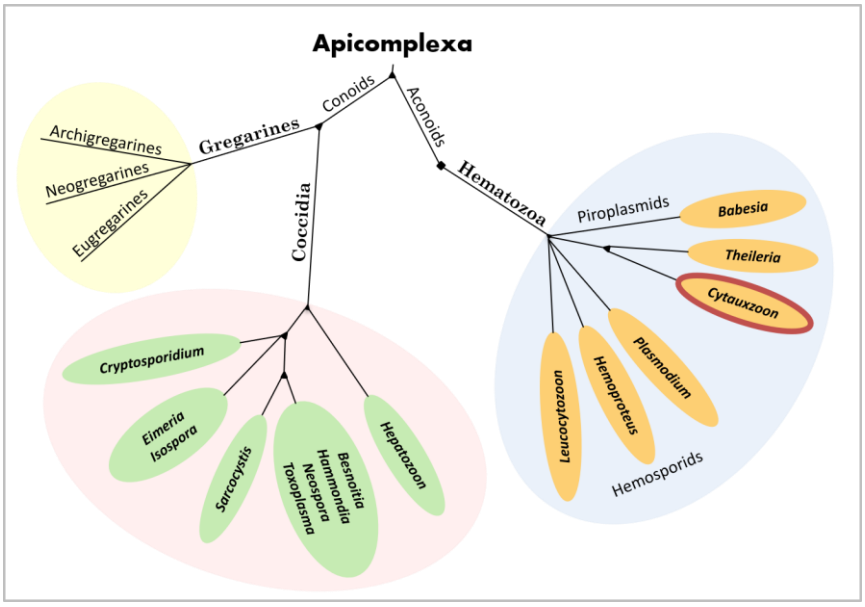


Figure 1.1: Apicomplexa phylogenetic tree with genus *Cytauxzoon* (circled in red) within the family Theileriidae, order Piroplasmida, subclass Hematozoa, and phylum Apicomplexa.

History.

The *Cytauxzoon* genus was first described in 1948, when W.O. Neitz and A.D. Thomas characterized a *Theileria*-like piroplasm that underwent schizogony in histiocytes of an African duiker (*Sylvicapra grimmia*) from South Africa [1]. Nearly thirty years later, *Cytauxzoon felis* was first described by J.E. Wagner of the University of Missouri when he characterized a *Cytauxzoon*-like piroplasm in four cats that suffered fatal disease outcomes from 1973-1975 [2]. Because *Cytauxzoon* organisms had previously only been described in African ungulates, the Plum Island Animal Disease Center of the United States Department of Agriculture (USDA) and the Animal and Plant Health Inspection Service (APHIS) endeavored to determine if the organism was a foreign disease and whether or not it posed a threat to the U.S. livestock industry [3]. To help address this question, over 500 cats were experimentally infected with *C. felis* in over 100 serial passages, and the course and outcome of the disease was studied [3]. Their findings suggested that the disease was: i) largely fatal for domestic cats, and ii) unlikely to be a foreign animal disease, thus not a concern for U.S. food production. Since that time, molecular diagnostic methods suggest that the organism found in the African duiker was likely a *Theileria* species [4]. The potential for interspecies transmission of *C. felis* was investigated further when four domestic livestock species, nine lab animal species, and 17 wildlife species were experimentally inoculated with blood or tissue homogenates from euthanized domestic cats with confirmed acute cytauxzoonosis [5]. One of the bobcats in the study developed clinical signs while another bobcat and two sheep developed low-grade persistent parasitemia without clinical signs. No studies have investigated whether or not sheep with parasitemia can act as a competent host to infect the tick vector of this disease. Since its initial description in the U.S., *C. felis* has been identified in a variety of felid species within the U.S. and South America [6].

Phylogeny.

In the past, taxonomic classification of organisms like *C. felis* was based on phenotypic similarities, but with the advent of molecular assays, genetic similarities have taken a greater role in phylogenetic organization [7]. Several genetic-based phylogenetic studies have been performed to flesh out the taxonomic organization of the order Piroplasmida within the phylum Apicomplexa. These have included the 18S gene, C1A-cysteine proteinase (a cell invasion-egress enzyme) gene, and a linked 18S ribosomal subunit-Cox1 mitochondrial amino acid sequence evaluation [7–9]. The results of these studies suggest five distinct Piroplasmida groups: (1) *Babesia* sensu strico, (2) *Theileria* and *Cytauxzoon*, (3) *Theileria equi*, (4) *Babesia conradae*, and (5) *Babesia microti* spp (Figure 1.2) [7].

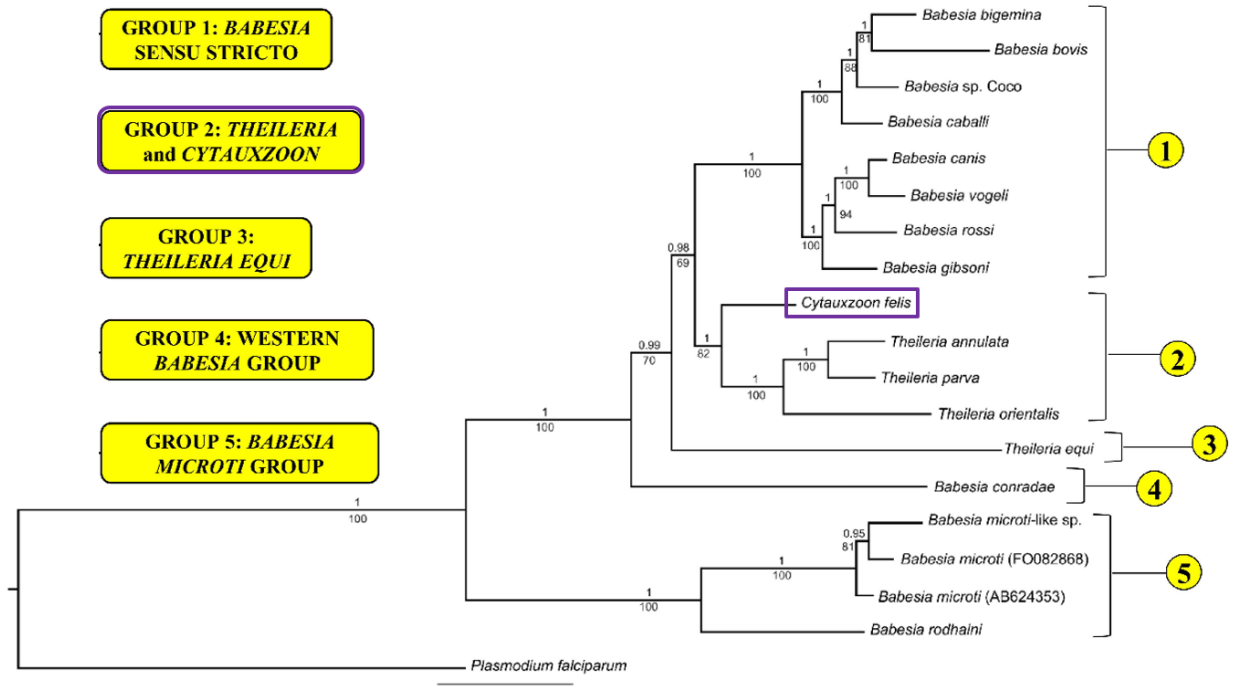


Figure 1.2: Taxonomic classification of the order Piroplasmida by phylogenetic organization with five distinct groups: (1) *Babesia* sensu strico, (2) *Theileria* & *Cytauxzoon*, (3) *Theileria equi*, (4) *Babesia conradae*, and (5) *Babesia microti* spp

Anatomy.

Like all Apicomplexa, each *C. felis* organism contains a nucleus, endoplasmic reticulum, Golgi apparatus, an apicoplast, a mitochondrion, and a pellicle with an apical complex (Figure 1.3) [10]. The first three organelles have functions similar to those in other unicellular or multicellular eukaryote organisms. Whereas the apicoplast, mitochondrion, and apical complex are unique to members of this phylum. The apicoplast contains its own circular DNA and is surrounded by triple or quadruple membranes, supporting an endosymbiotic origin. Although the exact function of this organelle is unclear, it has been proposed that it may take part in several potential pathways including the synthesis of fatty acids, heme breakdown, amino acid synthesis, isoprenoid precursors, and/or iron-sulfur clusters [8]. More studies are needed to fully elucidate the function of apicoplasts in these organisms.

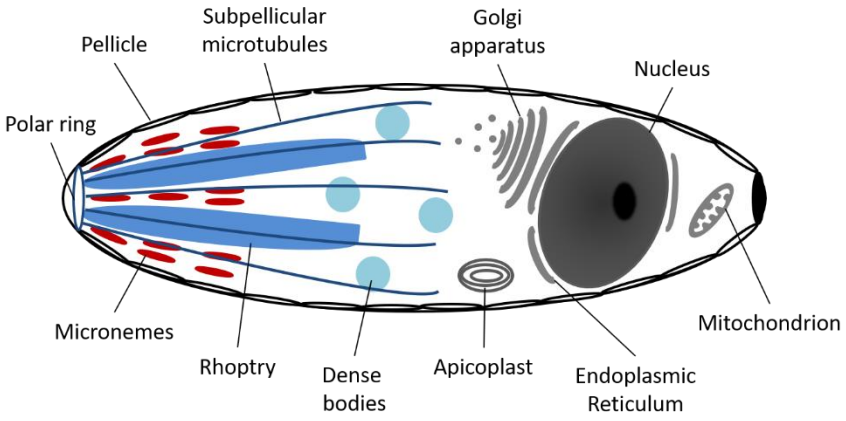


Figure 1.3: Anatomy of an aconoid apicomplexan sporozoite containing a nucleus, endoplasmic reticulum, Golgi apparatus, mitochondrion with multiple DNA copies, apicoplast, cytoskeletal components (pellicle, subpellicular microtubules, polar ring) and secretory organelles (micronemes, rhoptry, dense bodies).

The one mitochondrion found in each apicomplexan organism generally contains multiple copies of short (6-kb) circular or linear strands of DNA (miDNA) [10]. The number of mitochondrial genome copies per mitochondrion in each apicomplexan species varies from a

handful to over 100 copies each, which may further vary based on apicomplexan life stage. This redundancy may be a survival mechanism developed to maintain nucleic acid sequence integrity by allowing the disposal of miDNA damaged by reactive oxygen species (ROS) or harmful mutations [11]. The number of mitochondrial genome copies per *C. felis* mitochondrion has yet to be determined. Interestingly, apicomplexan miDNA contains genes for only three electron transport chain (ETC) proteins: cytochrome b (Cytb), cytochrome c oxidase subunit I (Cox1), and cytochrome c oxidase subunit III (Cox3) [10]. As such, they are missing the necessary genes for at least two of the needed mitochondrial ETC subunits to perform oxidative phosphorylation and may derive all their energy via anaerobic glycolysis [12]. It has been proposed that mitochondrion ETC functions in these organisms include: i) providing an electron sink for ubiquinone-dependent dehydrogenase used in cell metabolism for mitochondrial protein degradation, ii) maintaining a transmembrane gradient for metabolite and protein transport, and/or iii) reducing ROS. Additional studies are needed to evaluate the specific function of this organelle in apicomplexan organisms.

The apical complex and pellicle consists of up to seven distinct cytoskeletal components, each with a specialized function (Figure 1.3) [8, 13]. The outer membrane and subpellicular microtubules of the pellicle provide a variably elastic cell shape to the organism [14]. The polar ring, made up of microtubular bands at the apical end of the organism, acts as an organizing center for the subpellicular microtubules and gives the cell polarity. The small dense bodies called micronemes secrete adhesive proteins allowing the hemoprotozoan to move along the host cell membrane in a gliding motion just prior to penetration [10, 15]. Rhoptries, large saccular electron dense bodies, and dense body vacuoles secrete dissolving enzymes to enable penetration into the host cell [8, 13]. The final apical complex structure lacking in all Hematozoans including *C. felis*,

is the conoid, a spiral cone of microtubules associated with the polar ring used as a feeding tube for cellular vampirism in some apicomplexans (not depicted in Figure 1.3) [8].

Life cycle.

Cytauxzoon felis has a complex lifecycle that starts, for the feline host, with the injection of *C. felis* sporozoite-laden saliva from a feeding tick [13] (Figure 1.4). Each sporozoite invades a monocyte and undergoes asexual division (schizogony) resulting in hundreds if not thousands of ring-shaped merozoites being released into the blood when the monocyte ruptures. Each merozoite, or piroplasm, invades a red cell and either develops into a non-replicating macro- and micro-gametocyte (gamogony) or undergoes asexual division (merogony) resulting in 2-4 piroplasms being released when the red cell ruptures. Repeating rounds of merogony can occur indefinitely while the felid is alive. When a vector-competent tick attaches to feed on an infected felid, they ingest the piroplasms along with the blood meal. The gametocyte piroplasms will then undergo sexual development (amphimixis) within the gut of the tick to form, first a zygote, then kinetes before migrating and encysting in the tick's salivary glands where they undergo additional replication (sporogony). Once the tick molts into a nymph or adult, the encysted sporozoites are ready to be released in the saliva of the feeding tick. Thus, the cycle begins anew.

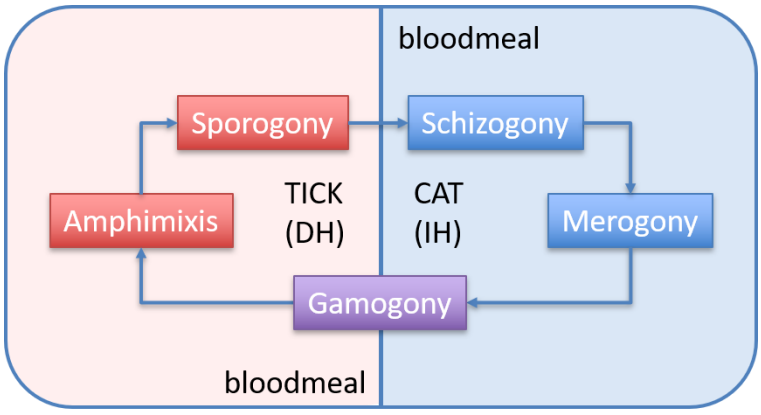


Figure 1.4: *Cytauxzoon felis* lifecycle schematic demonstrating asexual reproduction in the feline intermediate host (IH) on the right (blue shaded area) and sexual reproduction (amphimixis) in the tick definitive host (DH) on the left (pink shaded area) with bloodmeal transmission to each host.

The following detailed *C. felis* life cycle description is based on what is known of *Theileria* spp., a close relative with a presumed similar cycle (Figure 1.5).

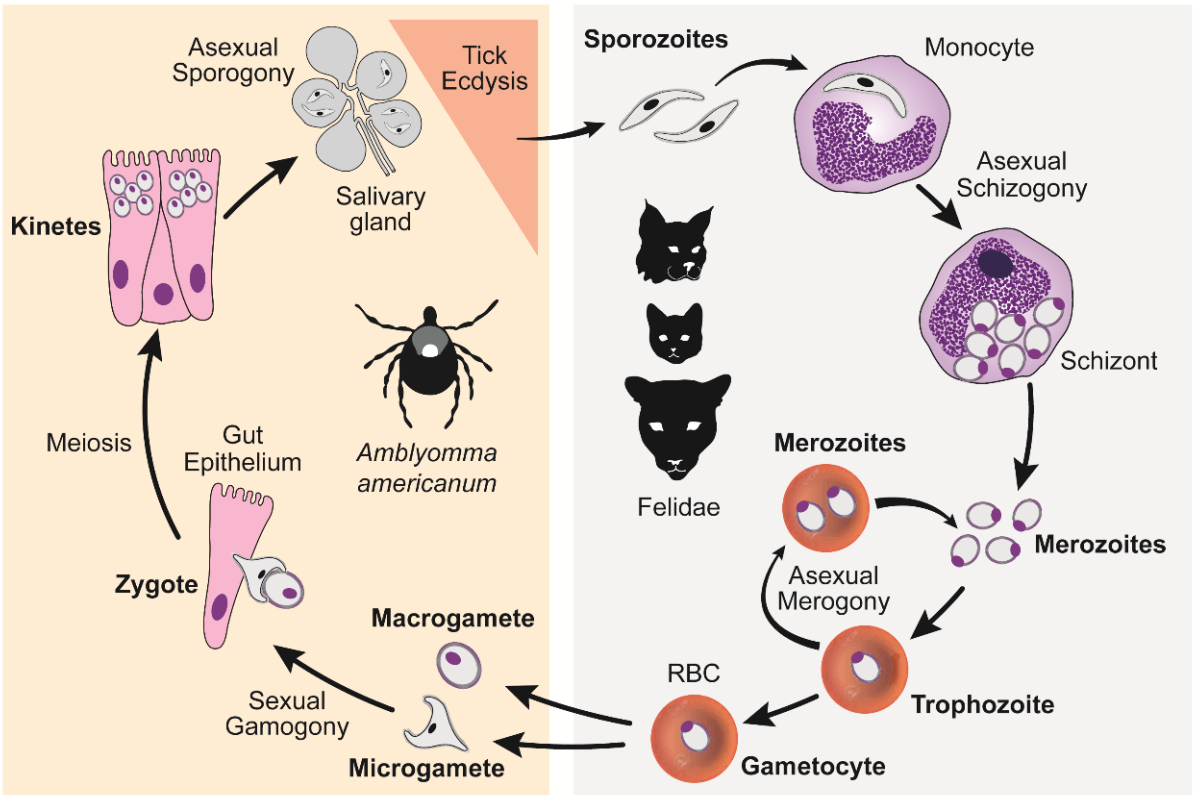


Figure 1.5: *Cytauxzoon felis* lifecycle demonstrating asexual reproduction within the host felid (right panel) and both sexual and asexual reproduction within the tick transmission vector (left panel). (Wikander, Y. M.; Anantatat, T.; Kang, Q.; Reif, K.E. Prevalence of *Cytauxzoon felis* infection reservoirs in the domestic cat population of eastern Kansas. MDPI *Pathogens*, 2020.)

Asexual schizogony.

The rapid multiplication of *C. felis* within a monocyte is called schizogony and the merozoite-laden monocytes are called schizonts or Koch's bodies [13]. Asexual schizogony begins after sporozoite-laden tick saliva is released into the feeding lesion around an embedded tick's mouthparts [8, 13]. The immotile sporozoites, which have an outer coat of fibrillar material and hypervariable surface proteins, rely on random contact with monocytes to begin their attachment and internalization. Unlike some apicomplexans, *Theileria* spp. and presumably *C. felis*, does not require an apical-end orientation with the host cell. It can enter the host cell in any orientation and does so within three minutes of contact [13]. The process of invasion is as follows; (1) the organism recognizes and attaches to the host membrane, (2) using gliding motility a junction is formed between the parasite and the host cell, (3) as the parasite internalizes via host membrane zippering, it's fuzzy coat is shed, (4) the host membrane surrounding the parasite is separated and dissolved leaving the organism free within the host cytoplasm, and (5) the parasite takes control of the host cell's microtubular network for its own development [13, 15] (Figure 1.6). The mechanism by which the parasite hijacks the host cell to multiply and/or evade the host's immune system is unknown [16]. In addition, it is unknown if *C. felis* blocks monocyte apoptosis as *Theileria parva* blocks lymphocyte apoptosis. Regardless, once established within a host monocyte, ultrastructural changes to the parasite's organelles and outer membrane and sequential fissions results in a multilobulated, multinucleated mass connected by cytoplasmic bridges [13, 17]. Each lobe has a nucleus, a mitochondrion, and related organelles [17]. Eventually the cytoplasmic bridges separate leaving multiple mature intracytoplasmic uninuclear merozoites [13, 17]. Mature schizonts (measuring 25-60 μm in diameter) rupture, releasing merozoites into the blood [18] (Figure 1.7).

As with all extracellular phases of *C. felis* development, merozoites form a fuzzy coat of fibrillar material to assist in its invasion of the next host cell, the erythrocyte [13, 17].

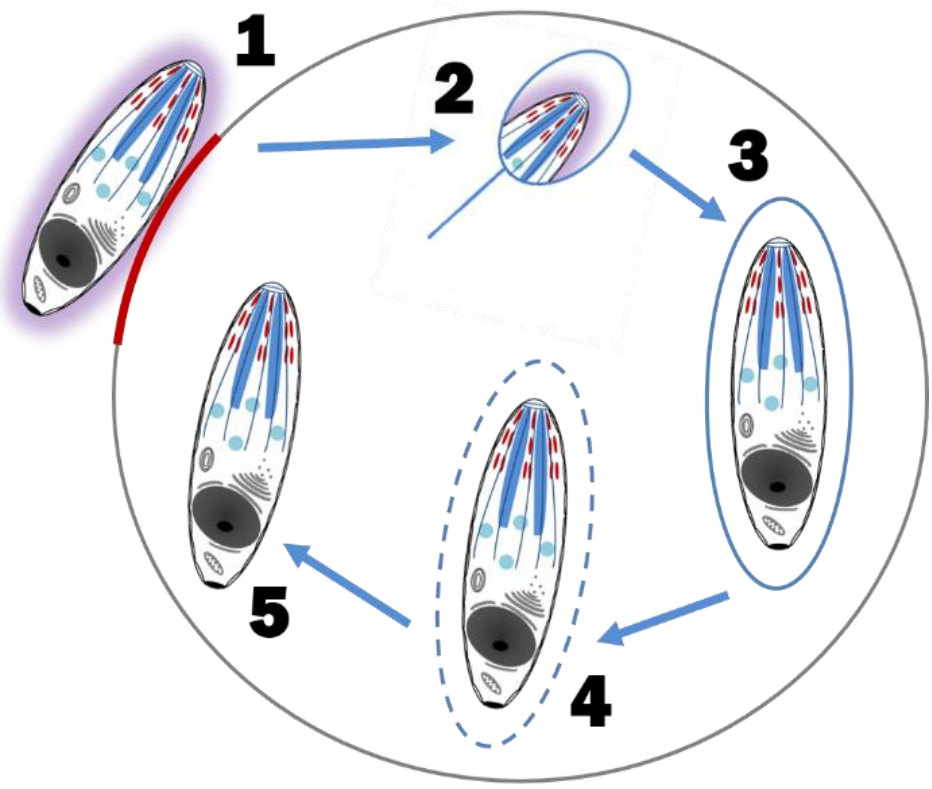


Figure 1.6: Sporozoite attachment and entry into a host cell. (1) a 'slug trail' of adhesive proteins (red) released from the micronemes of the apical complex allow actomyosin molecules of the sporozoite cell membrane to attach to the host cell resulting in a gliding motion, (2) sporozoite internalization via host cell membrane zippering with concurrent loss of its fibrillar coat (purple), (3) sporozoite withing the host cell membrane-bound vacuole, (4) host membrane separated and dissolved by the sporozoite, (5) sporozoite free within the host cytoplasm coopts the host cell microtubule network for replication.

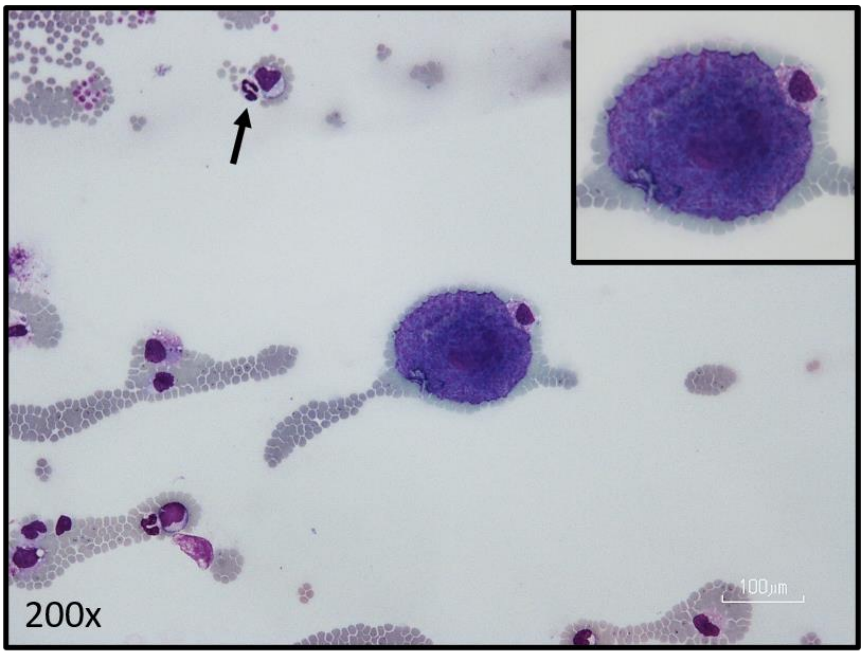


Figure 1.7: Merozoite-laden schizont or Koch's body (insert) at the feathered edge of a blood smear from a cat with acute cytauxzoonosis (Modified Wright stain). Mature schizonts (measuring 25-60 µm in diameter) rupture, releasing merozoites into the blood. Note: A 12-14 microns in diameter neutrophil (black arrow) allows sizing of this schizont, approximately 4.5 times larger than the neutrophil, to be 54-63 microns in diameter.

Asexual merogony.

The budding fission of *C. felis* within erythrocytes is called merogony and occurs when a trophozoite divides into a pair or tetrad of merozoites [13]. Merozoites enter erythrocytes in the same fashion that sporozoites enter monocytes [13, 17]. The internalized parasite develops into a trophozoite, phagocytoses or pinocytoses host cytoplasm through their pellicular micropores, and then asexually divides into 2-4 merozoites. Erythrocyte rupture releases the merozoites into host blood to begin the asexual merogony cycle again. That said, not all trophozoites produce merozoites. Some develop into haploid gametocytes (gamogony), which do not reproduce within red cells [13]. Gametocytes are larger than merozoites and have unusual shapes that are not visible

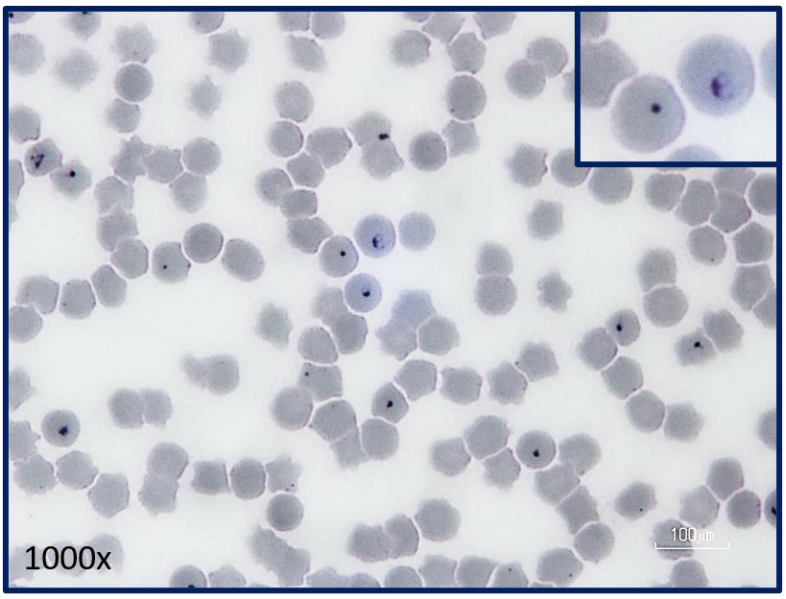


Figure 1.8: Intra-erythrocytic signet ring piroplasms in a blood smear from a cat with acute cytauxzoonosis (Modified Wright stain). Note the pair of merozoites in one of the erythrocytes demonstrating active merogony (inset). The remaining intra-erythrocytic piroplasms could be trophozoites, merozoites, or gametocytes which cannot be differentiated via light microscopy.

via light microscopy. As such, trophozoites, merozoites, and gametocytes all appear as 1-2 μm signet ring piroplasms within erythrocytes microscopically (Figure 1.8).

Sexual reproduction.

Amphimixis, the fusion of two gametes of *Theileria* spp. and, presumably *C. felis*, occurs within the gut of the tick vector [13]. Ticks coat their food bolus with organized chitin microfibrils held together with specific proteins (peritrophins) within a thick proteoglycan matrix. This porous coating, called the peritrophic matrix, acts as a barrier protecting the tick's gut epithelium from infectious organisms and mechanical and chemical damage while improving the efficiency of digestion [19–21]. Intra-erythrocyte merozoites, trophozoites, and gametocytes enter the tick gut lumen along with the bloodmeal coated with the previously described peritrophic matrix [13]. Within five days of ingestion, and as the erythrocytes are lysed, the merozoites and trophozoites are destroyed and digested whereas the gametocytes reorganize their microtubules and cytoplasm

forming haploid anisogametes. Macrogametes (female form) take on a rounded shape, whereas microgametes (male form) stretch out and form a ray body, which has the shape of an arrowhead with trailing arms. When a micro- and macrogamete come into close contact, they attach to each other via small fibrils, a small tube forms from one gamete to the other, the nucleus of the microgamete fuses with the nucleus of the macrogamete, and a motile diploid zygote is born. The arrowhead of the zygote releases chitinases and proteinases to dissolve a path through the peritrophic matrix, allowing its escape into the ecto-peritrophic space next to the intestinal epithelial cells. The zygote immediately enters an intestinal epithelial cell via the same mechanisms previously described. Once inside the host cell's cytoplasm, the zygote spheres, its arrowhead disappears, and multiple motile haploid kinetes are formed via meiosis. The kinetes then exit the cell and enter the tick's hemolymph, an open circulatory system equivalent to mammalian blood. This process occurs within 13-34 days of tick ingestion.

Asexual sporogony.

Sporogony is the production of infective sporozoites. This stage starts with the kinetes travelling to the tick's salivary acini and gaining entry in the same way they entered monocytes, erythrocytes, and intestinal epithelial cells [13]. The intracellular kinete then forms a sporont, a large multinucleated syncytial cell that further develops into a sporoblast, a sporont with a three-dimensional branching network. During this process, the tick's salivary acinar cell hypertrophies to accommodate the enlarging sporoblast. At this point the tick undergoes ecdysis (molting of its cuticle) and the sporoblast remains dormant. In this way, the *C. felis* organisms remain within their tick host from one life stage to the next, also called trans-stadial or horizontal transmission. Within 48 hours of tick attachment to a host, the dormant sporoblast matures, apical complexes are formed, and sporozoites 'bud' off via multiple fission. These sporozoites are released into the tick saliva

and inoculated into the host. If the host is a felid, the life cycle is repeated. Sporogony is an asynchronous event with various stages of sporozoite development occurring at the same time within salivary acinar cells. The end result is a nearly continuous production, and release, of sporozoites into the tick's saliva and thus, the felid host.

Transmission vectors & definitive host.

Thus far, two ixodid ticks have been identified as competent biological vectors of *C. felis*, *Amblyomma americanum* and *Dermacentor variabilis* [22–25] (Figure 1.9). Like all ixodid ticks, they have a life cycle with four stages, each of which can survive several months waiting for a host, (1) egg, (2) larvae, (3) nymph, and (4) adult, with the latter three stages requiring a bloodmeal prior to transitioning to the next stage [26, 27] (Figure 1.10). Depending on environmental conditions, mainly temperature and humidity, the life cycle of these ticks can take 2-3 years to complete [26]. To identify a host, ticks engage in a behavior called 'questing' which includes climbing vegetation to an appropriate height, extending is two rostral legs for host attachment, and responding to several potential factors, e.g. movement, carbon dioxide, size, color, odor, touch, and sweat [26, 27]. Once on its host, the tick will take up to several hours to find an appropriate feeding site [27]. To obtain a bloodmeal, the tick uses toothed chelicerae to cut through the dermis and allow for the barbed hypostome to penetrate the skin. The mouthparts secrete a cement or latex-like material to hold the hypostome in place during the several days of feeding [26, 27]. During feeding the tick releases many substances in its saliva, including anti-coagulants, anti-inflammatories, analgesics, and pathogens like *C. felis* [28, 29]. These salivary substances may mitigate the host immune responses allowing pathogens like *C. felis* to become established. In addition, the salivary composition changes over time to ensure an uninterrupted bloodmeal and reaches peak volumes during the final 24-48 hours of attachment. It is unknown how this

composition change affects *C. felis* sporozoite transmission to the felid host, if at all. Adult male ticks remain on their host taking several small bloodmeals to produce spermatophores in order to mate with as many females as possible [26]. Whereas the adult female only mates once while taking a bloodmeal. Regardless of life stage, once feeding is complete, the mouthpart cement dissolves, the tick falls off, digests its bloodmeal, and molts into the next life stage in preparation to overwinter or lay an egg mass of several thousand eggs [27]. The larval, nymph, and adult forms of vector ticks may become infected with *C. felis* during acquisition of a bloodmeal if their host is a piroplasm-carrying felid. Only after molting into the nymph or adult forms can these ticks transmit *C. felis* to their next felid host. Although both tick vectors have similar life cycles and a preference for geographic areas with ground debris, long grasses, and brushy to wooded areas, they also have important differences [30].

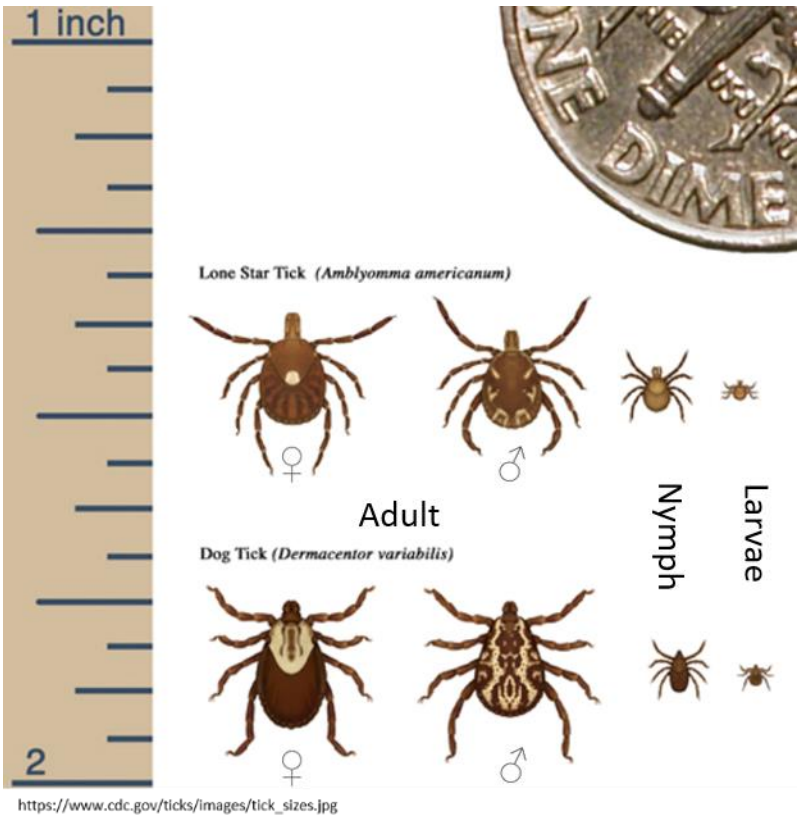


Figure 1.9: Known *Cytauxzoon felis* competent transmission vectors include *Amblyomma americanum* and *Dermacentor variabilis*.

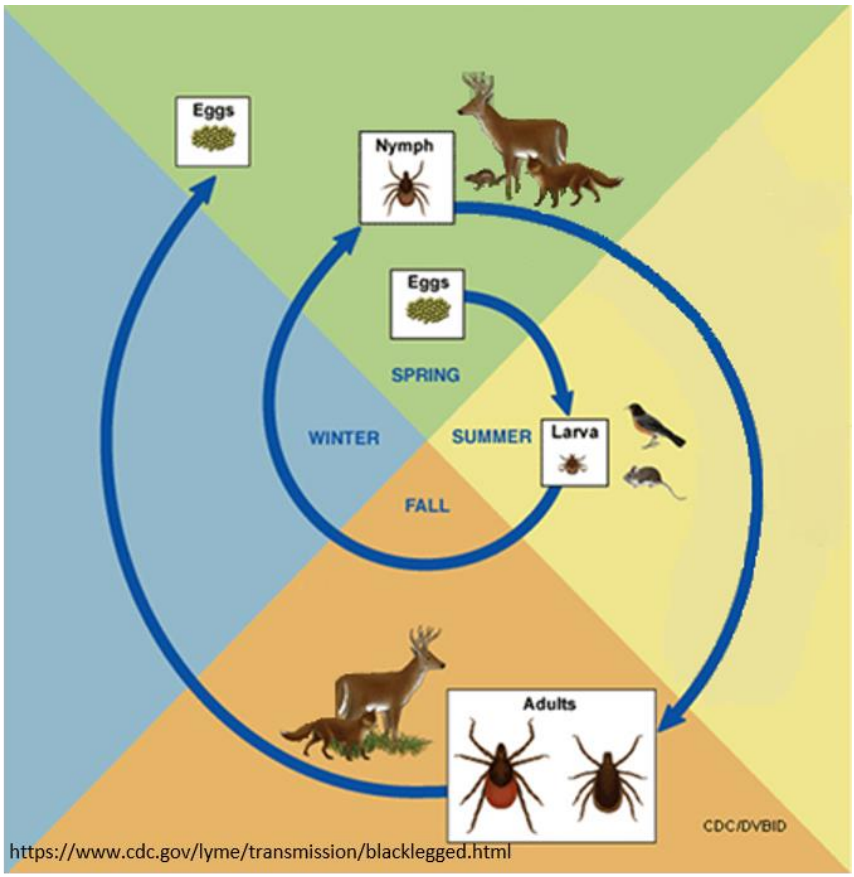


Figure 1.10: Representative seasonal ixodid tick lifecycle with four stages (egg, larvae, nymph, adult), each of which can survive several months waiting for a host. The latter three stages requiring a bloodmeal prior to transitioning to the next stage. Depending on environmental conditions, mainly temperature and humidity, the life cycle of these ticks can take 2-3 years to complete

***Dermacentor variabilis*.**

Dermacentor variabilis, the American Dog tick, has a large geographic distribution extending over the entire eastern half of the U.S. with a focal region on the west coast [30–32] (Figure 1.11). It is a brown tick with grey or silver markings on its scutum. As in all ixodid ticks species, the males have a scutum that covers most of their dorsum, while the females have a short scutum to accommodate engorgement from a bloodmeal [30] (Figure 1.9). Larvae of this species prefer to feed on rodent-sized hosts and are most active in early spring through mid-summer, nymphs prefer opossum-sized hosts and are most active in early summer to early fall, and adult *D. variabilis* ticks feed most commonly on dog to deer-sized mammalian hosts with their greatest activity in early spring to early fall. Tick infestation studies have determined that *D. variabilis* ticks are the third most common tick species found on cats, most being adult females (59.2%) with fewer larvae and nymphs, and are generally found in the dorsal regions of the cat, especially the head and ears [33, 34] (Figure 1.12).

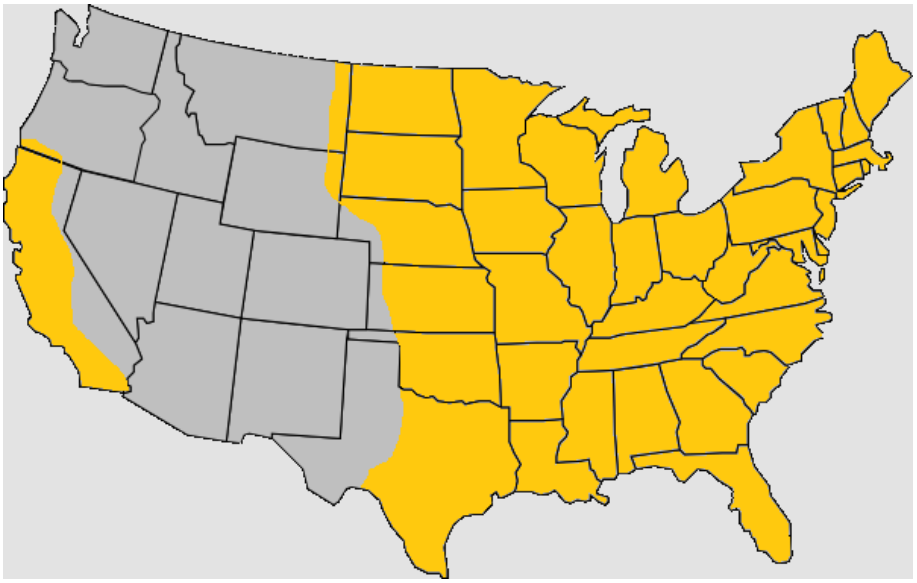
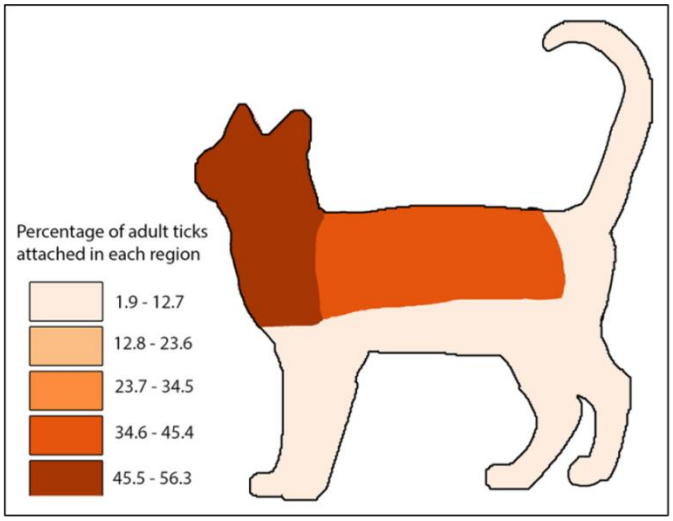


Figure 1.11: *Dermacentor variabilis,* the American Dog tick, has a large geographic distribution extending over the entire eastern half of the U.S. with a focal region on the west coast (orange). (map recreated based on data from CDC: https://www.cdc.gov/ticks/geographic_distribution.html)



Saleh, M. N.; Sundstrom, K. D.; Duncan, K. T.; Ientile, M. M.; Jordy, J.; Ghosh, P.; Little, S. E. Show Us Your Ticks: A Survey of Ticks Infesting Dogs and Cats across the USA. *Parasites and Vectors*, **2019**, *12* (1), 1–11. https://doi.org/10.1186/s13071-019-3847-3.

Figure 1.12: *Dermacentor variabilis* infestation pattern on domestic cats. *Dermacentor variabilis* ticks are the third most common tick species found on cats and are generally found on the dorsal regions of the cat, especially the head and ears.

Two studies have demonstrated *D. variabilis* is a competent vector for *C. felis* transmission. In the first study, *D. variabilis* nymphs were allowed to feed to repletion on a splenectomized chronically *C. felis*-infected wild-caught bobcat with 40% *C. felis* parasitemia [35]. Once the ticks molted into adults, they were allowed to feed on a splenectomized cat, resulting in typical acute cytauxzoonosis signs and findings. Another study used laboratory-reared *D. variabilis* nymphs allowed to feed to repletion on *C. felis*-infected cats [17]. After molting into adults, they were allowed to feed on *C. felis* naïve cats. As with the previous experiment, the *C. felis* naïve cats also demonstrated typical acute cytauxzoonosis signs and findings. To this author's knowledge, transmission time studies have not been performed to determine the transfer time of *C. felis* to felids by *D. variabilis*.

***Amblyomma americanum*.**

Amblyomma americanum, the Lone Star tick, is indigenous to the southeastern and mid-central U.S. with a range that overlaps that of *D. variabilis* and is expanding west and north with

climate changes [26, 36] (Figure 1.13). It is a brown sexually dimorphic species in which the females demonstrate a white spot on their central caudal scutum, while the males demonstrate small white spots along the margin of their scutum [26] (Figure 1.9). This is an aggressive, indiscriminate tick species in which all life stages will feed on any size animal, including cats. Generally speaking, adults are most active in early spring to mid-summer, nymphs in late spring to early fall, and larvae in late summer to early fall. Additionally, tick infestation studies have determined that *A. americanum* ticks are the second most common tick found on cats, most being larval forms (39.1%) with fewer adults and nymphs, and are generally found on the ventral regions of the cats, especially the tail and perianal regions [33, 34] (Figure 1.14). Although feline tick infestations peak in mid-summer, studies have identified cats with ticks year around, including on indoor exclusive cats. As such, *C. felis* infections could occur in any season and in any cat regardless of their lifestyle.

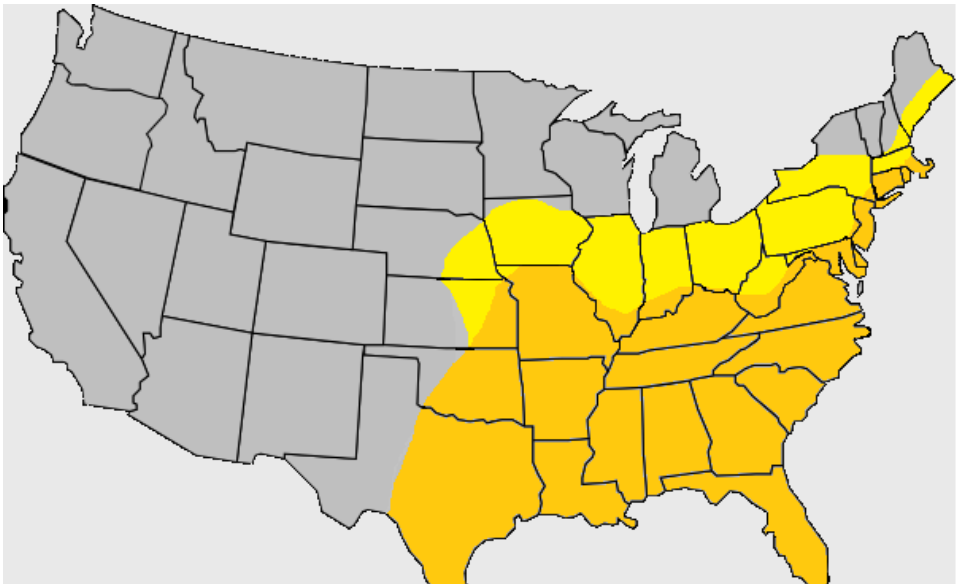
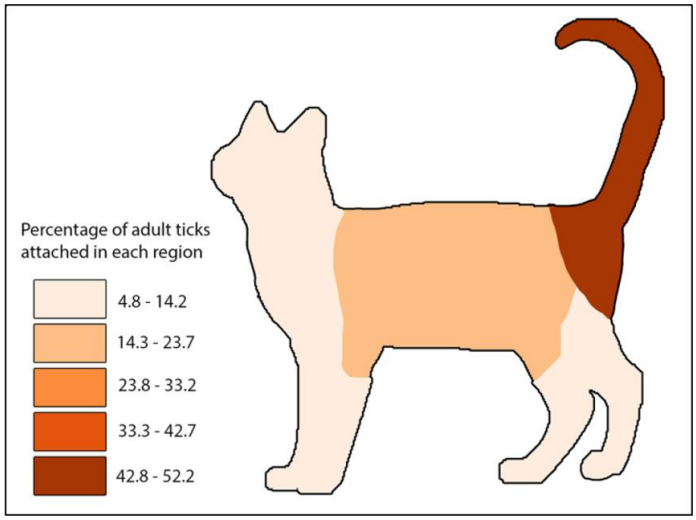


Figure 1.13: *Amblyomma americanum*, the Lone Star tick, is indigenous to the southeastern and mid-central U.S. (orange) with a range that is expanding west and north with climate changes (yellow). (map recreated based on data from CDC: https://www.cdc.gov/ticks/geographic_distribution.html and https://www.cdc.gov/grand-rounds/pp/2017/20170321-presentation-tickborne-diseases-H.pdf)



Saleh, M. N.; Sundstrom, K. D.; Duncan, K. T.; Ientile, M. M.; Jordy, J.; Ghosh, P.; Little, S. E. Show Us Your Ticks: A Survey of Ticks Infesting Dogs and Cats across the USA. *Parasites and Vectors*, **2019**, *12* (1), 1–11. https://doi.org/10.1186/s13071-019-3847-3.

Figure 1.14: *Amblyomma americanum* infestation pattern on domestic cats. *Amblyomma americanum* ticks are the second most common tick found on cats and are generally found on the ventral regions of cats, especially the tail and perianal regions

Two studies have demonstrated *A. americanum* is a competent vector for *C. felis* transmission. The first study used *A. americanum* adults that had been fed to repletion as nymphs on a chronically *C. felis*-infected cat with 0.9% *C. felis* parasitemia to infect four *C. felis* naïve cats [22]. All four *C. felis* naïve cats exhibited typical cytauxzoonosis signs with evidence of infection on blood and tissue evaluation. Another study used *A. americanum* nymphs that had been fed to repletion as larvae on a chronically *C. felis*-infected cat with 0.004% *C. felis* parasitemia to infect three *C. felis* naïve cats [24]. All three C. felis naïve cats exhibited typical cytauxzoonosis signs with evidence of infection on blood and tissue evaluation. Transmission studies have determined *C. felis* sporozoite transfer can occur within 36-72 hours of feeding initiation by a tick of this species [23, 25]. As this ticks' range expands so too will the number of cytauxzoonosis cases seen.

Felid intermediate hosts.

Members of the family Felidae serve as intermediate hosts of *C. felis* and infection reservoirs. Examples of natural felid infection carriers in the U.S. include, bobcat (*Lynx rufus*) [38–42], domestic cat (*Felis catus*) [43, 44] cougar (*Puma concolor*) [45–47], and a captive tiger [48]. Of these, only the bobcat and domestic cat have been studied to confirm they are competent reservoirs for transmission of *C. felis* to other felids [22, 35, 49, 50].

For many decades, the bobcat was assumed to be the primary or main reservoir for *C. felis* in the U.S. Studies demonstrated that most bobcats show few clinical signs with a shortened schizont phase [38–40]. One prevalence study that evaluated the distribution and prevalence of *C. felis* carriers in wild felids (n = 705) using nested PCR and sequence analysis, found that 138 of 696 bobcats over 14 states tested positive for *C. felis* [42]. The individual state prevalence of *C. felis* in bobcats varied from 0-79% with a strong association between the *C. felis* prevalence and the distribution of *A. americanum*. That said, there are also reports of acute cases leading to death [39, 51]. It is possible, that acute cases of this disease occur more frequently than thought in bobcats but are never seen due to the hidden life of these small predators.

At one time, domestic cats were considered a dead-end host for *C. felis* as infection commonly ended in death [2, 3, 52, 53]. However, as more studies explored the transmission and prevalence of *C. felis* carriers in domestic cats, this assumption came under scrutiny [6, 25, 49, 50, 54–58] (Figure 1.15). Four studies between 2007 and 2020 looked at the prevalence of *C. felis* carrier domestic cats using molecular diagnostic assays. One study evaluated a total of 961 trap-neuter-release cats including 494 cats in Florida, 392 cats in North Carolina, and 75 cats in Tennessee . They identified only 3 positive cases (0.3% overall prevalence) [56]. Another identified 56 positive cases out of 902 healthy client-owned cats in Arkansas (25/161 cats; 15.5% prevalence), Missouri

(8/62 cats; 12.9% prevalence), and Oklahoma (23/679 cats; 3.4% prevalence) [50]. A third study identified 3 positives of 672 healthy free-roaming (presumed trap-and-release) cats in Oklahoma (3/380 cats; 0.8% prevalence) and Iowa (0/292 cats: 0% prevalence) [25]. The last study, evaluated 1,104 feral (n=216), owned (n=351) and rescued (n=537) domestic cats with no known history of *Cytauxzoon* infection in eastern Kansas identifying 270 positive cases (25.8% overall prevalence) [59]. These studies demonstrate cats can act as infection carriers and could be the primary reservoir for domestic cat infections.

Figure 1.15: U.S. states with confirmed feline cytauxzoonosis cases (beige and orange) and states in which carrier cat populations have been identified (orange).

Cytauxzoonosis.

Cytauxzoonosis can present as either an acute life-threatening or as a subclinical carrier condition, with the subclinical carrier form generally diagnosed incidentally. The acute life-threatening form is clinically evident during the leukocyte, or schizogenous, phase of *C. felis* infection and is most typically seen in late spring with fewer cases identified in early fall,

corresponding with tick vector activity [60, 61]. The mortality rate for acute feline infection is very high (40-100%) depending on whether appropriate and timely treatment is initiated or not. The minimum sporozoite load needed to cause infection, how sporozoite load affects the length and/or severity of the schizogenous phase, and whether or not it correlates with clinical disease symptoms is not yet known.

Diagnosis.

The differential diagnosis for the acute (schizogenous) cytauxzoonosis clinical signs of fever, lethargy, icterus, dyspnea, and anemia could include cholangiohepatitis, hepatic lipidosis, pancreatitis, triaditis, sepsis, immune-mediated hemolytic anemia, oxidative damaging toxins (e.g. acetaminophen, *Allium* spp), neoplasia, tularemia, feline infectious peritonitis, and hemotropic mycoplasma to name a few. Since no rapid, in-clinic test is available to diagnosis acute cytauxzoonosis, it is generally diagnosed by schizont (Figure 1.7) and/or intra-erythrocytic signet ring (Figure 1.8) identification on antemortem blood smear review, or schizont identification on postmortem tissue sample histology (Figure 1.16). Blood smear review and tissue histology are equally diagnostic for this disease [59]. When a blood smear demonstrates signet ring (aka piroplasm) erythroid hemoparasites in a cat, the three main differentials include: i) *Mycoplasma hemofelis*, ii) *Cytauxzoon felis*, and iii) *Babesia felis*. *Mycoplasma hemofelis* is generally associated with a strongly regenerative anemia, and several coccoid or signet ring shaped organisms located on the red cells membrane and/or in the background [62]. *Cytauxzoon felis* infections are often associated with a variable non-regenerative anemia, 1-2 signet ring shaped organisms within erythrocytes and rarely in the background, and/or schizonts at the blood smear feathered edge or in tissue sample histology. *Babesia felis* is associated with a regenerative anemia and intra-erythrocytic signet rings shaped organisms often arranged in tetrads [63]. Although *B.*

felis has not been reported in the U.S., it should be a differential for any felid with a history of travel to Africa, especially the southern coastal regions. PCR is readily available to differentiate *M. hemofelis* and *Babesia* spp, but less available for *C. felis*. Since subacute and early acute infections may not present with visible intra-erythrocytic signet rings and/or schizonts in *C. felis* infected cats, an in-hospital test needs to be developed for rapid diagnoses of acute cytauxzoonosis cases to initiate potentially lifesaving treatment for these cats. That said, a diagnosis of acute cytauxzoonosis in any cat living in or near *C. felis* endemic areas with typical clinical signs can be made by visualization of either schizonts and/or intra-erythrocytic signet rings via histology or blood smear samples. In locations where the aforementioned feline piroplasm diseases overlap, DNA sequencing may be necessary for species identification.

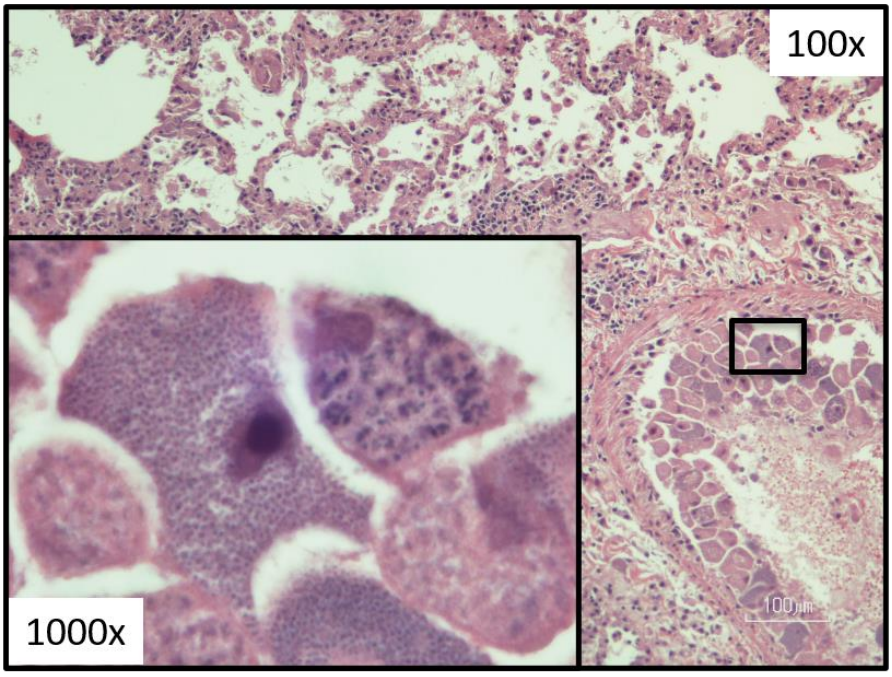


Figure 1.16: Schizont identification on postmortem histology of feline lung tissue confirming a diagnosis of acute cytauxzoonosis. The merozoite-laden monocytes are seen within a pulmonary vessel in various stages of development (inset). (Hematoxylin and eosin stain).

Acute disease.

Acute cytauxzoonosis cats commonly present to the veterinary clinic for acute lethargy, anorexia, depression, and fever approximately 11 days post infection (dpi) [2, 49, 52, 54, 55]. On physical exam, these cats are dehydrated, pyrexic (103-106 F; 39.4-41.1 C), have pale mucous membranes, and splenomegaly [2, 49, 52, 54, 55, 64]. As the disease progresses, pyrexia resolves and drops to subnormal with icterus and dyspnea develop shortly thereafter (16-21 dpi) [3, 49, 55, 60]. Complete blood count and serum biochemistry changes are seen at around 13 dpi. Pancytopenia or bicytopenia are variable and can include: i) marked non-regenerative anemia (0.10-0.18 L/L; RI 0.29-0.48 L/L), ii) leukopenia or leukocytosis (1.3-6.5 x 10^9/L; RI 2.3-5.4 x 10^9/L), with iii) variable neutrophilia (10.45-22.09 x 10^9/L; RI 2.5-8.5 x 10^9/L), and/or iv) variable lymphocytosis (21.2-31.63 x 10^9/L; RI 1.2-8.0 x 10^9/L), and v) moderate to marked thrombocytopenia (12.9-73.3 x 10^9/L; RI 300-800 x 10^9/L) [49, 52, 64]. Serum biochemistry and urinalysis findings commonly include decreased albumin, increased glucose (7.94-12.15 mmol/L; RI 3.50-8.32 mmol/L), increased ALT, increased total bilirubin (15.39-141.93 mmol/L; RI 0.0-8.55 mmol/L), and bilirubinuria. When blood smears are evaluated, schizont-laden monocytes may or may not be seen at the feathered edge (Figure 1.7), but 1-2 μm signet ring piroplasms within erythrocytes will be seen by 18 dpi [49] (Figure 1.8). Blood samples submitted for real time PCR will test positive for *C. felis* at around 17 dpi.

Death, generally occurring at about 21 dpi, has long been assumed to be due to vascular obstruction by engorged schizonts resulting in multiorgan failure due to hypoxic injury. However, recent studies have suggested that along with hypoxic injury, local and systemic immune responses to proinflammatory substances released by neutrophils and schizogenous monocytes are responsible for much of the morbidity and mortality seen in cytauxzoonosis [18, 65–67].

Leukocyte activation results in proinflammatory cytokine release which affect platelets and endothelial cells resulting in a hypercoagulable state that may culminate in disseminated intravascular coagulation (DIC) [67, 68]. Studies have measured significantly higher systemic concentration of the proinflammatory cytokine TNF-α in cats that eventually died of the disease versus those that survived [65, 66]. In addition, CD18, an adhesion molecule that likely attaches infected monocytes to activated endothelium, was also upregulated in cats that died versus those that recovered. It is not yet known whether the immune dysregulation seen is caused by the parasites themselves or by the secondary responses to proinflammatory cytokines. Cats that succumbed to cytauxzoonosis had typical interstitial pneumonia findings on lung histology that included thickened pulmonary interstitium due to edema and neutrophilic infiltrates, neutrophilic alveolar exudate, and evidence of vasculitis. Immunohistochemistry demonstrated "a significant, widespread, qualitative increase in the expression of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, as well as inducible nitric oxide synthase (iNOS),"[66] while uninfected cat tissues demonstrated no or minimal expression of iNOS. The expression of iNOS is commonly found in human and animals with acute pulmonary distress syndrome. In the aforementioned studies, schizonts stained positive for iNOS, suggesting they were producing reactive nitrogen intermediates. These intermediates can then activate TNF-α, IL-1β, and/or IL-6. TNF-α and IL-1β upregulate CD18, the adhesion molecule previously mentioned, while TNF-α activates endothelial cells and induces them to express MHC-II. Activated endothelial cells contract, causing local edema, and release nitrous oxide (NO), which contributes to local tissue inflammation and damage, and results in hypotensive shock and microvascular damage.

On necropsy, felids that have succumbed to acute cytauxzoonosis demonstrate generalized icterus, splenomegaly, lymphadenopathy, petechia and ecchymosis on the heart and lungs, clear

yellow serous pericardial effusion, interstitial pneumonia, and large numbers of schizonts within the vasculature attached to endothelial cells of most organs; with the liver, lung, spleen, and lymph nodes most effected [2, 18, 55, 64] (Figure 1.16). When examined, the brain demonstrates schizogenous vascular occlusion with secondary ischemia and necrosis of neurons as well [55, 69, 70]. The intravascular mega-schizonts are a prominent diagnostic feature in these cases and typically measure 25-60 μm in diameter but may reach 250 μm in diameter with an enlarged nucleus, prominent nucleolus, and cytoplasm full of variably mature basophilic merozoites [18]. The cause of death in acute *Cytauxzoon* cases is a combination of hypoxic injury and local and systemic immune responses to proinflammatory cytokines leading to poor pulmonary ventilation, decreased gas exchange, and/or a procoagulant state resulting in DIC. Additional studies are needed to determine how the host immune response to this pathogen differs during schizogenous and erythrocytic parasite phases, what factors mitigate those host immune responses, and how we might manipulate the feline immune response to improve survival of these patients.

Subclinical carrier disease.

Felids that survive the schizogenous phase of this disease, show evidence of erythrocyte regeneration at 18-22 dpi with resolution of subnormal body temperature and clinical signs shortly thereafter (23-24 dpi) [49]. Normalization of red cell indices is complete by 43 dpi. These survivors manifest the chronic subclinical carrier form of this disease by entering a persistently parasitized erythrocyte stage [56, 64]. It is unknown if the intra-erythrocytic piroplasms are predominantly trophozoites, merozoites, or gametocytes, or if the distinction matters. Felids with chronic disease are completely asymptomatic and remain persistently infected for years, if not life [54]. They appear to be protected from re-developing clinical disease with additional *C. felis* challenges, provided they survived a previous schizogenous phase. However, this reprieve may not be lifelong

or may not be equally protective against heterologous strains of the pathogen. A study evaluating the prevalence of *C. felis* in southern Illinois wild-caught bobcats, identified one individual that appeared to have been infected with a different strain (ITS-1 single nucleotide polymorphism) of *C. felis* between captures [41]. More recently, a domestic shorthair cat successfully treated for acute cytauxzoonosis seven years prior, was presented with a repeat *C. felis* infection confirmed on splenic histology [71]. Because survival of the schizogenous phase is required for future protection, cats transfused with piroplasm-laden blood will harbor piroplasms as a chronic carrier but are not immune to *C. felis* challenge [38, 72]. Due to their asymptomatic nature, carrier cats are typically only diagnosed with *C. felis* if: i) they have a known history of surviving acute cytauxzoonosis, ii) piroplasms are identified on a blood smear exam, and/or iii) they test positive for *C. felis* via PCR. Further studies are needed to determine if *C. felis* reservoir cats are more likely to have co-infections with other organisms and, if so, how that might affect the cat's carrier status.

Treatment options.

All treatments for acute *C. felis* infections have incorporated basic supportive care methods including intravenous fluids for dehydration and hypotension, antimicrobials to treat concurrent septicemia, and heparin to prevent thrombus formation and DIC. Several targeted anti-protozoal treatments have been proposed and attempted for *C. felis* with variable success. Parvaquone and buparvaquone, used to treat *Theileria parva* in cattle, were investigated, but found to be ineffective in treating experimentally induced cytauxzoonosis cases [72, 73]. Imidocarb dipropionate, a urea derivative used to treat *Babesia canis*, demonstrated limited, inconsistent results, and was also abandoned as a treatment candidate [54, 72, 74]. Dimethazine aceturate, used to treat

trypanosomiasis in livestock, was equally ineffective in the treatment of naturally occurring acute and chronic *C. felis* infections in cats [75, 76].

Currently, the most effective treatment is a combination of atovaquone (15 mg/kg PO q 8 hours x 10 days) and azithromycin (10 mg/kg PO q 24 hours x 10 days) which provides a 60% success rate in treated cats, if initiated in a timely manner [77]. Atovaquone, a malaria treatment candidate, is a ubiquinone (Coenzyme Q) analogue that targets the cytochrome b subunit of the mitochondrial electron transport chain [12, 78–80]. Early studies of atovaquone as a potential anti-malarial medication, demonstrated parasite mitochondrial membrane potential collapsed within minutes of treatment, while leaving the host mammalian mitochondria unaffected [81]. Subsequent studies explored energy metabolism in *Plasmodium spp.* and found their mitochondrial energy production in the erythrocytic stage was very slow, possibly explaining the poor *Plasmodium* response to atovaquone [82]. As stated previously, Apicomplexans appear to be missing at least two of the necessary mitochondrial ETC subunits needed to perform oxidative phosphorylation [12]. As such, atovaquone may not inhibit a vital function of their mitochondrion resulting in a less than optimal response to this drug. Azithromycin, a macrolide antibiotic, inhibits protein translation at the parasite mitochondrial ribosome level and, thus, the parasite's growth [12, 78–80]. With the best treatment resulting in 40% mortality, improved treatment protocols are needed. Many factors may influence patient response to treatment including the timing of treatment initiation, patient immune response to the parasite, sporozoite load injected, and parasite strain virulence to name a few [54, 83]. Although further investigation is needed to assess each of these factors and their contribution to the disease process and patient response, no *C. felis* strain banks nor in vitro experimental systems exist to facilitate the needed research. Thus far, studies trying to identify different pathogenic or virulent strains using the ITS region of the 18S rRNA gene have

determined there is no genotype association with geography or clinical outcome of this disease [43, 84].

Control & Prevention.

Since cats surviving the acute schizogenous phase appear to have protection from re-developing clinical signs with future infections for an undetermined duration, it is assumed they form a protective immune response [38, 72, 85]. As such, a vaccine could potentially be developed if an appropriate protein candidate were found. To that end, Tarigo et al. sequenced the entire *C. felis* genome and identified 4,300 potential protein-coding genes [86]. To date, two potential vaccine candidates have been examined. The highly conserved Cf76 gene, like the *T. parva* p67 gene, encodes a protein expressed during the schizont phase and is recognized by the feline humoral immune system and the AMA-1 gene encoding an apical membrane antigen [6, 85, 87]. To date, no vaccine has been developed. To produce a successful vaccine, more research is needed to identify and confirm immunogenic and protective antigens. In addition, more studies are needed to better understand genetic determinants of virulence, transmission, and treatment success, especially in the context of *C. felis* strain heterogeneity.

Since no *C. felis* vaccine exists and there is no consistently effective treatment, control of this disease is based on transmission prevention by eliminating, or limiting, exposure to the vectors *A. americanum* and *D. variabilis*. As such, limiting cats' outdoor time during peak tick season, manual tick removal, and/or the application of prophylactic acaricides remain the mainstay of control for this disease [6, 50, 88, 89]. Two acaricides have demonstrated good to excellent efficacy in preventing *C. felis* transmission by *A. americanum* under experimental conditions. The first product, imidacloprid 10%/flumethrin 4.5% collar (Seresto, Bayer Animal Health (a subsidiary of Elanco), Greenfield, IN), demonstrated 100% efficacy in preventing tick attachment

and feeding, and thus transmission of *C. felis*, in 10 cats [88]. The flumethrin, a synthetic pyrethroid, in this product affects the neuronal sodium channels of mites and ticks resulting in their death. Although cats are generally sensitive to pyrethroids, this synthetic form does not require glucuronidation, rendering it safe for use in cats. The second product, selamectin/sarolaner (Revolution Plus, Zoetis Inc., Parsippany, NJ), demonstrated >90% efficacy in reducing *A. americanum* and *D. variabilis* tick counts 72 hours after infestation and preventing transmission of *C. felis* in 8 cats when applied monthly [89]. The sarolaner, an isoxazoline compound, in this product causes paralysis and death of ticks via neural blockade. Since *C. felis* can be transmitted from the tick to its felid host within as little as 36 hours, it is critical to use preventative products that either repel ticks or kill the ticks prior to parasite transmission. Additional research is needed to improve preventative treatments including considering developing a molecule that would block earlier parasite developmental stages (e.g. sporozoite development in the tick), or development of a vaccine against the tick vector.

Summary & Future Considerations.

Cytauxzoonosis is caused by *Cytauxzoon felis*, a protozoal apicomplexan hemoparasite of felids in the *Theileria* & *Cytauxzoon* clade of Piroplasmidae, endemic to North America. In the last four decades, much has been elucidated about this organism's complex life cycle, transmission, and the disease it causes in felids. Although it appears that most, if not all, felids can become infected, the known competent hosts include bobcats (*Lynx rufus*) and domestic cats (*Felis catus*). Once within a host, *C. felis* begins the schizogenous phase of asexual replication, which can cause severe illness and death in its host due to proinflammatory cytokine stimulated damage, DIC secondary to endothelial damage, hypoxic injury of multiple organs, and interstitial pneumonia with poor gas exchange exacerbating hypoxemia. If the host animal survives the

schizogenous phase, the *C. felis* organism enters a perpetual erythrocytic phase causing the asymptomatic host to remain persistently parasitemic acting as a disease reservoir for years. Although clinical disease upon subsequent re-infection is uncommon, it appears that infection with a different *C. felis* strain can lead to additional acute life-threatening schizogenous phases in the same host. The cornerstone of *C. felis* prevention is avoidance of transmission via tick control. As such, all cats living in endemic areas, including indoor exclusive cats, should be treated year around with acaricide products that are known to repel or kill ticks rapidly.

A vaccine and more effective, affordable treatments are desperately needed. As is an affordable rapid diagnostic test to confirm diagnosis early in the disease process and to identify *C. felis* carrier cats. In addition, more studies are needed to better understand factors affecting infection, disease, and treatment. These factors include the complex interactions of the pathogen life cycle stages with the tick vector life cycle stages and immune responses, as well as with the felid host immune responses, and the physical environment where each of these organisms live. The genetic diversity within the *C. felis* population needs to be explored along with how this diversity might affect strain virulence, pathogen transmission to and from the tick and felid hosts, disease progression within felid hosts, and treatment options and outcomes for felids. This includes identifying the minimum sporozoite load necessary for infection, what tick and/or felid factors might affect sporozoite load, what factors might impact sporozoite tropism for monocytes and/or merozoite tropism for erythrocytes, *C. felis* virulence factors, how treatment timing effects outcomes for the felid host, and developing *C. felis* strain banks, to mention a few. Genetic differences among cats that survive versus those that succumb to *C. felis* infection is another area that needs to be explored. This includes determining whether sporozoite load affects the length and severity of the schizogenous phase within the felid host, how the felid immune system

responds to the parasite to help or harm the felid, and how those responses can be manipulated to improve feline survival. Research of this caliber would require significant funds; however, funding opportunities for researching this organism are currently limited. Additionally, there is no *in vitro* experimental system available for *C. felis*, so investigating many of these questions requires the use of deliberately infected cats, raising ethical concerns. As such, much of the current knowledge about *C. felis* assumes it is similar to related Piroplasmidae species. These assumptions may be partially, or wholly, inaccurate. A good starting point would be to form a large coalition including multiple institutions (e.g. universities, research facilities, humane societies, rescue organizations, feral cat groups, and private practitioners) across several disciplines (e.g. clinicians, researchers, pathologists, entomologists, climatologists) across multiple states and/or internationally with the goal of answering many of these questions through an integrated collaborative approach in which detailed client and patient information could be collected and shared with the greatest efficiency and the least negative impact on the feline population involved.

References

[1] Neitz, W. O.; Thomas, A. D. *Cytauxzoon sylvicaprae* Gen. Nov., Spec. Nov., a Protozoon Responsible for a Hitherto Undescribed Disease in the Duiker, *Sylvicapra grimmia* (Linné). *Onderstepoort J. Vet. Sci. Anim. Ind.*, 1948, *23* (1–2), 63–76.

[2] Wagner, J. E. A Fatal Cytauxzoonosis like Disease in Cats. *J. Am. Vet. Med. Assoc.*, 1976, *168* (7), 585–588.

[3] Ferris, D. H. A Progress Report on the Status of a New Disease of American Cats: Cytauxzoonosis. *Comp. Immunol. Microbiol. Infect. Dis.*, 1979, *1*, 269–276.

[4] Nijhof, A. M.; Pillay, V.; Steyl, J.; Prozesky, L.; Stoltsz, W. H.; Lawrence, J. A.; Penzhorn, B. L.; Jongejan, F. Molecular Characterization of *Theileria* Species Associated with Mortality in Four Species of African Antelopes. *J. Clin. Microbiol.*, 2005, *43* (12), 5907–5911. https://doi.org/10.1128/JCM.43.12.5907-5911.2005.

[5] Kier, A. B.; Wightman, S. R.; Wagner, J. E. Interspecies Transmission of *Cytauxzoon felis*. *Am. J. Vet. Res.*, 1982.

[6] Wang, J. L.; Li, T. T.; Liu, G. H.; Zhu, X. Q.; Yao, C. Two Tales of *Cytauxzoon felis* Infections in Domestic Cats. *Clinical Microbiology Reviews*. 2017. https://doi.org/10.1128/CMR.00010-17.

[7] Schreeg, M. E.; Marr, H. S.; Tarigo, J. L.; Cohn, L. A.; Bird, D. M.; Scholl, E. H.; Levy, M. G.; Wiegmann, B. M.; Birkenheuer, A. J. Mitochondrial Genome Sequences and Structures Aid in the Resolution of Piroplasmida Phylogeny. *PLoS One*, 2016, *11* (11), 1–27. https://doi.org/10.1371/journal.pone.0165702.

[8] O'Donoghue, P. Haemoprotozoa: Making Biological Sense of Molecular Phylogenies. *Int. J. Parasitol. Parasites Wildl.*, 2017, *6*, 241–256. https://doi.org/10.1016/j.ijppaw.2017.08.007.

[9] Ascencio, M.; Florin-Christensen, M.; Mamoun, C.; Weir, W.; Shiels, B.; Schnittger, L. Cysteine Proteinase C1A Paralog Profiles Correspond with Phylogenetic Lineages of Pathogenic Piroplasmids. *Vet. Sci.*, 2018, *5* (41), 1–12. https://doi.org/10.3390/vetsci5020041.

[10] Wilson, R. J.; Williamson, D. H. Extrachromosomal DNA in the Apicomplexa. *Microbiol. Mol. Biol. Rev.*, 1997, *61* (1), 1–16. https://doi.org/10.1128/.61.1.1-16.1997.

[11] Alexeyev, M.; Shokolenko, I.; Wilson, G.; LeDoux, S. The Maintenance of Mitochondrial DNA Integrity - Critical Analysis and Update. *Cold Spring Harb. Perspect. Biol.*, 2013, *5* (5), 1–17. https://doi.org/10.1101/cshperspect.a012641.

[12] Mather, M.; Henry, K.; Vaidya, A. Mitochondrial Drug Targets in Apicomplexan Parasites. *Curr. Drug Targets*, 2006, *8* (1), 49–60. https://doi.org/10.2174/138945007779315632.

[13] Jalovecka, M.; Hajdusek, O.; Sojka, D.; Kopacek, P.; Malandrin, L. The Complexity of Piroplasms Life Cycles. *Front. Cell. Infect. Microbiol.*, 2018, *8* (July), 1–12. https://doi.org/10.3389/fcimb.2018.00248.

[14] Tran, JQ, de Leon, JC, Li, Catherine, Huynh, My-Hang, Beatty, Wandy, Morrissette, N. RNG1 Is a Late Marker of the Apical Polar Ring in *Toxoplasma gondii*. *Cytoskeleton*, 2010, *67* (9), 586–598. https://doi.org/doi:10.1002/cm.20469.

[15] Sibley, L. D. How Apicomplexan Parasites Move in and out of Cells. *Curr. Opin. Biotechnol.*, 2010, *21* (5), 592–598. https://doi.org/10.1016/j.copbio.2010.05.009.

[16] Schreeg, M. E.; Marr, H. S.; Griffith, E. H.; Tarigo, J. L.; Bird, D. M.; Reichard, M. V.; Cohn, L. A.; Levy, M. G.; Birkenheuer, A. J. PCR Amplification of a Multi-Copy Mitochondrial Gene (Cox3) Improves Detection of *Cytauxzoon felis* Infection as Compared to a Ribosomal Gene (18S). *Vet. Parasitol.*, 2016, *225*, 123–130. https://doi.org/10.1016/j.vetpar.2016.06.013.

[17] Kocan, A. A.; Kocan, K. M.; Blouin, E. F.; Mukolwe, S. W. A Redescription of Schizogony of Cytauxzoon Felis in the Domestic Cat. *Ann. N. Y. Acad. Sci.*, 1992. https://doi.org/10.1111/j.1749-6632.1992.tb19639.x.

[18] Snider, T. A.; Confer, A. W.; Payton, M. E. Pulmonary Histopathology of *Cytauxzoon felis* Infections in the Cat. *Vet. Parasitol.*, 2010, *47* (4), 698–702. https://doi.org/DOI: 10.1177/0300985810364527.

[19] Lehane, M. J. Peritrophic Matrix Structure and Function. *Annu. Rev. Entomol.*, 1997, *42* (1), 525–550. https://doi.org/10.1146/annurev.ento.42.1.525.

[20] Hegedus, D.; Erlandson, M.; Gillott, C.; Toprak, U. New Insights into Peritrophic Matrix Synthesis, Architecture, and Function. *Annu. Rev. Entomol.*, 2009, *54* (1), 285–302. https://doi.org/10.1146/annurev.ento.54.110807.090559.

[21] Bolognesi, R.; Terra, W. R.; Ferreira, C. Peritrophic Membrane Role in Enhancing Digestive Efficiency. Theoretical and Experimental Models. *J. Insect Physiol.*, 2008, *54* (10–11), 1413–1422. https://doi.org/10.1016/j.jinsphys.2008.08.002.

[22] Reichard, M. V; Edwards, A. C.; Meinkoth, J. H.; Snider, T. A.; Meinkoth, K. R.; Heinz, R. E.; Little, S. E. Confirmation of *Amblyomma americanum* (Acari: Ixodidae) as a Vector for *Cytauxzoon felis* (Piroplasmorida: Theileriidae) to Domestic Cats. *J. Med. Entomol.*, 2010. https://doi.org/10.1603/ME10013.

[23] Thomas, J. E.; Ohmes, C. M.; Payton, M. E.; Hostetler, J. A.; Reichard, M. V. Minimum Transmission Time of *Cytauxzoon felis* by *Amblyomma americanum* to Domestic Cats in Relation to Duration of Infestation, and Investigation of Ingestion of Infected Ticks as a Potential Route of Transmission. *J. Feline Med. Surg.*, 2018, *20* (2), 67–72. https://doi.org/10.1177/1098612X17691172.

[24] Allen, K. E.; Thomas, J. E.; Wohltjen, M. L.; Reichard, M. V. Transmission of *Cytauxzoon felis* to Domestic Cats by *Amblyomma americanum* Nymphs. *Parasites and Vectors*, 2019, *12* (1), 1–6. https://doi.org/10.1186/s13071-018-3276-8.

[25] Nagamori, Y.; Slovak, J. E.; Reichard, M. V. Prevalence of *Cytauxzoon felis* Infection in Healthy Free-Roaming Cats in North-Central Oklahoma and Central Iowa. *J. Feline Med. Surg. Open Reports*, 2016. https://doi.org/10.1177/2055116916655174.

[26] Holderman, C. J.; Kaufman, P. E. common name : lone star tick scientific name : *Amblyomma americanum* (Linnaeus) (Acari : Ixodidae) http://entnemdept.ufl.edu/creatures/urban/medical/lone_star_tick.htm (accessed Mar 15, 2020).

[27] Adams, D.; Anderson, B.; Ammirati, C.; Helm, K. Identification And Diseases Of Common U.S. Ticks http://ispub.com/IJD/2/1/7515 (accessed Mar 16, 2020).

[28] Nuttall, P. A. Tick Saliva and Its Role in Pathogen Transmission. *Wiener Klin. Wochenschrift Cental Eur. J. Med.*, 2019, No. May. https://doi.org/10.1007/s00508-019-1500-y.

[29] Chmelar, J.; Kotál, J.; Kovaríková, A.; Kotsyfakis, M. The Use of Tick Salivary Proteins as Novel Therapeutics. *Front. Physiol.*, 2019, *10* (JUN), 1–10. https://doi.org/10.3389/fphys.2019.00812.

[30] Chan, W.-H.; Kaufman, P. E. common name : American dog tick scientific name : *Dermacentor variabilis* (Say) (Arachnida : Ixodida : Ixodidae) http://entnemdept.ufl.edu/creatures/urban/medical/american_dog_tick.htm (accessed Mar 24, 2020).

[31] Kaufman, E. L.; Stone, N. E.; Scoles, G. A.; Hepp, C. M.; Busch, J. D.; Wagner, D. M. Range-Wide Genetic Analysis of *Dermacentor variabilis* and Its *Francisella*-like Endosymbionts Demonstrates Phylogeographic Concordance between Both Taxa. *Parasites and Vectors*, 2018, *11* (1), 1–11. https://doi.org/10.1186/s13071-018-2886-5.

[32] Minigan, J. N.; Hager, H. A.; Peregrine, A. S.; Newman, J. A. Current and Potential Future Distribution of the American Dog Tick (*Dermacentor variabilis*, Say) in North America. *Ticks Tick. Borne. Dis.*, 2018, *9* (2), 354–362. https://doi.org/10.1016/j.ttbdis.2017.11.012.

[33] Saleh, M. N.; Sundstrom, K. D.; Duncan, K. T.; Ientile, M. M.; Jordy, J.; Ghosh, P.; Little, S. E. Show Us Your Ticks: A Survey of Ticks Infesting Dogs and Cats across the USA. *Parasites and Vectors*, 2019, *12* (1), 1–11. https://doi.org/10.1186/s13071-019-3847-3.

[34] Little, S. E.; Barrett, A. W.; Nagamori, Y.; Herrin, B. H.; Normile, D.; Heaney, K.; Armstrong, R. Ticks from Cats in the United States: Patterns of Infestation and Infection with Pathogens. *Vet. Parasitol.*, 2018, *257* (May), 15–20. https://doi.org/10.1016/j.vetpar.2018.05.002.

[35] Blouin, E. F.; Kocan, A. A.; Glenn, B. L.; Kocan, K. M.; Hair, J. A. Transmission of *Cytauxzoon felis* Kier, 1979 from Bobcats, *Felis rufus* (Schreber), to Domestic Cats by *Dermacentor veriabilis* (Say). *J. Wil*, 1984, *20* (3), 241–242.

[36] Monzón, J. D.; Atkinson, E. G.; Henn, B. M.; Benach, J. L. Population and Evolutionary Genomics of *Amblyomma americanum*, an Expanding Arthropod Disease Vector. *Genome Biol. Evol.*, 2016, *8* (5), 1351–1360. https://doi.org/10.1093/gbe/evw080.

[37] Sonenshine, D. E. Range Expansion of Tick Disease Vectors in North America: Implications for Spread of Tick-Borne Disease. *Int. J. Environ. Res. Public Health*, 2018, *15* (3), 1–9. https://doi.org/10.3390/ijerph15030478.

[38] Glenn, B. L.; Kocan, A. A.; Blouin, E. F. Cytauxzoonosis in Bobcats. *J. Am. Vet. Med. Assoc.*, 1983.

[39] Blouin, E. F.; Kocan, A. A.; Kocan, K. M.; Hair, J. Evidence of a Limited Schizogonous Cycle for *Cytauxzoon felis* in Bobcats Following Exposure to Infected Ticks. *J. Wildl. Dis.*, 1987. https://doi.org/10.7589/0090-3558-23.3.499.

[40] Birkenheuer, A. J.; Marr, H. S.; Warren, C.; Acton, A. E.; Mucker, E. M.; Humphreys, J. G.; Tucker, M. D. *Cytauxzoon felis* Infections Are Present in Bobcats (*Lynx rufus*) in a Region Where Cytauxzoonosis Is Not Recognized in Domestic Cats. *Vet. Parasitol.*, 2008, *153*, 126–130. https://doi.org/10.1016/j.vetpar.2008.01.020.

[41] Zieman, E. A.; Nielsen, C. K.; Jiménez, F. A. Chronic *Cytauxzoon felis* Infections in Wild-Caught Bobcats (*Lynx rufus*). *Vet. Parasitol.*, 2018, *252*, 67–69. https://doi.org/10.1016/j.vetpar.2018.01.022.

[42] Shock, B. C.; Murphy, S. M.; Patton, L. L.; Shock, P. M.; Olfenbuttel, C.; Beringer, J.; Prange, S.; Grove, D. M.; Peek, M.; Butfiloski, J. W.; et al. Distribution and Prevalence of *Cytauxzoon felis* in Bobcats (*Lynx rufus*), the Natural Reservoir, and Other Wild Felids in Thirteen States. *Vet. Parasitol.*, 2011, *175* (325–330). https://doi.org/10.1016/j.vetpar.2010.10.009.

[43] Brown, H. M.; Lockhart, J. M.; Latimer, K. S.; Peterson, D. S. Identification and Genetic Characterization of *Cytauxzoon felis* in Asymptomatic Domestic Cats and Bobcats. *Vet. Parasitol.*, 2010, *172*, 311–316. https://doi.org/10.1016/j.vetpar.2010.04.041.

[44] Lewis, K. M.; Cohn, L. A.; Marr, H. S.; Birkenheuer, A. J. Diminazene Diaceturate for Treatment of Chronic *Cytauxzoon felis* Parasitemia in Naturally Infected Cats. *J. Vet. Intern. Med.*, 2012. https://doi.org/10.1111/j.1939-1676.2012.01003.x.

[45] Butt, M. T.; Bowman, D.; Barr, M. C.; Roelke, M. E. Iatrogenic Transmistion of *Cytauxzoon felis* from a Florida Panther (*Felix concolor coryi*) to a Domestic Cat. *J. Wildl. Dis.*, 1991, *27* (2), 342–347.

[46] Rotstein, D. S.; Taylor, S. K.; Harvey, J. W.; Bean, J. Hematologic Effects of Cytauxzoonosis in Florida Panthers and Texas Cougars in Florida. *J. Wildl. Dis.*, 1999. https://doi.org/10.7589/0090-3558-35.3.613.

[47] Harvey, J. W.; Dunbar, M. R.; Norton, T. M.; Yabsley, M. J. Laboratory Findings in Acute *Cytauxzoon felis* Infection in Cougars (*Puma concolor couguar*) in Florida. *J. Zoo Wildl. Med.*, 2007. https://doi.org/10.1638/1042-7260(2007)038[0285:LFIACF]2.0.CO;2.

[48] Lewis, K. M.; Cohn, L. A.; Downey, M. E.; Whitney, M. S.; Birkenheuer, A. J. Evaluation of *Cytauxzoon felis* Infection Status in Captive-Born Wild Felids Housed in an Area Endemic for the Pathogen. *J. Am. Vet. Med. Assoc.*, 2012. https://doi.org/10.2460/javma.241.8.1088.

[49] Reichard, M. V.; Meinkoth, J. H.; Edwards, A. C.; Snider, T. A.; Kocan, K. M.; Blouin, E. F.; Little, S. E. Transmission of *Cytauxzoon felis* to a Domestic Cat by *Amblyomma americanum*. *Vet. Parasitol.*, 2009, *161* (1–2), 110–115. https://doi.org/10.1016/j.vetpar.2008.12.016.

[50] Rizzi, T. E.; Reichard, M. V.; Cohn, L. A.; Birkenheuer, A. J.; Taylor, J. D.; Meinkoth, J. H. Prevalence of *Cytauxzoon felis* Infection in Healthy Cats from Enzootic Areas in Arkansas, Missouri, and Oklahoma. *Parasites and Vectors*, 2015, *8* (13), 1–6. https://doi.org/10.1186/s13071-014-0618-z.

[51] Nietfeld, J. C.; Pollock, C. Fatal Cytauxzoonosis in a Free-Ranging Bobcat (*Lynx rufus*). *J. Wildl. Dis.*, 2002, *38* (3), 607–610. https://doi.org/10.7589/0090-3558-38.3.607.

[52] Hoover, J. P.; Walker, D. B.; Hedges, J. D. Cytauxzoonosis in Cats: Eight Cases (1985-1992). *J. Am. Vet. Med. Assoc.*, 1994.

[53] Meinkoth, J. H.; Kocan, A. A. Feline Cytauxzoonosis. *Vet. Clin. North Am. - Small Anim. Pract.*, 2005. https://doi.org/10.1016/j.cvsm.2004.08.003.

[54] Meinkoth, J.; Kocan, A. A.; Whitworth, L.; Murphy, G.; Fox, J. C.; Woods, J. P. Cats Surviving Natural Infection with *Cytauxzoon felis*: 18 Cases (1997-1998). *J. Vet. Intern. Med.*, 2000. https://doi.org/10.1111/j.1939-1676.2000.tb02270.x.

[55] Jackson, C. B.; Fisher, T. Fatal Cytauxzoonosis in a Kentucky Cat (Felis Domesticus). *Vet. Parasitol.*, 2006, *139*, 192–195. https://doi.org/10.1016/j.vetpar.2006.02.039.

[56] Haber, M. D.; Tucker, M. D.; Marr, H. S.; Levy, J. K.; Burgess, J.; Lappin, M. R.; Birkenheuer, A. J. The Detection of *Cytauxzoon felis* in Apparently Healthy Free-Roaming Cats in the USA. *Vet. Parasitol.*, 2007, *146*, 316–320. https://doi.org/10.1016/j.vetpar.2007.02.029.

[57] Brown, H. M.; Latimer, K. S.; Erikson, L. E.; Cashwell, M. E.; Britt, J. O.; Peterson, D. S. Detection of Persistent *Cytauxzoon felis* Infection by Polymerase Chain Reaction in Three Asymptomatic Domestic Cats. *J. Vet. Diagnostic Investig.*, 2008. https://doi.org/10.1177/104063870802000411.

[58] MacNeill, A. L.; Barger, A. M.; Skowronski, M. C.; Lanka, S.; Maddox, C. W. Identification of *Cytauxzoon felis* Infection in Domestic Cats from Southern Illinois. *J. Feline Med. Surg.*, 2015, *17* (12), 1069–1072. https://doi.org/10.1177/1098612X14567158.

[59] Wikander, Y.; Anantatat, T.; Kang, Q. Reif, K.E. Prevalence of *Cytauxzoon felis* Infection-Carriers in Eastern Kansas Domestic Cats. *MDPI Pathog.*, 2020, *9* (10), 854–868.

[60] Reichard, M. V.; Baum, K. A.; Cadenhead, S. C.; Snider, T. A. Temporal Occurrence and Environmental Risk Factors Associated with Cytauxzoonosis in Domestic Cats. *Vet. Parasitol.*, 2008. https://doi.org/10.1016/j.vetpar.2007.12.031.

[61] Snider, T. A.; Confer, A. W.; Payton, M. E. Pulmonary Histopathology of *Cytauxzoon felis* Infections in the Cat. *Vet. Pathol.*, 2010, *47* (4), 698–702. https://doi.org/10.1177/0300985810364527.

[62] Sykes, J. E. Feline Hemotropic Mycoplasmas. *Vet. Clin. North Am. - Small Anim. Pract.*, 2010, *40* (6), 1157–1170. https://doi.org/10.1016/j.cvsm.2010.07.003.

[63] Penzhorn, B. L.; Oosthuizen, M. C. *Babesia* Species of Domestic Cats: Molecular Characterization Has Opened Pandora's Box. *Front. Vet. Sci.*, 2020, *7* (March), 1–10. https://doi.org/10.3389/fvets.2020.00134.

[64] Birkenheuer, A. J.; Le, J. A.; Valenzisi, A. M.; Tucker, M. D.; Levy, M. G.; Breitschwerdt, E. B. *Cytauxzoon felis* Infection in Cats in the Mid-Atlantic States: 34 Cases (1998–2004). *J. Am. Vet. Med. Assoc.*, 2006. https://doi.org/10.2460/javma.228.4.568.

[65] Frontera-Acevedo, K. Feline Immune Response To Infection With *Cytauxzoon felis* and the Role of CD18 in the Pathogenesis of Cytauxzoonosis, University of Georgia, 2013.

[66] Frontera-Acevedo, K.; Sakamoto, K. Local Pulmonary Immune Responses in Domestic Cats Naturally Infected with *Cytauxzoon felis. Vet. Immunol. Immunopathol.*, 2015. https://doi.org/10.1016/j.vetimm.2014.10.012.

[67] Ridgway, M. D. Feline Cytauxzoonosis https://vetmed.illinois.edu/wp-content/uploads/2015/08/22.-Feline-Cytauxzoonosis.pdf (accessed Sep 3, 2018).

[68] Conner, B. J.; Hanel, R. M.; Brooks, M. B.; Cohn, L. A.; Birkenheuer, A. J. Coagulation Abnormalities in 5 Cats with Naturally Occurring Cytauxzoonosis. *J. Vet. Emerg. Crit. Care*, 2015, *25* (4), 538–545. https://doi.org/10.1111/vec.12326.

[69] Clarke, L. L.; Rissi, D. R. Neuropathology of Natural *Cytauxzoon felis* Infection in Domestic Cats. *Vet. Pathol.*, 2015, *52* (6), 1167–1171. https://doi.org/10.1177/0300985814564986.

[70] Clarke, L. L.; Krimer, P. M.; Rissi, D. R. Glial Changes and Evidence for Apoptosis in the Brain of Cats Infected by *Cytauxzoon felis*. *J. Comp. Pathol.*, 2017. https://doi.org/10.1016/j.jcpa.2016.11.268.

[71] Cohn, L. A.; Shaw, D.; Shoemake, C.; Birkenheuer, A. J. Second Illness Due to Subsequent *Cytauxzoon felis* Infection in a Domestic Cat. *J. Feline Med. Surg. Open Reports*, 2020, *6* (1), 1–5. https://doi.org/10.1177/2055116920908963.

[72] Lewis, K. *Cytauxzoon felisS* : An Emerging Feline Pathogen and Potential Therapy, University of Missouri, 2011.

[73] Motzel, S. L.; Wagner, J. E. Treatment of Experimentally Induced Cytauxzoonosis in Cats with Parvaquone and Buparvaquone. *Vet. Parasitol.*, 1990. https://doi.org/10.1016/0304-4017(90)90122-R.

[74] Brown, H. M.; Modaresi, S. M.; Cook, J. L.; Latimer, K. S.; Peterson, D. S. Genetic Variability of Archived *Cytauxzoon felis* Histologic Specimens from Domestic Cats in Georgia, 1995-2007. *J. Vet. Diagnostic Investig.*, 2009, *21* (4), 493–498. https://doi.org/10.1177/104063870902100410.

[75] Lewis, K. M.; Cohn, L. A.; Marr, H. S.; Birkenheuer, A. J. Diminazene Diaceturate for Treatment of Chronic *Cytauxzoon felis* Parasitemia in Naturally Infected Cats. *J. Vet. Intern. Med.*, 2012, *26* (6), 1490–1493. https://doi.org/10.1111/j.1939-1676.2012.01003.x.

[76] Lewis, K. M.; Cohn, L. A.; Marr, H. S.; Birkenheuer, A. J. Failure of Efficacy and Adverse Events Associated with Dose-Intense Diminazene Diaceturate Treatment of Chronic *Cytauxzoon felis* Infection in Five Cats. *J. Feline Med. Surg.*, 2014. https://doi.org/10.1177/1098612X13502974.

[77] Cohn, L. A.; Birkenheuer, A. J.; Brunker, J. D.; Ratcliff, E. R.; Craig, A. W. Efficacy of Atovaquone and Azithromycin or Imidocarb Dipropionate in Cats with Acute Cytauxzoonosis. *J. Vet. Intern. Med.*, 2011, *25* (1), 55–60. https://doi.org/10.1111/j.1939-1676.2010.0646.x.

[78] Vaidya, A. B.; Mather, M. W. Mitochondrial Evolution and Functions in Malaria Parasites. *Annu. Rev. Microbiol.*, 2009, *63* (1), 249–267. https://doi.org/10.1146/annurev.micro.091208.073424.

[79] Schreeg, M. E.; Marr, H. S.; Tarigo, J.; Cohn, L. A.; Levy, M. G.; Birkenheuer, A. J. Pharmacogenomics of *Cytauxzoon felis* Cytochrome b: Implications for Atovaquone and Azithromycin Therapy in Domestic Cats with Cytauxzoonosis. *J. Clin. Microbiol.*, 2013. https://doi.org/10.1128/JCM.01407-13.

[80] Schreeg, M. E.; Marr, H. S.; Tarigo, J. L.; Cohn, L. A.; Levy, M. G.; Birkenheuer, A. J. Rapid High-Resolution Melt Analysis of *Cytauxzoon felis* Cytochrome b to Aid in the Prognosis of Cytauxzoonosis. *J. Clin. Microbiol.*, 2015, *53* (8), 2517–2524. https://doi.org/10.1128/JCM.00635-15.

[81] Srivastava, I. K.; Rottenberg, H.; Vaidya, A. B. Atovaquone, a Broad Spectrum Antiparasitic Drug, Collapses Mitochondrial Membrane Potential in a Malarial Parasite. *J. Biol. Chem.*, 1997, *272* (7), 3961–3966. https://doi.org/10.1074/jbc.272.7.3961.

[82] Hikosaka, K.; Komatsuya, K.; Suzuki, S.; Kira, K. Mitochondria of Malaria Parasits as a Drug Target. In *An Overview of Tropical Diseases*; Chiba, Japan; Tokyo, Japan; Nagasaki, Japan, 2012; pp 17–38.

[83] Tarigo, J. The *Cytauxzoon felis* Genome: A Guide to Vaccine Candidate Antigen Discovery for Cytauxzoonosis, North Carolina State University, 2013.

[84] Pollard, D. A.; Reichard, M. V.; Cohn, L. A.; James, A. M.; Holman, P. J. Genetic Variability of Cloned *Cytauxzoon felis* Ribosomal RNA ITS1 and ITS2 Genomic Regions from Domestic Cats with Varied Clinical Outcomes from Five States. *Vet. Parasitol.*, 2017. https://doi.org/10.1016/j.vetpar.2017.08.002.

[85] Tarigo, J. L.; Scholl, E. H.; Bird, D. M. K.; Brown, C. C.; Cohn, L. A.; Dean, G. A.; Levy, M. G.; Doolan, D. L.; Trieu, A.; Nordone, S. K.; et al. A Novel Candidate Vaccine for Cytauxzoonosis Inferred from Comparative Apicomplexan Genomics. *PLoS One*, 2013, *8* (8), 1–9. https://doi.org/10.1371/journal.pone.0071233.

[86] Tarigo, J. The *Cytauxzoon felis* Genome: A Guide To Vaccine Candidate Antigen Discovery For Cytauxzoonosis. *ProQuest Diss. Theses*, 2013.

[87] Tarigo, J. L.; Kelly, L. S.; Brown, H. M.; Peterson, D. S. Limited Genetic Variability of *Cytauxzoon felis* Apical Membrane Antigen-1 (Ama1) from Domestic Cats and Bobcats. *Parasites and Vectors*, 2019, *12* (1), 1–7. https://doi.org/10.1186/s13071-019-3347-5.

[88] Reichard, M. V.; Thomas, J. E.; Arther, R. G.; Hostetler, J. A.; Raetzel, K. L.; Meinkoth, J. H.; Little, S. E. Efficacy of an Imidacloprid 10 % / Flumethrin 4.5 % Collar (Seresto®, Bayer) for Preventing the Transmission of *Cytauxzoon felis* to Domestic Cats by *Amblyomma americanum*. *Parasitol. Res.*, 2013, *112*, S11–S20. https://doi.org/10.1007/s00436-013-3277-7.

[89] Reichard, M. V.; Rugg, J. J.; Thomas, J. E.; Allen, K. E.; Barrett, A. W.; Murray, J. K.; Herrin, B. H.; Beam, R. A.; King, V. L.; Vatta, A. F. Efficacy of a Topical Formulation of Selamectin plus Sarolaner against Induced Infestations of *Amblyomma americanum* on Cats and Prevention of *Cytauxzoon felis* Transmission. *Vet. Parasitol.*, 2019, *270*, S31–S37. https://doi.org/10.1016/j.vetpar.2018.10.018.

Chapter 2 - **Acute *Cytauxzoon felis* cases in domestic cats from eastern Kansas, a retrospective study (2006-2019).**

Introduction.

Cytauxzoonosis is an often-fatal disease of domestic cats caused by *Cytauxzoon felis*, a tick-borne hemoprotozoal pathogen of felids. In the United States (U.S.), cytauxzoonosis cases most frequently occur in southeastern and south-central regions. This protozoal organism has a complex lifecycle that includes asexual reproduction in felid hosts and both asexual and sexual reproduction in competent ixodid tick vectors [1] (Figure 2.1). Briefly, *C. felis* sporozoites are transferred via tick saliva into a felid host during a blood meal. Once within the felid host, sporozoites enter monocytes and begin replicating asexually (schizogony) forming many 1-2 μm diameter signet ring merozoites. When the monocyte ruptures, these merozoites are released into the blood where they enter host erythrocytes and begin either replicating asexually (merogony) or develop into gametocytes. Ticks become infected when they feed on an infected felid host and ingest gametocytes. The ingested gametocytes undergo sexual reproduction to form zygotes in the lumen of the tick gut. Zygotes invade the tick gut epithelium, transform into kinetes, and migrate to the salivary glands where they transform into sporozoites. Transstadial maintenance of the parasite through the larvae-to-nymph or nymph-to-adult ecdysis is required for transmission of the infectious sporozoites to a new felid host during the subsequent tick bloodmeal. The competent biological transmission vectors of *C. felis* in the U.S. include *Dermacentor variabilis* (American dog tick) and *Amblyomma americanum* (Lone star tick) [2–5]. The range of *D. variabilis* encompasses the eastern U.S. as well as focal regions in the west [6, 7]. The continuously expanding range of *A. americanum* covers the southeastern and mid-central U.S., largely overlapping with the range of *D. variabilis* [8, 9]. Larval and nymphal *D. variabilis* prefer to feed

on small to medium-sized mammals whereas adults feed on larger mammals, including cats. In contrast, *A. americanum* are less discriminating, such that all life stages will seek out and feed on many mammals, including cats. Because of intensifying populations and its aggressive nature and willingness to feed on cats at all life stages, it is likely that *A. americanum* is a more significant vector of *C. felis* compared to *D. variabilis* in areas where both tick species reside.

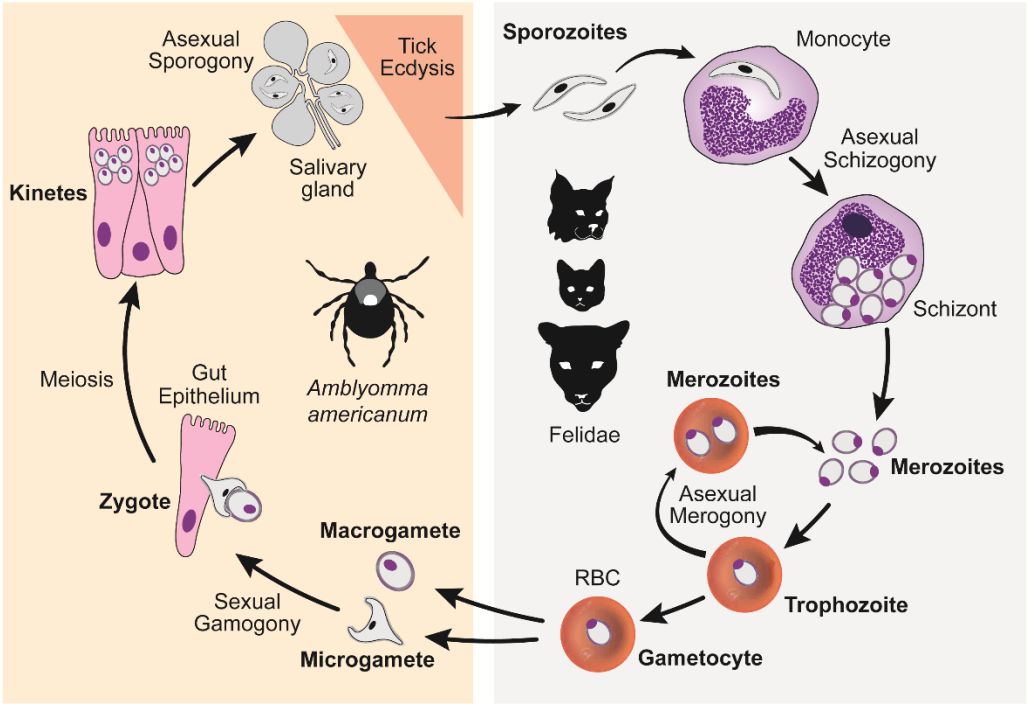


Figure 2.1: *Cytauxzoon felis* lifecycle. Right panel: *C. felis* reproduction within a felid host, asexual schizogony and merogony. Left panel: *C. felis* reproduction within a tick vector, sexual gamogony and asexual sporogony. (Wikander et al. Prevalence of *Cytauxzoon felis* infection carriers in the domestic cat population of eastern Kansas. *Pathogens* 2020, *9*(10), 854; https://doi.org/10.3390/pathogens9100854.) (Reproduced with permission from Yvonne Wikander, Pathogens; published by MDPI, 2020.)

Clinical cytauxzoonosis cases are most commonly observed in domestic cats (*Felis catus*), which serve as intermediate host carriers for *C. felis* along with other members of the Felidae family. Domestic cat cytauxzoonosis cases typically peak in late spring and early fall when *A. americanum* adult and nymph life stages are most active [10]. For many decades, domestic cats

were considered a dead-end host as those with observed clinical disease commonly died [11–13]. Clinical disease signs for acute cytauxzoonosis appear during the schizogenous phase of *C. felis* asexual replication and can cause severe illness and death [2, 11]. Cats commonly present with anorexia, depression, lethargy, pyrexia, dehydration, pale mucous membranes, and splenomegaly 11-13 days post infection (dpi) [2, 14, 15]. As the disease progresses, the pyrexia resolves and drops to subnormal, with icterus and dyspnea developing shortly thereafter. Complete blood count and serum biochemistry changes are seen around 13 dpi and can include variable cytopenias, hypoalbuminemia, hyperglycemia, and increased ALT (alanine aminotransferase) and total bilirubin [2, 13, 15]. Intra-erythrocytic signet rings (aka: piroplasms) and/or schizont-laden monocytes are often observed at the feathered edge of thin blood smear preparations [2]. The organs most affected include the lung, liver, spleen, and lymph nodes [12, 13, 15–17]. Death occurs around 21 dpi due to inflammatory cytokine-mediated injury of multiple organs, interstitial pneumonia, hypoxic injury, and disseminated intravascular coagulation [2, 11, 16–20]. If initiated early in the disease process, treatment for ten days with a combination of atovaquone (15 mg/kg) and azithromycin (10 mg/kg) may result in the recovery of up to 60% of patients [21]. For survivors, clinical signs begin to resolve around 23-24 dpi after which they become subclinical infected carriers that act as reservoirs for further *C. felis* transmission via competent tick vectors [2, 22]. Domestic cat carriers of *C. felis* may be more common than previously thought as we recently identified *C. felis* carrier infection in 25% of cats in eastern Kansas [23]. Similar clinical signs and disease progression have been reported with *Cytauxzoon* spp. in South America, Europe, and Asia [24–27].

Only a few studies have examined the incidence and risk factors associated with acute cytauxzoonosis cases in U.S. domestic cats; however, the conclusions of these studies vary. For

example, investigation into cytauxzoonosis incidence trends varied from stable [18] to increasing [28], while evaluation of specific risk factors varied from identifying a predisposition of disease in young male cats [15] to tick climate and habitat conditions being more predictive of the disease rather than cat age and gender [29]. Anecdotal reports of increasing acute cytauxzoonosis cases, as well as heightened public awareness of this disease makes understanding cytauxzoonosis and associated patient risk factors important for mitigating disease transmission among high-risk cat populations. To address these concerns in eastern Kansas, the objectives of this study were to: i) determine if acute cytauxzoonosis cases in eastern Kansas increased between 2006-2019; and, ii) examine if specific feline risk factors are correlated with acute disease or disease outcomes, by performing a retrospective records search and review of acute cytauxzoonosis cases submitted to the Kansas State Veterinary Diagnostic Laboratory (KSVDL). Based on the high density of *A. americanum* ticks in eastern Kansas and feline risk factors reported as important by cytauxzoonosis studies in other states, we hypothesized that cases of acute cytauxzoonosis would: i) show an increasing trend over time; ii) peak in seasons of peak *A. americanum* activity; and, iii) be reported more commonly in young, intact, male cats. Updated information on acute cytauxzoonosis incidence and risk factors will help identify and focus *C. felis* education and intervention efforts for high-risk domestic cat populations.

Materials & Methods

Study design.

A retrospective study with individual cats as the study subject was designed using case records from the Kansas State Veterinary Diagnostic Laboratory (KSVDL) in Manhattan, Kansas. The KSVDL case database was queried for the term "*Cytauxzoon felis*" among records between May 2006 through October 2019 using the laboratory management software VetView v1.6.12

(University of Georgia, Athens, GA, USA). All search-populated records were individually reviewed to confirm cases met study inclusion criteria – i) domestic cats from eastern Kansas, and ii) a confirmed diagnosis of cytauxzoonosis. Cases that came from within the Kansas State University teaching hospital, as well as, outside submissions of blood and/or tissue samples were included in the study. Exclusion criteria included: i) any cat not from eastern Kansas; or, ii) cases where cytauxzoonosis diagnosis was not confirmed. Data collected from each feline patient record included: specimen receipt date (year, month, season); geographic information (county, state); diagnostic method (necropsy vs blood smear); blood smear diagnosis (schizont vs signet rings); signet ring frequency (occasional vs frequent); patient age (years); patient sex (male vs female) and sterilization status (neutered vs intact); feline lifestyle (stray, owned, or rescue/rescinded); patient live/dead status within 30 days of initial case record; and, mention of similar household cat deaths in the medical record (yes vs no). Historical data of 123 control cases from Raghavan et al. [29] which consisted of "cats with a history of fever, malaise, icterus, and anorexia but not *C. felis* on blood film examination or schizonts within macrophages from fresh tissue or within multiple organs" were included as controls for this study [29].

Statistical analysis.

Yearly feline cytauxzoonosis incidence was analyzed under the Poisson log linear model with year block (2005~2009, 2010~2014, 2015~2019) being the fixed effect. Tukey's multiplicity adjustment was applied when comparing incidence rates among year blocks. For assessing risk factors including age (<1 year, 1-3 year, 3-5 year, $\geq$5 years) and season, (Winter (Dec, Jan, Feb), Spring (March, April, May), Summer (June, July, August), Fall (September, October, November)) the present study data of 170 cases was combined with the previously published 123 non-cytauxzoonosis control cases [29]. The resulting case-control data gave rise to two-way

contingency tables where the Pearson chi-square test for difference in risk factor distributions was applied. For assessing sex (male, female) and lifestyle (feral, owned, rescue/rescinded) risk factors; diagnostic method used (blood smear, necropsy); *C. felis* life stages observed (signet rings, schizonts); and, the relative amount of signet rings observed (frequent vs. occasional), one-way tables were created from the study data. A chi-square goodness-of-fit test was then performed to compare the observed percentages against the hypothesized distributions. When the cell count(s) in a contingency table were less than five, *P* value was computed using the exact method instead of asymptotic approximation. The confidence interval for mortality rate was estimated using the Clopper-Pearson (exact) method. The confidence interval for percentage of owners of cats diagnosed with cytauxzoonosis reporting similar loss of other cats was estimated using the Wald method. Missing data was excluded from the statistical analyses. Statistical analyses were performed using the GENMOD and FREQ procedures of SAS/STAT® software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

Results.

Annual acute cytauxzoonosis incidence in domestic cats from eastern Kansas.

A total of 183 domestic cat records with a diagnosis of acute cytauxzoonosis from May 2006 through October 2019 were identified. Thirteen records were excluded from the study because they had no identifiable state of origin (n=3) or they came from adjacent states (n=10), leaving records from 170 domestic cats diagnosed with cytauxzoonosis from eastern Kansas available for analysis. These 170 records were evaluated to determine if the incidence of acute cytauxzoonosis cases was changing over time. Of those cases with a reported live-dead status (n=140) only one cat survived. The greatest number of acute cytauxzoonosis cases were reported in 2006, 2009, 2012, 2017, 2018, and 2019 (Figure 2.2, Table A2.1). Because the number of cases varied from

year-to-year, testing results were evaluated by year blocks: 2005-2009, 2010-2014, and 2015-2019. No significant difference was detected between any of the year blocks (P=0.754) (Table 2.1, Table A2.2). Although acute cytauxzoonosis inter-annual variation was observed over time, collectively the incidence was stable over a broader time scale.

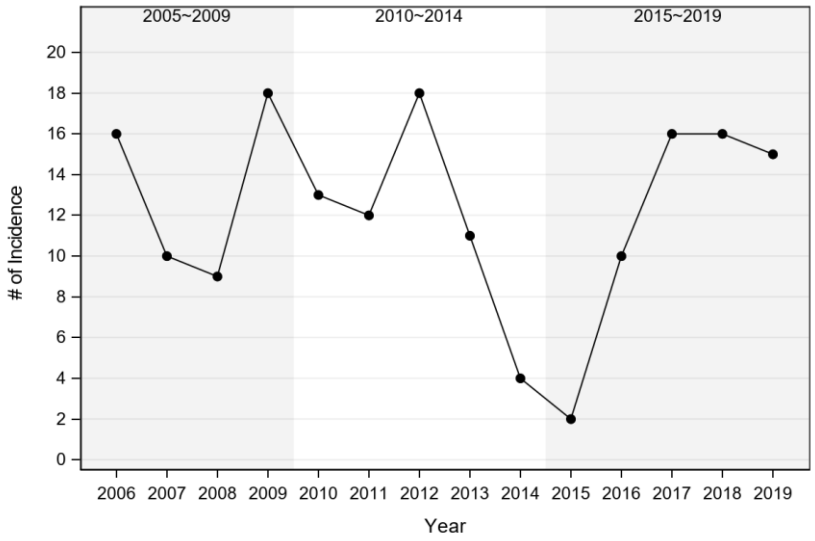


Figure 2.2: Incidence of acute cytauxzoonosis cases by year. Numbers represent raw acute cytauxzoonosis case counts.

Table 2.1: Statistical analysis of year block effect on acute cytauxzoonosis incidence.

	# of Incidence per Year		**Ratio to (Adj. *P* value of Testing for Ratio≠1)**	
Year Block	**Mean**	**SE[1]**	2010-2014	2015-2019
2005~2009	13.3	1.8	1.14 (0.764)	1.12 (0.813)
2010~2014	11.6	1.5		0.98 (0.995)
2015~2019	11.8	1.5		

[1] Standard Error.

County-level location of identified acute cytauxzoonosis cases in Kansas are presented in Figure 2.3. Acute cytauxzoonosis case samples were submitted to KSVDL from cats living in 31 eastern Kansas counties, with >50% of the samples coming from the county in which the KSVDL resides (Riley) and those immediately adjacent (Pottawatomie, Wabaunsee, Geary, Clay, Marshall). Statistical analysis of location data for acute cytauxzoonosis cases in specific counties was not possible because the sampling method across counties was not standardized.

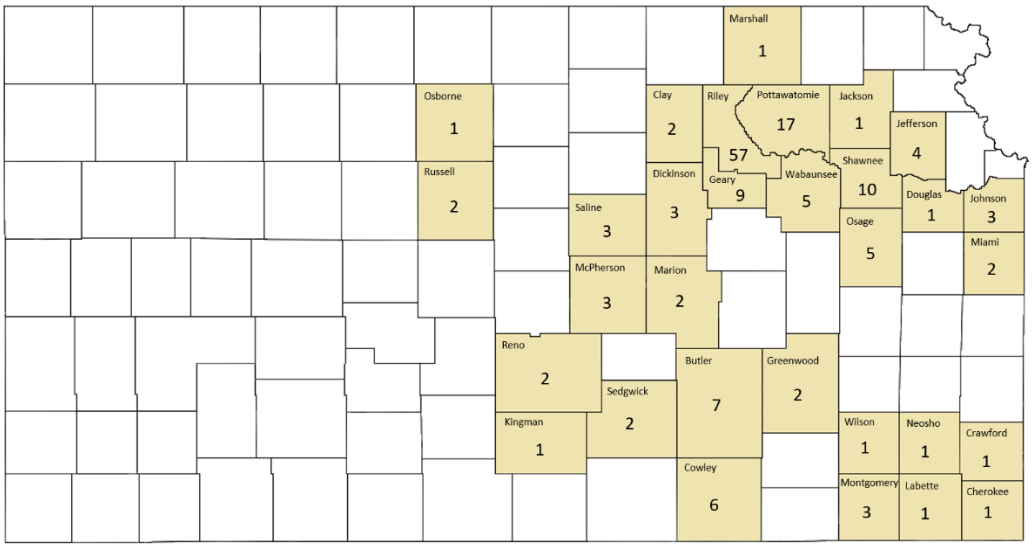


Figure 2.3: County-level location of acute cytauxzoonosis diagnosed domestic cat records in Kansas. Acute cytauxzoonosis case samples were submitted from cat(s) in shaded Kansas counties while no cat records were identified from unshaded counties. Numbers represent raw acute cytauxzoonosis case counts.

Season variation of acute cytauxzoonosis incidence in domestic cats from eastern Kansas.

To examine the intra-annual acute cytauxzoonosis case incidence, the incidence of acute cytauxzoonosis cases diagnosed in different months and seasons were evaluated (Figure 2.4). A bimodal distribution of cases was observed, with the greatest case peak between May (n=40) to June (n=51) and a second smaller case peak in September (n=21). Similarly, the greatest

proportion of acute cases were observed in summer (54.1%) then spring (30.6%) and fall (15.3%). The seasonal distribution of acute cytauxzoonosis cases was significantly different from that of ill (*C. felis*-unrelated) control case incidence ($P<0.001$) (Table 2.2, Figure A2.1). The seasons that contributed most to the significance (i.e. large Pearson chi-square statistic) are fall with 15.3% for the acute cytauxzoonosis cases vs. 38.2% for the ill control cases as well as winter with 0% for the acute cytauxzoonosis cases vs. 7.3% for the ill control cases.

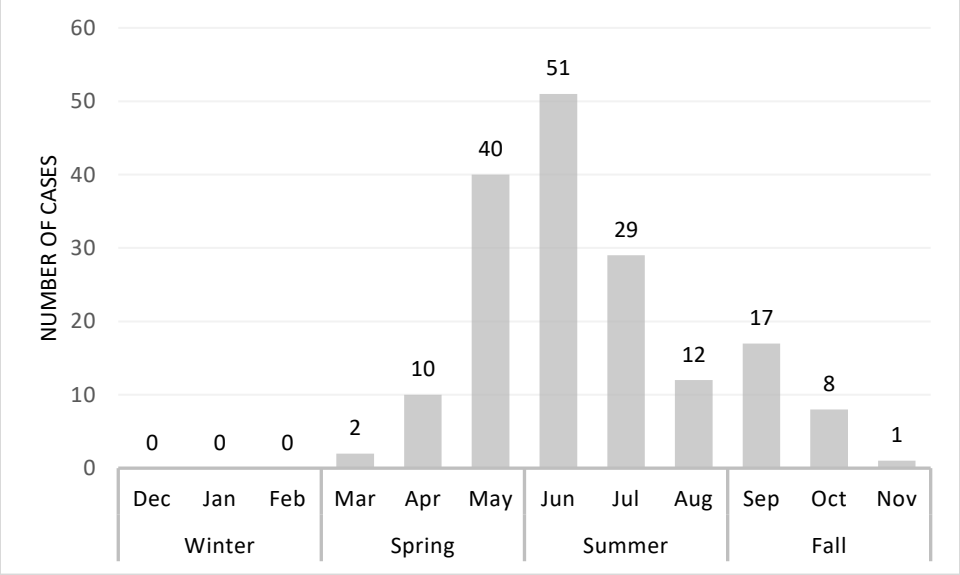


Figure 2.4: Acute cytauxzoonosis cases by month from May 2006 to Oct 2019. Case numbers represent the sum of total number of cases for each month from all years. These numbers represent raw acute cytauxzoonosis case counts.

Table 2.2: Statistical analysis of season effect on acute cytauxzoonosis and control case incidence.

		Case		Control		
Effect	**Season**	**Count**	**Percent**	**Count**	**Percent**	***P* value of Exact Chi-Square Test**
Season	Spring	52	30.6%	21	17.1%	<0.001
	Summer	92	54.1%	46	37.4%	
	Fall	26	15.3%	47	38.2%	
	Winter	0	0.0%	9	7.3%	

Evaluation of acute cytauxzoonosis incidence among cats with different ages, sex, and lifestyle.

To examine if a correlation exists between specific cat risk factors with acute cytauxzoonosis case incidence, cat age, sex, and lifestyle were investigated. The greatest number of acute cytauxzoonosis cases was observed in cats 1-3 years old (40.4%) with fewer cases observed in ≥5 years old (25.6%), 3-5 years old (21.8%), and <1 year old (12.2%) age groups (Table 2.3, Figure A2.2). The mean age of cats diagnosed with acute cytauxzoonosis was 3.4 years (range 1.4 months to 13 years) (Figure 2.5). The age distribution of acute cytauxzoonosis cases was significantly different from that of ill control case incidence (P<0.001). The age group that contributed most to the significance (i.e. large Pearson chi-square statistic) are <1 year old with 12.2% for the acute cytauxzoonosis cases vs. 47.0% for the ill control cases.

Table 2.3: Statistical analysis of age effect on acute cytauxzoonosis and control case incidence.

		Case		Control		
Effect	**Age Group[1]**	**Count**	**Percent**	**Count**	**Percent**	***P* value of Chi-Square Test**
Age	<1	19	12.2%	47	47.0%	<0.001
	1-3	63	40.4%	24	24.0%	
	3-5	34	21.8%	18	18.0%	
	≥5	40	25.6%	11	11.0%	

[1] Years

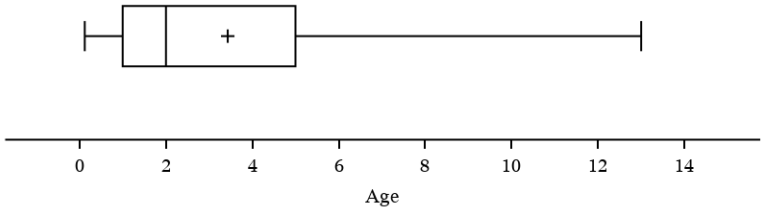


Figure 2.5: Acute cytauxzoonosis cases by age (years). The mean age of cats diagnosed with acute cytauxzoonosis was 3.4 years (range 1.4 months to 13 years).

A diagnosis of acute cytauxzoonosis was observed significantly more in male (70.4%) compared to female (29.6%) cats (P<0.001) (Table 2.4, Figure A2.3). Statistical analysis of sterilization status data was not possible as the sterilization status was not reported in the control group data [29] and data regarding sterilization status among the general Kansas cat population is unknown or unreported.

Table 2.4: Statistical analysis of sex effect on acute cytauxzoonosis case incidence.

		Case			
Effect		**Count**	**Percent**	**Percent tested against**	***P* value of Chi-Square Test**
Sex	F	48	29.6%	50.0%	<0.001
	M	114	70.4%	50.0%	

Acute cytauxzoonosis cases were predominantly owned cats (96.5%) with fewer feral and rescue/rescinded cats (2.4% and 1.2% respectively) (Table 2.5). The distribution of lifestyle for acute cytauxzoonosis was significantly different (P<0.001) to that for the general cat population of Kansas with 49.9% feral, 47.1% owned and 3.0% rescue cats [30–33]. Collectively, acute cytauxzoonosis was most commonly observed in ≥1 year old, male, owned cats.

Table 2.5: Statistical analysis of lifestyle effect on acute cytauxzoonosis case incidence.

		Case			
Effect		**Count**	**Percent**	**Percent tested against**	***P* value of Exact Chi-Square Test**
Lifestyle	feral	4	2.4%	3.0%	<0.001
	owned	164	96.5%	47.1%	
	rescue	2	1.2%	49.9%	

Evaluation of method used to diagnose acute cytauxzoonosis in domestic cats from eastern Kansas.

Lastly, the method used to diagnose acute cytauxzoonosis was evaluated (Table 2.6). Means of diagnosis did not vary significantly among acute cytauxzoonosis cases (*P*=0.092); however, a small majority of cases were diagnosed via blood smear (56.5%) versus via necropsy results (43.5%). Necropsy diagnosis was by visual identification of *C. felis* schizont(s) within vessels of any histological tissue sample. The tissues most commonly affected included lung, liver, spleen, and lymph node. Blood smear diagnosis was made by visually identifying either the schizont (49%) or intra-erythrocyte signet ring life stages (51%) (aka: piroplasms) (Table 2.6). The blood smear diagnosis via schizont vs signet rings did not vary significantly among acute cytauxzoonosis cases (*P*=0.838). For those diagnosed through the identification of signet rings, the quantity of signet rings observed was generally classified as frequent (61.2%) or occasional (38.8%), which did not differ significantly from the 1:1 ratio (*P*=0.116) (Table 2.6). Based on cases with a known disease outcome (1 survivor and 139 deaths), the survival rate in acute cytauxzoonosis was 0.7% with 95% confidence interval of (0.02%,3.92%). The sole survivor was diagnosed by schizont identification using the blood smear method.

Table 2.6: Statistical analysis of diagnostic method, blood smear diagnosis, and relative number of signet ring form effects on acute cytauxzoonosis case mortality.

		Case			
Effect	**Method**	**Count**	**Percent**	**Percent tested against**	***P* value of Chi-Square test**
Method of Diagnosis	blood smear	96	56.5%	50%	0.092
	necropsy	74	43.5%	50%	
Blood smear rings vs schizonts	rings	49	51%	50%	0.838
	schizonts	47	49%	50%	
Relative number of rings observed	frequent	30	61.2%	50%	0.116
	occasional	19	38.8%	50%	

Discussion

In *C. felis* endemic regions, acute cytauxzoonosis is one of the most fatal feline diseases. Several retrospective studies have demonstrated a bimodal pattern of seasonal cytauxzoonosis cases that correlates with *A. americanum* activity; young male cats being over-represented [8, 10, 15, 29, 34, 35]. As a disease with a high fatality rate, increased public concern, and practitioner belief of increased incidence, understanding factors contributing to acute cytauxzoonosis can help focus efforts on preventing *C. felis* transmission among high-risk cat populations. In the present retrospective study, we demonstrate: (1) acute cytauxzoonosis case incidence in domestic cats from eastern Kansas has an overall stable trend; (2) acute cytauxzoonosis cases are more common in the spring and summer seasons; (3) young male cats are more likely to be diagnosed with acute cytauxzoonosis; and, (4) schizont and/or intra-erythrocytic signet ring identification on histology or blood smear samples are reliable identifiers of acute cytauxzoonosis when associated with typical cytauxzoonosis clinical signs.

This study did not support anecdotal reports of an increasing acute cytauxzoonosis case trend in eastern Kansas cats based on KSVDL case sample submissions over the study period (Figure 2.2). Rather, the anecdotal reports of increasing acute cytauxzoonosis cases may be more attributed to individual year-to-year case fluctuations instead of a general increasing trend over the evaluated 14-year period (2006-2019). This finding agrees with a study that determined there was no significant year-to-year difference in 232 acute cytauxzoonosis cases identified over a 12-year period (1995-2006) in Oklahoma [10]. In contrast, another study determined there was an overall increase in acute cytauxzoonosis cases over a 10-year period (2001-2011) in western Kentucky [34]. However, the latter study consisted of only 56 cases and no statistical analysis method was reported. It is important to note that all three studies, including ours, were based on retrospectively evaluated data with limited patient details and histories. The discrepancy between anecdotal reports of increasing case numbers and data presented here may be due to a variety of reasons. First, more clinicians may be diagnosing cytauxzoonosis with in-hospital microscopic blood smear evaluation versus sending out samples to a diagnostic laboratory, like KSVDL. Alternatively, an increased awareness of the disease may make the number of cases appear more prominent in the eyes of the clinicians, diagnosticians, and/or owners, while not actually being increased. Third, vector tick populations – especially *A. americanum* - may be increasing or expanding their ranges with changing environmental conditions, increasing clinician index of suspicion resulting in more suspected *C. felis* infection diagnoses without definitive diagnosis. Lastly, there may be an increasing incidence of acutely infected cats surviving infection without veterinary care, while the incidence of those presented to veterinary hospitals may be unchanged. Interestingly with regard to this last point, we recently conducted a study investigating the prevalence of *C. felis* carrier (reservoir) cats in eastern Kansas and found that 25.8% of sampled domestic cats were actively

infected with *C. felis* [23]. As such, more cats may survive the schizogenous phase of cytauxzoonosis than previously expected, thus contributing to an expanding domestic cat reservoir population. The extent of *C. felis* genetic diversity and how these differences may affect virulence, transmissibility, and potential for treatment success is largely unexplored, with investigations hampered by lack of methods to preserve or manipulate *C. felis* isolates outside of living cat or tick hosts. Attempts at identifying genetic markers of virulence have yet to find definitive virulence determinants [21, 24, 36–38].

A seasonal pattern of acute cytauxzoonosis incidence in domestic cats from eastern Kansas demonstrated a bimodal distribution, similar to those reported in other studies, peaking in early summer (June) with a smaller peak in early fall (September) [10, 15, 29, 34]. This pattern corresponds with *A. americanum* peak activity in this area, which is predominantly dependent on environmental conditions like diurnal temperature range, precipitation, and humidity [8, 29]. Adult ticks are most active in early spring to mid-summer, nymphs in late spring to mid-summer as well as late summer to early fall, and larvae in late summer to early fall [8]. This would suggest that *C. felis* may have been transmitted to the cats in this study predominantly by adults/nymphs in early summer with fewer transmissions occurring in early fall by nymphs. Previous tick transmission studies have demonstrated that both adult and nymphal *A. americanum* can successfully transmit *C. felis* [2, 10]. However, since tick findings or tick preventative use practices were not identified in case records, further evaluation regarding the tick vector was not possible in this study. In addition, late spring through early fall may be times when cats spend more time outdoors actively roaming, resulting in a greater chance of encountering tick vectors. The combined seasonal cat and tick activity could explain why cats in our study area with evidence of fever, malaise, icterus, and anorexia were more likely to be diagnosed with acute cytauxzoonosis in spring/summer than other

diseases with a similar presentation. A larger, long-range, prospective study evaluating both predisposing environmental conditions and habitat niches would provide insight into the timing of increased tick activity which could be used to anticipate or forecast times of increased risk for *C. felis* transmission, as well as other tick-borne diseases.

As expected, young cats in their first and second year of life were more likely to have samples submitted and diagnosed with acute cytauxzoonosis. Our findings that young cats had a predisposition for acute cytauxzoonosis diagnosis supports previous data regarding age distribution for cats diagnosed with acute cytauxzoonosis [15] and tick infestation [35]. The first study, conducted in the mid-Atlantic states, found cats diagnosed with acute cytauxzoonosis (n=34) had a mean age of 4 years (range 2 months to 14 years) [15]. Interestingly, another study investigating tick infestation of cats found cats with tick infestations were a mean age of 4.4 years (range 18 days to 18 years) (n=336), with ticks recovered more frequently on young cats [35]. Combined, both studies support our study results of most commonly observing acute cytauxzoonosis cases in younger cats. In our study, a greater proportion of sample submissions came from young cats, for which there are several possible explanations. First, young cats may explore their environment more aggressively and/or more frequently than older cats resulting in potentially greater exposure to *C. felis* infected ticks. Second, owners may be more likely to present samples from younger cats with illness or an unexpected death, thinking an older cat died of old age. Third, older cats may lack the energy or drive to return home when feeling ill and expire where their body is less likely to be retrieved. Lastly, older cats maybe be more likely to have encountered and survived a previous *C. felis* challenge, resulting in an asymptomatic reservoir stage. It is reasonable to assume that most cats that contract cytauxzoonosis do so through their outdoor activities because that is where the tick vector is most commonly located.

Among cats diagnosed with acute cytauxzoonosis in our study, male cats were over-represented (Table 2.4) supporting a previous study that also found male cats to be over-represented (20/31; 64.5% male cats in other study) [15]. Interestingly, male cats were also previously found to be more likely to have tick infestations than female cats (59% and 41% respectively) [35]. At one time it was believed that male cats had territories up to ten times larger than female cats, which could explain these findings [39]. However, more recent studies determined there is no significant difference in home range sizes between male and female cats [40–42]. As such, it is unclear why male cats had a higher incidence of disease compared to female cats in our study and others. A larger sample size as well as more complete patient background information would have been helpful in more thoroughly evaluating acute cytauxzoonosis risk factors; however, we were limited by the information provided in the case records. Overall, our findings regarding acute cytauxzoonosis seasonality and feline age/sex predilections in Kansas provide additional support to age and sex being important risk factors.

Not surprisingly, most of the sample submissions in this study came from owned cats (96.5%) with rare rescue/rescinded or feral cat samples. Since owned cats generally benefit from a stronger human-animal bond, more diagnostics are performed either to understand the cause of a pet's demise or to ascertain if a zoonotic or infectious potential exists for the owner and/or other household pets. Additionally, 26.5% (45/170) of the patient records evaluated included mention of multiple cats in the household dying of acute cytauxzoonosis or a cat that died in a similar but undiagnosed manner. These pet owners may be more sensitive to the potential for this disease in their pets resulting in increased diagnostic efforts. Feral cats, barn cats, and/or outdoor 'pets' which are fed but lack a strong human-animal bond with the owners are less likely to be submitted for diagnoses due to cost and/or difficulty in obtaining samples. This population of 'wild' cats may

also stray further afield, dying far from the owned property, or the property owners may be less attentive to these cats' cause of death or disappearance. Rescue and humane society organizations have limited medical care budgets and are more likely to perform on-site diagnostics for treatment decisions, and less likely to submit tissue samples to an outside diagnostic laboratory after death.

Rapid, patient-side diagnostics are not currently available for cytauxzoonosis diagnosis. Instead, diagnosis of acute cytauxzoonosis is most commonly accomplished by review of blood smears by a clinical pathologist or review of tissue histology after necropsy. A diagnosis of acute cytauxzoonosis via identification of *C. felis* schizonts or intra-erythrocytic signet rings in blood smears, or schizonts in histology tissue samples was equally diagnostic (Figure 2.6). Additionally, the density of intra-erythrocytic signet ring forms seen in blood smears did not determine the severity of this disease as nearly all cats in this cohort died or were euthanized (99.3%) due to their illness. Different methods and criteria have been used in studies to diagnose acute cytauxzoonosis, including the identification of: i) schizonts only [29]; ii) schizonts or intra-erythrocytic signet rings [34]; or, iii) either schizonts or intra-erythrocytic signet rings with expected clinical signs [10]. Schizonts, when identified, are specific for acute cytauxzoonosis because this is the first life stage that develops in the feline host after the tick inoculates the sporozoites (Figure 2.1). In this study, observation of schizonts was noted in all case records where histologic tissue samples were evaluated (Figure 2.6C). That said, tissue sampling for histologic evaluation is far more costly and invasive than obtaining a blood sample, and requires a sedated, anesthetized, or deceased patient. Additionally, it does not demonstrate/confer a diagnostic advantage over a blood smear sample. Indeed, schizonts are not always identified on blood smear evaluation (Figure 2.6B). Approximately half of our blood smear samples lacked schizont identification. Piroplasms, 1-2 μm diameter intra-erythrocyte signet rings, are found in both the schizogenous and erythrocytic phase

of cytauxzoonosis and are too small to be reliably seen on histology (Figure 2.6A). Blood smear cases lacking schizonts in this study had either multiple intra-erythrocytic signet rings per erythrocyte (active merogony), a high density of intra-erythrocytic signet rings, or occasional intra-erythrocytic signet rings in patients exhibiting clinical signs consistent with acute cytauxzoonosis. These presenting cats were unlikely to be merely a *C. felis* reservoir, which can also have low parasite loads (generally 0.045-1.27% infected erythrocytes), because they also exhibited clinical signs of cytauxzoonosis [22]. In subacute and early acute infections, intra-erythrocytic signet rings and schizonts may not be immediately apparent on blood smears. Blood or tissue samples submissions to a diagnostic laboratory for pathologist evaluation takes time that these patients may not have. Ideally, a rapid chairside test for in-house veterinary use would be developed to identify these cases very early in the disease process, allowing for early treatment initiation that may prove lifesaving for many of these cats.

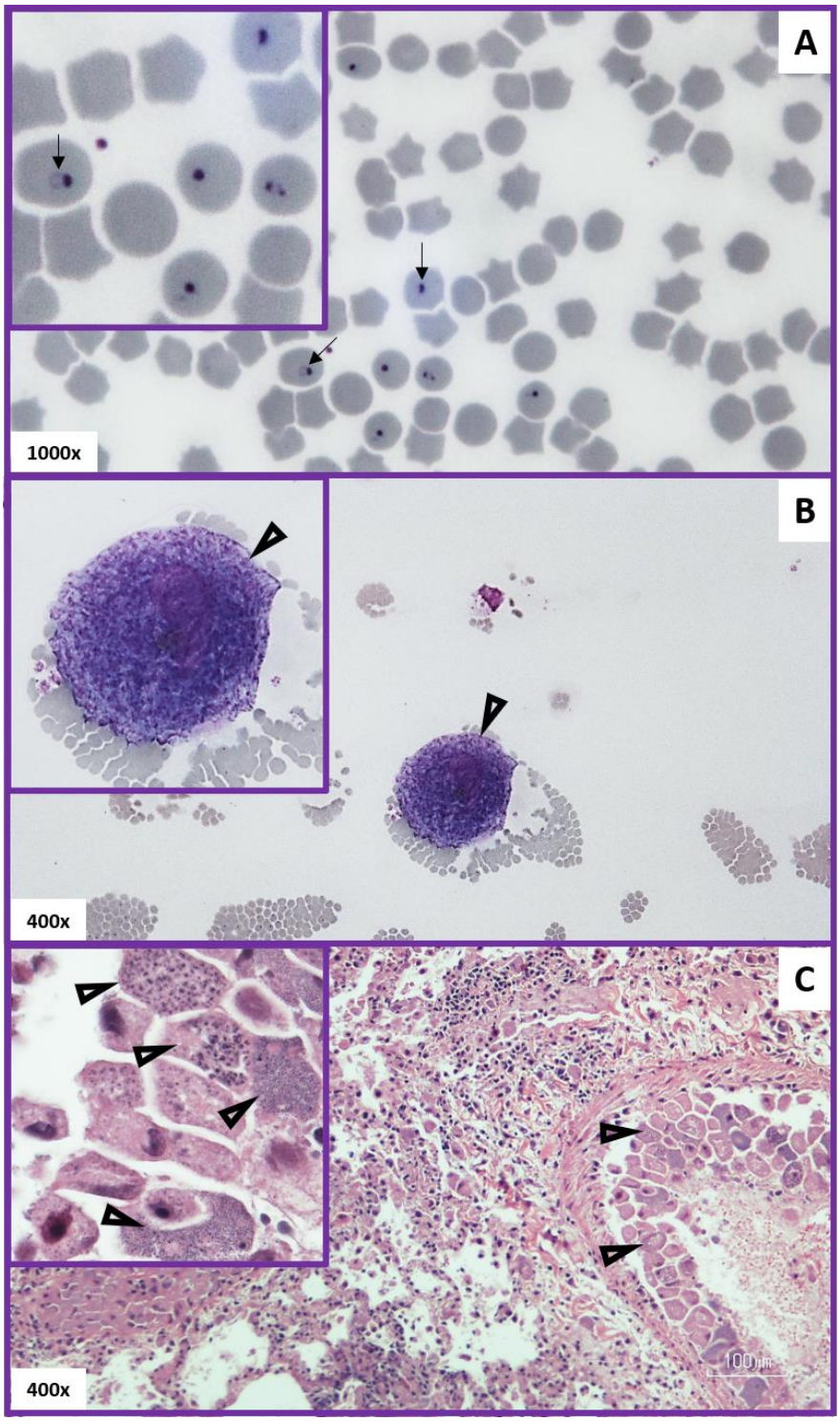


Figure 2.6: Diagnostic pathology of acute cytauxzoonosis. Panel A (top): Blood smear with intra-erythrocytic signet rings (arrows) (Modified Wright stain). Panel B (middle): Blood smear with a schizogenous monocyte (schizont) at the feathered edge (open arrowheads) (Modified Wright stain). Panel C (bottom): Histologic lung tissue with schizonts attached to endothelium (open arrowheads) within a vessel. Note the variable schizont developmental stages seen in the Panel C inset (Hematoxylin and Eosin stain).

The differential diagnosis for the nonspecific clinical signs seen in cytauxzoonosis (lethargy, fever, icterus, dyspnea, +/- anemia) could include cholangiohepatitis, triaditis, pancreatitis, hepatic lipidosis, sepsis, immune-mediated hemolytic anemia, toxins causing oxidative damage (e.g. *Allium* spp,, acetaminophen), neoplasia, and infectious agents (tularemia, feline infectious peritonitis, hemotropic mycoplasma) to name a few [43, 44]. When intra-erythrocytic hemoparasites with a signet ring or piroplasm appearance are seen in cats, there are three main differential diagnoses: i) *Cytauxzoon felis*, ii) *Mycoplasma hemofelis*, and iii) *Babesia felis*. *C. felis*, discussed herein, typically presents with a variable non-regenerative anemia, intra-erythrocytic signet rings, rare to occasional signet rings in the background, and/or schizonts at the feathered edge of blood smears or on histology of tissue samples. *M. hemofelis* commonly presents with a strong regenerative anemia, and signet ring and/or coccoid forms of epierythrocytic organism located on the red cells and/or in the background [45]. *B. felis* presents with a regenerative anemia and intra-erythrocytic signet rings often arranged in tetrads [46]. Considering our study area is within the U.S., *B. felis* is unlikely to be the cause of infection in these cats as *B. felis* has not been reported in the U.S. However, it should be considered for any feline patient with a travel history to Africa, particularly the southern coastal regions [46]. While *M. hemofelis* and *Babesia* spp. PCR is readily available for differentiation, *C. felis* PCR is less available. That said, identification of either schizonts and/or intra-erythrocytic signet rings via histology or blood smear samples are equally diagnostic for acute cytauxzoonosis when associated with typical acute cytauxzoonosis clinical signs for cats living in or near endemic areas. In locations where these feline piroplasm diseases overlap, DNA sequencing may be necessary to resolve piroplasm species identity.

Conclusion

In conclusion, we determined the incidence of acute cytauxzoonosis in domestic cats of eastern Kansas has remained stable over the last 14 years, commonly occurs in young male cats, and correlates with the expected *A. americanum* activity and life cycle. Clinicians practicing in or near endemic areas should consider acute cytauxzoonosis as a differential for any cat exhibiting depression, lethargy, fever, anorexia, icterus, anemia, cytopenias, or sudden death with confirmation via histologic or blood smear identification of schizonts or intra-erythrocytic signet rings and/or PCR. Since no vaccine exists and effective treatment options are limited, *C. felis* tick transmission should be mitigated by use of aggressive, year-around, acaricide products for all domestic cats living in endemic areas, regardless of age, sex, or lifestyle. More studies are needed to further elucidate factors affecting cytauxzoonosis disease progression and presentation, and treatment options and outcomes within the U.S. and globally.

References

[1] Jalovecka, M.; Hajdusek, O.; Sojka, D.; Kopacek, P.; Malandrin, L. The Complexity of Piroplasms Life Cycles. *Front. Cell. Infect. Microbiol.*, 2018, *8* (July), 1–12. https://doi.org/10.3389/fcimb.2018.00248.

[2] Reichard, M. V.; Meinkoth, J. H.; Edwards, A. C.; Snider, T. A.; Kocan, K. M.; Blouin, E. F.; Little, S. E. Transmission of *Cytauxzoon felis* to a Domestic Cat by *Amblyomma americanum*. *Vet. Parasitol.*, 2009, *161* (1–2), 110–115. https://doi.org/10.1016/j.vetpar.2008.12.016.

[3] Thomas, J. E.; Ohmes, C. M.; Payton, M. E.; Hostetler, J. A.; Reichard, M. V. Minimum Transmission Time of *Cytauxzoon felis* by *Amblyomma americanum* to Domestic Cats in Relation to Duration of Infestation, and Investigation of Ingestion of Infected Ticks as a Potential Route of Transmission. *J. Feline Med. Surg.*, 2018, *20* (2), 67–72. https://doi.org/10.1177/1098612X17691172.

[4] Allen, K. E.; Thomas, J. E.; Wohltjen, M. L.; Reichard, M. V. Transmission of *Cytauxzoon felis* to Domestic Cats by *Amblyomma americanum* Nymphs. *Parasites and Vectors*, 2019, *12* (1), 1–6. https://doi.org/10.1186/s13071-018-3276-8.

[5] Little, S. E.; Barrett, A. W.; Nagamori, Y.; Herrin, B. H.; Normile, D.; Heaney, K.; Armstrong, R. Ticks from Cats in the United States: Patterns of Infestation and Infection with Pathogens. *Vet. Parasitol.*, 2018. https://doi.org/10.1016/j.vetpar.2018.05.002.

[6] Chan, W.-H.; Kaufman, P. E. common name : American dog tick scientific name : *Dermacentor variabilis* (Say) (Arachnida : Ixodida : Ixodidae) http://entnemdept.ufl.edu/creatures/urban/medical/american_dog_tick.htm (accessed Mar 24, 2020).

[7] Minigan, J. N.; Hager, H. A.; Peregrine, A. S.; Newman, J. A. Current and Potential Future Distribution of the American Dog Tick (*Dermacentor variabilis*, Say) in North America. *Ticks Tick. Borne. Dis.*, 2018, *9* (2), 354–362. https://doi.org/10.1016/j.ttbdis.2017.11.012.

[8] Holderman, C. J.; Kaufman, P. E. common name : lone star tick scientific name : *Amblyomma americanum* (Linnaeus) (Acari : Ixodidae) http://entnemdept.ufl.edu/creatures/urban/medical/lone_star_tick.htm (accessed Mar 15, 2020).

[9] Monzón, J. D.; Atkinson, E. G.; Henn, B. M.; Benach, J. L. Population and Evolutionary Genomics of *Amblyomma americanum*, an Expanding Arthropod Disease Vector. *Genome Biol. Evol.*, 2016, *8* (5), 1351–1360. https://doi.org/10.1093/gbe/evw080.

[10] Reichard, M. V.; Baum, K. A.; Cadenhead, S. C.; Snider, T. A. Temporal Occurrence and Environmental Risk Factors Associated with Cytauxzoonosis in Domestic Cats. *Vet. Parasitol.*, 2008. https://doi.org/10.1016/j.vetpar.2007.12.031.

[11] Ferris, D. H. A Progress Report on the Status of a New Disease of American Cats: Cytauxzoonosis. *Comp. Immunol. Microbiol. Infect. Dis.*, 1979. https://doi.org/10.1016/0147-9571(79)90028-6.

[12] Wagner, J. E. A Fatal Cytauxzoonosis-like Disease in Cats. *J. Am. Vet. Med. Assoc.*, 1976.

[13] Hoover, J. P.; Walker, D. B.; Hedges, J. D. Cytauxzoonosis in Cats: Eight Cases (1985-1992). *J. Am. Vet. Med. Assoc.*, 1994.

[14] Meinkoth, J.; Kocan, A. A.; Whitworth, L.; Murphy, G.; Fox, J. C.; Woods, J. P. Cats Surviving Natural Infection with *Cytauxzoon felis*: 18 Cases (1997-1998). *J. Vet. Intern. Med.*, 2000, *14*, 521–525.

[15] Birkenheuer, A. J.; Le, J. A.; Valenzisi, A. M.; Tucker, M. D.; Levy, M. G.; Breitschwerdt, E. B. *Cytauxzoon Felis* Infection in Cats in the Mid-Atlantic States: 34 Cases (1998–2004). *J. Am. Vet. Med. Assoc.*, 2006. https://doi.org/10.2460/javma.228.4.568.

[16] Jackson, C. B.; Fisher, T. Fatal Cytauxzoonosis in a Kentucky Cat (*Felis domesticus*). *Vet. Parasitol.*, 2006, *139*, 192–195. https://doi.org/10.1016/j.vetpar.2006.02.039.

[17] Snider, T. A.; Confer, A. W.; Payton, M. E. Pulmonary Histopathology of *Cytauxzoon felis* Infections in the Cat. *Vet. Parasitol.*, 2010, *47* (4), 698–702. https://doi.org/DOI: 10.1177/0300985810364527.

[18] Reichard, M. V.; Baum, K. A.; Cadenhead, S. C.; Snider, T. A. Temporal Occurrence and Environmental Risk Factors Associated with Cytauxzoonosis in Domestic Cats. *Vet. Parasitol.*, 2008, *152* (3–4), 314–320. https://doi.org/10.1016/j.vetpar.2007.12.031.

[19] Frontera-Acevedo, K. Feline Immune Response To Infection With *Cytauxzoon felis* and the Role of CD18 in the Pathogenesis of Cytauxzoonosis, University of Georgia, 2013.

[20] Conner, B. J.; Hanel, R. M.; Brooks, M. B.; Cohn, L. A.; Birkenheuer, A. J. Coagulation Abnormalities in 5 Cats with Naturally Occurring Cytauxzoonosis. *J. Vet. Emerg. Crit. Care*, 2015, *25* (4), 538–545. https://doi.org/10.1111/vec.12326.

[21] Cohn, L. A.; Birkenheuer, A. J.; Brunker, J. D.; Ratcliff, E. R.; Craig, A. W. Efficacy of Atovaquone and Azithromycin or Imidocarb Dipropionate in Cats with Acute Cytauxzoonosis. *J. Vet. Intern. Med.*, 2011, *25* (1), 55–60. https://doi.org/10.1111/j.1939-1676.2010.0646.x.

[22] Wang, J. L.; Li, T. T.; Liu, G. H.; Zhu, X. Q.; Yao, C. Two Tales of *Cytauxzoon fe lis* Infections in Domestic Cats. *Clin. Microbiol. Rev.*, 2017, *30* (4), 861–885. https://doi.org/10.1128/CMR.00010-17.

[23] Wikander, Y.; Anantatat, T.; Kang, Q.; Reif, K. Prevalence of *Cytauxzoon felis* Infection-Carriers in Eastern Kansas Domestic Cats. *MDPI Pathog.*, 2020, *9* (10), 854–868.

[24] Zou, F.; Li, Z.; Yang, J.; Chang, J.; Liu, G.; Lv, Y.; Zhu, X. *Cytauxzoon felis* Infection in Domestic Cats, Yunnan Province, China, 2016. *Emerg. Infect. Dis.*, 2019, *25* (2), 353–354.

[25] Furtado, M. M.; Taniwaki, S. A.; Metzger, B.; dos Santos Paduan, K.; O'Dwyer, H. L.; de Almeida Jácomo, A. T.; Porfírio, G. E. O.; Silveira, L.; Sollmann, R.; Tôrres, N. M.; et al. Is the Free-Ranging Jaguar (Panthera Onca) a Reservoir for *Cytauxzoon felis* in Brazil? *Ticks Tick. Borne. Dis.*, 2017, *8* (4), 470–476. https://doi.org/10.1016/j.ttbdis.2017.02.005.

[26] Legroux, J.-P.; Halos, L.; Rene-Martellet, M.; Servonnet, M.; Pingret, J.-L.; Bourdoiseau, G.; Baneth, G.; Chabanne, L. First Clinical Case Report of *Cytauxzoon* Sp. Infection in a Domestic Cat in France. *BMC Vet. Res.*, 2017. https://doi.org/https://dx.doi.org/10.1186/s12917-017-1009-4.

[27] Veronesi, F.; Ravagnan, S.; Cerquetella, M.; Carli, E.; Olivieri, E.; Santoro, A.; Pesaro, S.; Berardi, S.; Rossi, G.; Ragni, B.; et al. First Detection of *Cytauxzoon* Spp. Infection in European Wildcats (*Felis silvestris silvestris*) of Italy. *Ticks Tick. Borne. Dis.*, 2016, *7* (5), 853–858. https://doi.org/10.1016/j.ttbdis.2016.04.003.

[28] Miller, J.; Davis, C. D. Increasing Frequency of Feline Cytauxzoonosis Cases Diagnosed in Western Kentucky from 2001 to 2011. *Vet. Parasitol.*, 2013. https://doi.org/10.1016/j.vetpar.2013.08.012.

[29] Raghavan, R. K.; Almes, K.; Goodin, D. G.; Harrington, J. A.; Stackhouse, P. W. Spatially Heterogeneous Land Cover/Land Use and Climatic Risk Factors of Tick-Borne Feline Cytauxzoonosis. *Vector-Borne Zoonotic Dis.*, 2014, *14* (7), 486–495. https://doi.org/10.1089/vbz.2013.1496.

[30] Pets by the numbers: U.S. pet ownership, community cat and shelter population estimates. https://www.humanesociety.org/resources/pets-numbers (accessed Aug 10, 2020).

[31] Pets by the numbers: Data and statistics on pet ownership, community cat, and shelter population in the United States. https://www.animalsheltering.org/page/pets-by-the-numbers (accessed Aug 10, 2020).

[32] Gedeon, J. Special Report: States with the Most and Least Cat Owners. https://247wallst.com/special-report/2017/07/19/states-with-the-most-and-least-cat-owners/ (accessed Aug 10, 2020).

[33] History of National Feral Cat Day. https://nationaltoday.com/national-feral-cat-day/ (accessed Aug 10, 2020).

[34] Miller, J.; Davis, C. D. Increasing Frequency of Feline Cytauxzoonosis Cases Diagnosed in Western Kentucky from 2001 to 2011. *Vet. Parasitol.*, 2013, *198* (1–2), 205–208. https://doi.org/10.1016/j.vetpar.2013.08.012.

[35] Saleh, M. N.; Sundstrom, K. D.; Duncan, K. T.; Ientile, M. M.; Jordy, J.; Ghosh, P.; Little, S. E. Show Us Your Ticks: A Survey of Ticks Infesting Dogs and Cats across the USA. *Parasites and Vectors*, 2019, *12* (1), 1–11. https://doi.org/10.1186/s13071-019-3847-3.

[36] Schreeg, M. E.; Marr, H. S.; Tarigo, J.; Cohn, L. A.; Levy, M. G.; Birkenheuer, A. J. Pharmacogenomics of *Cytauxzoon felis* Cytochrome b: Implications for Atovaquone and Azithromycin Therapy in Domestic Cats with Cytauxzoonosis. *J. Clin. Microbiol.*, 2013, *51* (9), 3066–3069. https://doi.org/10.1128/JCM.01407-13.

[37] Brown, H. M.; Modaresi, S. M.; Cook, J. L.; Latimer, K. S.; Peterson, D. S. Genetic Variability of Archived *Cytauxzoon felis* Histologic Specimens from Domestic Cats in Georgia, 1995-2007. *J. Vet. Diagnostic Investig.*, 2009, *21* (4), 493–498. https://doi.org/10.1177/104063870902100410.

[38] Shock, B. C.; Birkenheuer, A. J.; Patton, L. L.; Olfenbuttel, C.; Beringer, J.; Grove, D. M.; Peek, M.; Butfiloski, J. W.; Hughes, D. W.; Lockhart, J. M.; et al. Variation in the ITS-1 and ITS-2 RRNA Genomic Regions of *Cytauxzoon felis* from Bobcats and Pumas in the Eastern United States and Comparison with Sequences from Domestic Cats. *Vet. Parasitol.*, 2012, *190*, 29–35. https://doi.org/10.1016/j.vetpar.2012.06.010.

[39] The territory of outdoor cats https://www.knowyourcat.info/info/teritory.htm.

[40] Meek, P. D. Home Range of House Cats Felis Catus Living within a National Park. *Aust. Mammal.*, 2003, *25* (1), 51–60. https://doi.org/10.1071/am03051.

[41] Horn, J. A.; Mateus-Pinilla, N.; Warner, R. E.; Heske, E. J. Home Range, Habitat Use, and Activity Patterns of Free-Roaming Domestic Cats. *J. Wildl. Manage.*, 2011, *75* (5), 1177–1185. https://doi.org/10.1002/jwmg.145.

[42] Hanmer, H. J.; Thomas, R. L.; Fellowes, M. D. E. Urbanisation Influences Range Size of the Domestic Cat (*Felis catus*): Consequences for Conservation. *J. Urban Ecol.*, 2017, *3* (1), 1–11. https://doi.org/10.1093/jue/jux014.

[43] L. Cohn, P. Bondy, P. W. Feline Cytauxzoonosis https://www.vetfolio.com/learn/article/feline-cytauxzoonosis.

[44] Cohn, L. Ctauxzoonosis https://veteriankey.com/cytauxzoonosis/.

[45] Sykes, J. E. Feline Hemotropic Mycoplasmas. *Vet. Clin. North Am. - Small Anim. Pract.*, 2010, *40* (6), 1157–1170. https://doi.org/10.1016/j.cvsm.2010.07.003.

[46] Penzhorn, B. L.; Oosthuizen, M. C. Babesia Species of Domestic Cats: Molecular Characterization Has Opened Pandora's Box. *Front. Vet. Sci.*, 2020, *7* (March), 1–10. https://doi.org/10.3389/fvets.2020.00134.

Chapter 3 - **Prevalence of *Cytauxzoon felis* infection carriers in eastern Kansas domestic cats**

Introduction.

Cytauxzoon felis is a tick-borne hemoprotozoal pathogen of felids and the agent of cytauxzoonosis, an often-fatal disease of domestic cats in the southeastern and south-central United States (U.S.). *C. felis* has a complex lifecycle that includes an asexual stage within a felid host and a sexual reproductive stage within a competent ixodid tick vector [1] (Figure 3.1). Briefly, *C. felis* sporozoites are transmitted to a felid via tick saliva during a blood meal. Within the felid host, sporozoites enter monocytes and begin a schizogenous, or leukocyte, phase of asexual replication resulting in the release of many 1–2 µm diameter signet ring merozoites. Merozoites enter host erythrocytes and either replicate asexually via merogony or develop into gametocytes. Ticks become infected again by ingesting gametocytes when feeding on a felid carrier host. In the lumen of the tick gut, sexual reproduction occurs among gametocytes resulting in the formation of zygotes. Zygotes invade the gut epithelium, transform into kinetes, migrate to the salivary gland, and transform into sporozoites, the life stage dispensed via saliva during the subsequent tick bloodmeal. Successful transmission of infectious sporozoites requires transstadial maintenance of the parasite through larvae-to-nymph or nymph-to-adult ecdysis. In the U.S., competent biological transmission vectors for *C. felis* include the ixodid ticks *Dermacentor variabilis* (American dog tick) and *Amblyomma americanum* (Lone star tick) [2–5]. The geographic distribution of *D. variabilis* extends throughout the eastern half of the U.S. and in focal regions along the western coast [6,7]. The geographic distribution of *A. americanum* largely overlaps with *D. variabilis* in the eastern U.S.; however, *A. americanum* occurs more densely in southeastern and mid-central regions [8,9]. Juvenile *D. variabilis* life stages prefer to feed on small to medium-sized rodents,

while adults feed on variety of medium to large mammals including cats. In contrast, *A. americanum* is more indiscriminate in host choice, and both juvenile and adult life stages will aggressively seek out and feed upon a wide diversity of small to large mammals including cats. Due to its aggressive and non-discriminating nature where cats may serve as a host for any life stage, *A. americanum* is likely a more significant vector for *C. felis* in Kansas than *D. variabilis*.

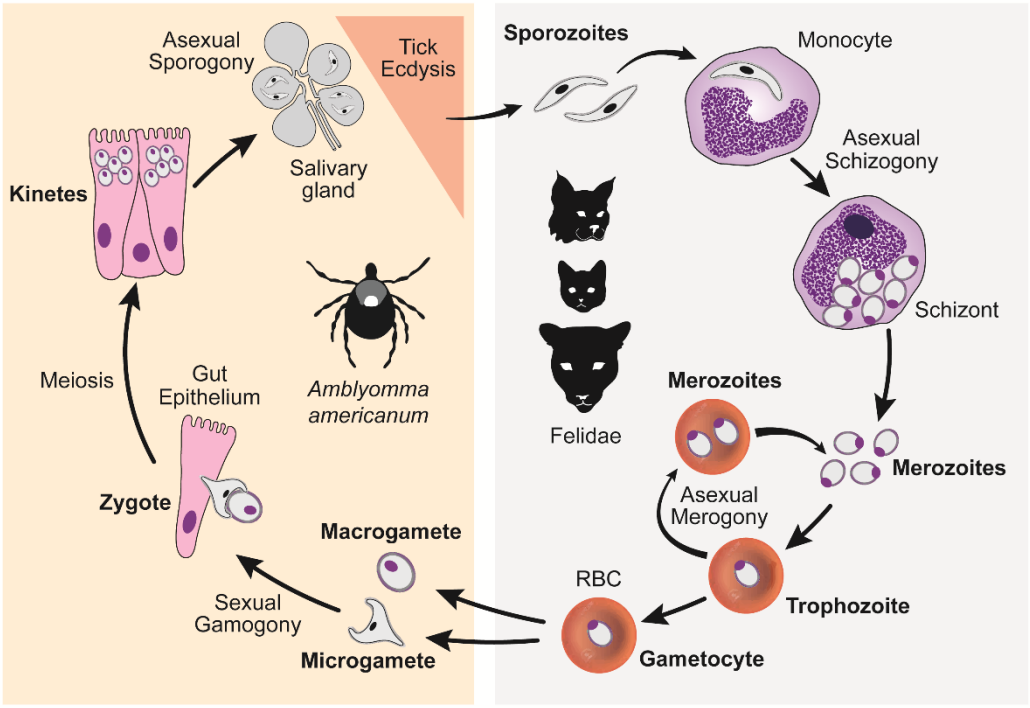


Figure 3.1: *Cytauxzoon felis* lifecycle. The right panel demonstrates asexual reproduction occurring within the host felid, while the left panel demonstrates the sexual and asexual reproduction occurring within the tick transmission vector.

The vertebrate intermediate hosts of *C. felis* are members of the family Felidae. In the U.S., clinical cases of cytauxzoonosis typically peak in late spring, with a smaller peak in early fall, corresponding with adult and nymph *A. americanum* life stage activity [10]. Acute cytauxzoonosis clinical signs appear during *C. felis* schizogenous asexual replication, which can cause severe illness and death [2,11]. Domestic cats with this disease frequently present with lethargy, anorexia, depression, fever, dehydration, icterus, pale mucous membranes, and splenomegaly [2,12,13],

which often culminates in death two–three weeks post-infection due to hypoxic and proinflammatory cytokine-mediated injury of multiple organs, interstitial pneumonia, and disseminated intravascular coagulation [14–17]. If presented early enough to initiate a ten-day treatment with atovaquone (15 mg/kg) and azithromycin (10 mg/kg), up to 60% of patients may recover [18]. Cats that survive the schizogenous phase of the parasite's life cycle become asymptomatic, persistently parasitemic infection carriers, contributing to continued *C. felis* transmission to other felids via tick vectors [19]. *Cytauxzoon* spp. infections with similar clinical signs and disease progression have also been reported in Asia, South America, and Europe [20–23].

Several felid species have been documented as natural *C. felis* infection reservoirs, including: bobcat (*Lynx rufus*) [24–29], domestic cat (*Felis catus*) [30, 31], cougar (*Puma concolor*) [32–34], and captive tigers [35]. For many decades, bobcats were presumed the main infection reservoir, while domestic cats were considered a dead-end host as those with observed clinical disease commonly died [11, 36, 37]. Recent *C. felis* transmission and prevalence studies however, challenge the domestic cat dead-end host assumption [19], with a combination of studies experimentally demonstrating and others documenting domestic cats as competent *C. felis*-carriers [2, 38, 39].

Several studies evaluating the distribution and prevalence of *C. felis* wild and domestic felid reservoirs have been conducted in the U.S. One study examining *C. felis* prevalence in wild felids found 20% (138/696) of bobcats over 14 states positive, with 79%, 65%, and 31% of sampled bobcats positive for *C. felis* in Missouri, Oklahoma, and Kansas, respectively [29]. Surveys of domestic cat populations demonstrate that *C. felis* prevalence can vary widely among these felid populations. Evaluation of *C. felis* infection among trap-neuter-release cats in Florida, North

Carolina, and Tennessee identified a 0.3% (3/961) overall prevalence [40], similar to another study that observed a 0.8% (3/380) *C. felis* infection prevalence among healthy free-roaming cats in Oklahoma [41]. The latter study also evaluated cats in Iowa but did not identify *C. felis* infection in any of the 292 free-roaming cats tested in that state. However, another study identified 6.2% (56/902) of healthy client-owned cats infected with *C. felis* in Arkansas (15.5%, 25/161), Missouri (12.9%, 8/62), and Oklahoma (3.4%, 23/679) [39].

Intensifying and expanding *A. americanum* populations, heighted public awareness and concern over cytauxzoonosis, and anecdotal reports of increasing cytauxzoonosis cases in eastern Kansas suggest that domestic cat reservoirs may be contributing to local *C. felis* transmission cycles. The prevalence of *C. felis*-domestic cat carriers in Kansas is unknown; however, studies in adjacent states (Oklahoma and Missouri) have identified 0.8–12.9% of domestic cats may be *C. felis*-carriers [39]. The objective of this study was to determine the prevalence of *C. felis*-carriers in the domestic cat population of eastern Kansas by obtaining blood samples from asymptomatic, predominantly outdoor cats (feral, rescue/rescinded, and/or owned), and evaluating samples for *C. felis* infection using a quantitative PCR (qPCR) assay targeting the multi-copy cytochrome oxidase 3 (Cox3) gene [42]. Based on previous lower Kansas bobcat *C. felis*-carrier prevalence and adjacent state domestic cat *C. felis*-carrier prevalence studies, we hypothesized: (i) the prevalence of *C. felis* reservoirs in eastern Kansas domestic cats would be less than that found in Missouri and Oklahoma cats; (ii) chronic *C. felis* infection prevalence would be stable despite collection season; and (iii) a greater percentage of feral cats would be carriers compared to rescued or owned cats. Knowing the prevalence of *C. felis*-carriers among local domestic cat populations is important for developing and recommending cytauxzoonosis transmission mitigation strategies.

Materials & Methods.

Study design.

A prospective study with individual cats as the experimental units was designed. Inclusion criteria included: (i) any cat old enough to have been outdoors during one tick season (March–November); (ii) healthy and not exhibiting overt signs of clinical disease; and (iii) from eastern Kansas. Exclusion criteria included: (i) any cat displaying overt signs of clinical disease; or (ii) not from eastern Kansas. Data collected from each cat blood sample included: collection date (year/month/day); collection organization; collection location (city/county); and felid lifestyle (feral, rescue/rescinded, owned).

Blood sample sources & collection.

All study activities involving animals were performed in accordance with an approved Kansas State University Institutional Animal Care and Use protocol, approved prior to study initiation. To collect blood samples, participation was solicited from 28 humane societies, animal shelters/rescues, and small animal practices in eastern Kansas. Eight organizations agreed to participate and six submitted samples from May 2018 through November 2019 (Table A3.4). EDTA blood samples (up to 2.0 mL) were opportunistically collected from domestic cats presented for routine procedures or illness unrelated to cytauxzoonosis at veterinary clinics, animal shelters/rescues/humane societies, or feral cat catch-and-release programs. Some samples were used for other blood tests prior to submission for this study. All samples submitted were stored at −20 °C prior to shipment to Kansas State University for study testing.

EDTA whole blood samples were defrosted at room temperature for 10–15 min within three days of arrival and vortexed for 15 s to mix. A 100 µL aliquot of blood was reserved for genomic DNA extraction and up to 1 mL of blood was saved for additional future testing.

DNA extraction.

Total DNA was extracted from 100 µL of whole blood using the Quick-DNA^{TM} Miniprep Kit according to manufacturer's instructions (Zymo Research, Irvine, CA, USA), with a final 35 µL elution volume. Extracted gDNA samples were stored at −20 °C.

Quantitative real-time polymerase chain reaction (qPCR).

Since *C. felis*-carrier cats can have very low parasite loads [19] and over 1000 samples were anticipated, a qPCR-based assay was optimal for efficient sample processing. An assay with at least comparable sensitivity to the commonly used 18S rRNA endpoint PCR assay that would target a highly conserved multi-copy gene and could identify *C. felis* regardless of pathogen developmental stage and potentially fluctuating parasitemia was sought. Thus, a sensitive qPCR assay targeting a 192 bp region *C. felis* cytochrome c oxidase subunit III (Cox3) gene [42] was chosen to evaluate samples for *C. felis* infection. PCR mastermix setups were prepared in a PCR template-clean workstation and gDNA template was added to individual reaction mixtures in a different room. Each PCR reaction mixture consisted of 12.5 µL of 2XSsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 1.25 µL of 10 µM *Cf* Cox3F (5′-GCATATCTTCAAATTACAGATACAC-3′), 1.25 µL 10 µM *Cf* Cox3R (5′-CCAGTAACTGTTTAGTGTAGTTAAC3′), 5 µL nuclease-free water, and 5 µL template for a 25 µL total reaction volume. Template consisted of DNA from blood samples, nuclease-free water as no template control (NTC), 1:100 gDNA from a clinical cytauxzoonosis cat blood sample (positive control), or 10-fold serial dilutions of pCR4-TOPO vector containing 192 bp *C. felis cox3* amplicon (standard curve). Samples were run on a CFX Connect Real-Time System (Bio-Rad Laboratories) using the following cycling parameters: initial denaturation at 98 °C for 3.5 min; 45

cycles of 60 °C for 30 s, 98 °C for 20 s; and a final melt curve (65–95 °C in 0.5 °C increments). The CFX Maestro Software (Bio-Rad Laboratories) was used to display results.

Cytauxzoon felis Cox3 qPCR assay limit of detection (LOD).

Genomic DNA was extracted from the blood of a cat that had died from acute cytauxzoonosis and was diluted to a 1:100 concentration in DNA elution buffer. The Cox3 qPCR assay was performed as described above. Eight 10-fold serial dilutions of the 1:100 diluted acute cat blood were prepared using uninfected cat blood (obtained from Kansas State University's Veterinary Teaching Hospital blood donor cat colony) as diluent. Genomic DNA was extracted as previously described. A similar procedure was performed using DNA elution buffer as diluent instead of uninfected cat blood. Duplicate PCR plates were assembled which included, in triplicate, 5 µL of the eight dilutions of DNA-extracted uninfected cat blood, 5 µL of the eight dilutions of elution buffer, 5 µL of nuclease-free water as NTC, and 5 µL of the 1:100 extracted infected cat blood as positive control. Cycling conditions were performed as previously described.

Cytauxzoon felis Cox3 qPCR assay limit of quantification (LOQ).

Cytauxzoon felis Cox3 amplicon-containing plasmid with a known concentration of 10^5 copies per µL DNA was used. The same process described for the above LOD was followed except for replacing the extracted infected cat DNA with the Cox3 plasmid and producing duplicate 10-fold serial dilutions a total of five times, resulting in a final dilution of 10^0 copies per µL DNA. A PCR plate was assembled which included duplicate sets of triplicate samples consisting of 5 µL of the five plasmid dilutions in DNA-extracted uninfected cat blood, 5 µL of the five plasmid dilutions of elution buffer, 5 µL of 10^5 copies per µL DNA *C. felis* Cox3 plasmid as positive control, three samples of 5 µL of nuclease-free water as NTC, and three samples of uninfected DNA-extracted cat blood. Cycling conditions were performed as previously described.

Amplicon cloning and sequence analysis.

To confirm amplicon identity, the resulting amplicon from approximately 10% of *C. felis cox3*-positive qPCR reactions was cloned in a pCR4-TOPO ™ cloning vector and transformed into TOP10 *E. coli* according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). A minimum of five resultant clones were screened for presence of a correctly sized insert and a minimum of two positive clones were submitted for rolling cycle amplification (RCA) and 5′ and 3′ sequencing using T3 or T7 sequencing primers (MC Lab, South San Francisco, CA, USA). The 142 bp region internal to the *C. felis cox3* primers was aligned using MEGA-X and alignments were presented using BoxShade v3.2.

Statistical Analysis.

PCR results for samples were treated as binary responses indicating the presence or absence of *C. felis* infection. These binary data were analyzed under the logit linear model. The full model includes season (winter (December to February), spring (March to May), summer (June to August), fall (September to November)), lifestyle (feral, owned, rescue), and their interaction as the fixed effects (Table A3.5, Table A3.6). Since the interaction was not significant (p-value = 0.13), the full model was simplified to a reduced model containing main effects of season and lifestyle only to quantify prevalence rates. The goodness-of-fit of the reduced model was verified based on the deviance-to-degrees-of-freedom ratio of 1.73 with a p-value of 0.109. Prevalence rates for each season were calculated by weighting model estimates according to the Kansas cat population of 49.9% for feral, 47.1% for owned, and 3.0% for rescue cats [53–57]. Tukey's multiplicity adjustment was applied when comparing among the four levels of season effect and among the three levels of lifestyle. Prevalence rates for each lifestyle were calculated by weighting model estimates according to 1:1:1:1 allocation of winter, spring, summer, and fall season.

Moreover, the overall prevalence rate was calculated based on 49.9%:47.1%:3.0% allocation of feral, owned, and rescue cats as well as 1:1:1:1 allocation of the four seasons. Statistical analysis was performed using the LOGISTIC procedure of the SAS/STAT® software, Version 9.4 (SAS Institute Inc., Cary, NC, USA).

Results.

Cox3 mitochondrial DNA (miDNA) qPCR assay limit of detection (LOD) and limit of quantification (LOQ).

Using blood from a cat acutely infected with *C. felis*, a series of assays was conducted to determine the LOD for the Cox3 qPCR assay used to evaluate domestic cat blood samples for *C. felis* infection in this study. The highest dilution of acute infected cat blood which consistently tested positive using the Cox3 qPCR assay was $1{:}10^5$, while still producing an amplicon-specific melt temperature of 76 °C ± 1 °C. Using the described Cox3 qPCR assay conditions, reagents, and equipment, a mean Ct value of 38.78 (95% CI 37.14–40.40) can reliably be interpreted as positive for *C. felis* assuming a correct melt peak is also detected (Table 3.1).

An LOQ study was performed for the Cox3 qPCR assay to determine the lowest copy number of the *cox3* target that could be reliably quantified from baseline with acceptable accuracy. The lowest dilution of amplicon-containing plasmid consistently detected using the Cox3 qPCR assay was 10^2 with a melt temperature of 76 °C ± 1 °C. From the LOQ study, using the described Cox3 qPCR assay conditions, reagents, and equipment, the lowest number of the *cox3* target that can be reliably quantified from baseline with acceptable accuracy is 12.46 copies per µL (95% CI 9.12–15.80) (Table 3.2).

Table 3.1: *Cytauxzoon felis* Cox3 qPCR assay limit of detection (LOD).

Sample Conc	Ct [1] Value	Melt Temp [2] (°C)
$1:10^5$	40.36	75.50
$1:10^5$	37.62	75.00
$1:10^5$	41.11	75.50
$1:10^5$	40.69	75.50
$1:10^5$	37.10	75.00
$1:10^5$	35.77	75.00

[1] Number of amplification cycles required to reach a fixed cycle threshold where the fluorescent signal exceeds the background level. [2] Temperature at which double-stranded DNA template disassociates into single strands.

Table 3.2: *Cytauxzoon felis* Cox3qPCR assay limit of quantification (LOQ).

Sample Conc	Ct [1] Value	SQ [2]	Melt Temp [3] (°C)
10^2	38.81	6	75.50
10^2	37.00	18	75.50
10^2	38.21	9	75.50
10^2	37.58	13	75.50
10^2	37.53	13	75.50
10^2	37.24	16	75.50

[1] Number of amplification cycles required to reach a fixed cycle threshold where the fluorescent signal exceeds the background level. [2] Starting quantity (SQ) is the number of DNA copies at the beginning of the reaction. [3] Temperature at which double-stranded DNA template disassociates into single strands.

Cytauxzoon felis infection prevalence in domestic cats in eastern Kansas.

A total of 1131 blood samples from domestic cats were received from May 2018 through November 2019. Twenty-seven samples were excluded from the study because they were collected from cats acutely ill with cytauxzoonosis (n = 7) or from cats from neighboring states (n = 20), leaving 1104 samples from domestic cats with no known history of cytauxzoonosis from eastern

Kansas available for *C. felis* infection carrier evaluation. In total, 270 of 1104 cats tested positive for *C. felis* (Table A3.1), resulting in a domestic cat *C. felis*-carrier prevalence of 25.8% (95% CI 23.7–27.9%) in eastern Kansas. To confirm the *C. felis* infection results, qPCR amplicons from approximately 10% (n = 28) of positive samples were cloned and sequenced. All sequenced amplicons shared 98.6 to 100% identity with *C. felis* GenBank Accession Number KC207821.1. Alignment of the 142 bp internal primer region produced six unique amplicon sequences (Figure 3.2).

```
CX0158 1    ATGAATGAGTATTCATCATTTTGAAAAGATAAAAACAATAAGCCAATTAAGACTAAGTTT
CX0487 1    ATGAATGAGTATTCATCATTTTGAAAAGATAAAAATAATAAGCCAATTAAGACTAAGTTT
CX0643 1    ATGAATGAGTATTCATCATTTTGAAAAGATAAAAATAATAAGCCAATTAAGACTAAGTTT
CX0832 1    ATGAATGAGTATTCATCATTCTGAAAAGATAAAAATAATAAGCCAATTAAGACTAAGTTT
CX0222 1    ATGAATGAGTATTCATCATTTTGAAAAGATAAAAACAATAAGCCAATTAAGACTAAGTTT
CX0665 1    ATGAATGAGTATTCATCATTTTGAAAAGATAAAAATAATAAGCCAATTAAGACTAAGTTT

CX0158 61   TCTATGTATAAATCATATTCACAACTTTTACTTTCTTGGATTTTTTGAAGTAAAACAGAT
CX0487 61   TCTATGTATAAATCATATTCACAACTTTTACTTTCTTGGATTTTTTGAAGTAAAACAGAT
CX0643 61   TCTATGTATAAATCATATTCACAACTTTTACTTTCTTGGATTTTTTGAAGTAAAACAGAT
CX0832 61   TCTATGTATAAATCATATTCACAACTTTTACTTTCTTGGATTTTTTGAAGTAAAACAGAT
CX0222 61   TCTATGTATAAATCATATTCACAACTTTTACTTTATTGGATTTTTTGAAGTAAAACAGAT
CX0665 61   TCTATGTATAAATCATATTCACAACTTTTACTTTCTTGGATTTTTTGAAGTAAAACAGAT

CX0158 121  ACTAAAGAACTACCTATATTTA (n=13)
CX0487 121  ACTAAAGAACTACCTATATTTA (n=7, 100% identity to KC207821.1)
CX0643 121  ACTAGAGAACTACCTATATTTA (n=5)
CX0832 121  ACTAAAGAACTACCTATATTTA (n=1)
CX0222 121  ACTAAAGAACTACCTATATTTA (n=1)
CX0665 121  ATTAGAGAACTACCTATATTTA (n=1)
```

Figure 3.2: Alignment of representative unique *C. felis cox3* amplicon sequences. Alignment of six unique *C. felis cox3* 142 bp amplicon sequences. Frequency of sequence recovery from 28 sequenced samples provided after sequences. The second most common sequence was identical to *C. felis* GenBank Accession #: KC207821.1.

County-level locations of identified *C. felis*-carrier cats in Kansas are presented in Figure 3.3. The number of samples received from each county varied (Figure 3.3), with >100 samples received

from three counties (Riley, Shawnee, Franklin) representing 11.5% of total samples, and <10 samples received from 16 counties. Of the 26 eastern Kansas counties from which cat blood samples were received, *C. felis*-infected blood samples were detected from cats in 18 counties. Statistical analysis of location data for reservoir population in specific counties was not possible because the sampling method across counties was not standardized as samples were opportunistically collected from cats undergoing routine procedures or bloodwork unrelated to our study.

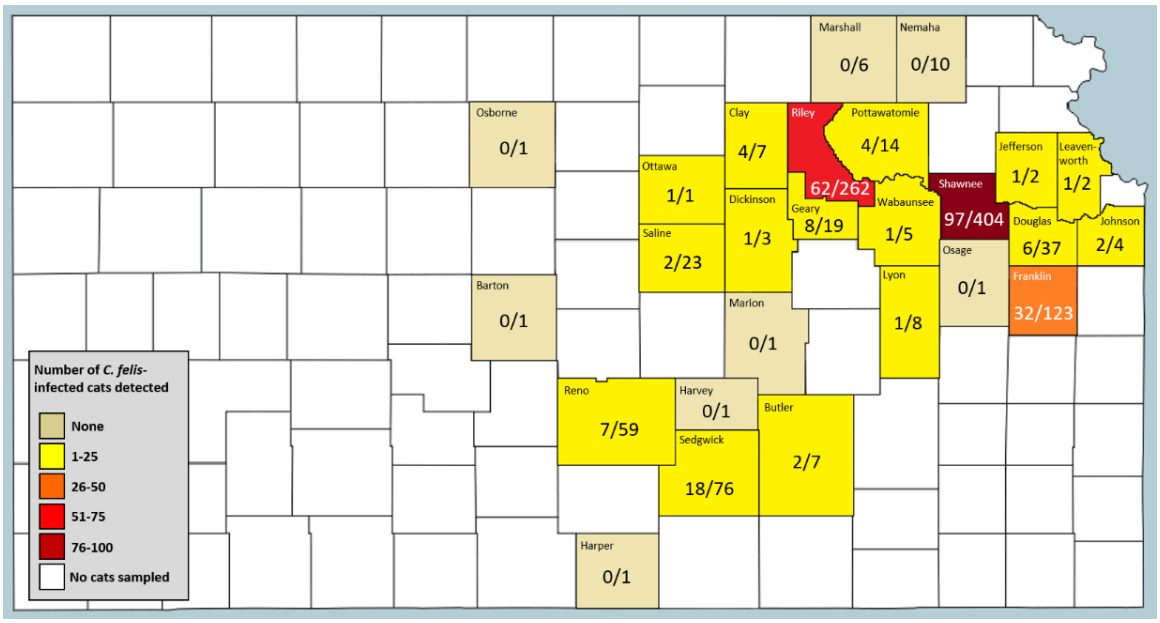
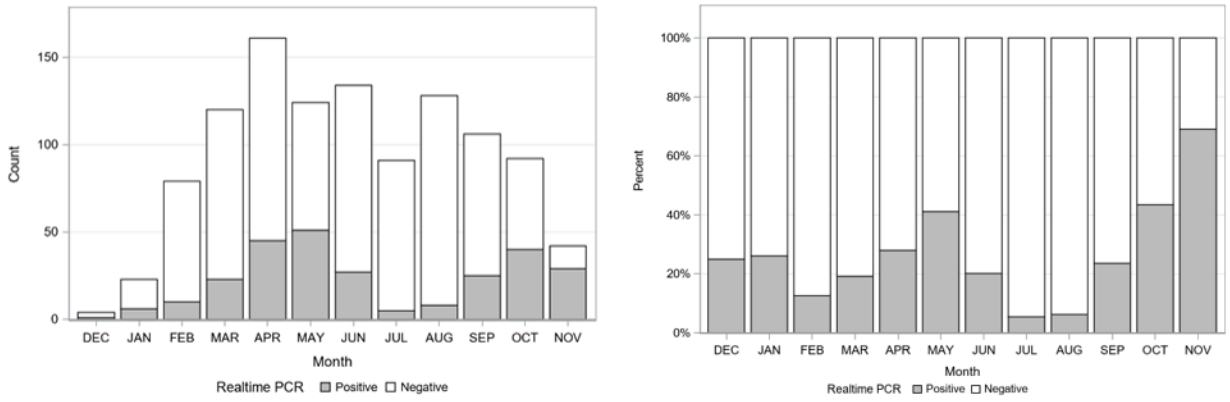


Figure 3.3: County-level location of identified *C. felis* carrier domestic cats in Kansas. *C. felis* carrier cats were identified in colored counties, with color legend indicating the overall incidence of *C. felis* carriers identified from that county. No cats were evaluated from unshaded counties. Numbers represent raw *C. felis*-infected cat counts in the numerator with total cat blood samples obtained from that county in the denominator.

Seasonal variation of detecting *C. felis* carrier cats.

Incidence of detecting *C. felis*-carrier cats during different months or seasons was investigated. The number of cats tested and the number of cats testing positive for *C. felis* are

presented in Figure 3.4 (Table A3.2, Figure A3.1). The greatest number of *C. felis*-carrier cats was observed in April (n = 45), May (n = 51), and October (n = 40), with the greatest percentage of *C. felis*-carrier cats observed in May (41.1%), October (43.5%), and November (69.0%). Since the number of cats tested varied from month to month, testing results were evaluated by season: winter, spring, summer, and fall. A significant seasonal pattern for identifying *C. felis*-carrier cats was observed ($p < 0.001$). A significantly greater percentage of *C. felis*-carrier cats were detected in fall compared to all other seasons, and in spring compared to summer and winter (Table 3.3, Figure 3.5).



(a) **(b)**

Figure 3.4: Number and percent of *C. felis* carrier cats identified by collection month. (a) Raw counts of the number of *C. felis*-infected cats (gray bars) among total cats tested (white bars). (b) Percentage of cats *C. felis*-infected (gray bars) among total tested by month (white bars).

Table 3.3: *Cytauxzoon felis*-infection prevalence by season.

			Odds Ratio to (Adj. *P*-value of Testing for Odds Ratio^=1)		
Season	**Prevalence**	**SE[1]**	**Spring**	**Summer**	**Fall**
Winter	18.2%	4.0%	0.42 (0.016)	1.46 (0.624)	0.27 (<0.001)
Spring	34.4%	3.0%		3.44 (<0.001)	0.64 (0.048)
Summer	13.3%	2.0%			0.19 (<0.001)
Fall	45.1%	3.8%			
Overall	25.8%	2.1%			

[1] Standard Error.

Evaluation of *C. felis* carriers among cats with different lifestyles.

The number (percentage) of individual blood samples obtained from cats with different lifestyles was (i) 537 (48.6%) rescue/rescinded cats from shelter/humane society/rescue organizations, (ii) 351 (31.8%) privately owned cats presenting to veterinary clinics, and (iii) 216 (19.6%) feral cats from catch-and-release programs (Table A3.3). Overall, *C. felis* infection prevalence significantly varied by cat lifestyle (p = 0.007). Of these different lifestyles, 21.8% (117/537) of rescue cats, 25.4% (89/351) of owned cats, and 29.6% (64/216) of feral cats were infected with *C. felis* (Table 3.4). Analyzing *C. felis* infection data by cat lifestyle, feral and owned cats were 1.7 and 1.5 times, respectively, more likely to be *C. felis*-carriers than rescue cats (Table 3.4). The prevalence of *C. felis*-carriers among feral and owned cats was not significantly different (Table 3.4). A similar seasonal trend in detecting *C. felis*-carriers among cats from lifestyles was

observed, with more *C. felis*-carriers observed from cats sampled in the spring and fall (Figure 3.5, Table A3.2).

Table 3.4: *Cytauxzoon felis*-infection prevalence by cat lifestyle.

			Odds Ratio to (Adj. P-value of Testing for Odds Ratio^=1)	
Lifestyle	**Prevalence**	**SE[1]**	**Owned**	**Rescue**
feral	27.2%	3.2%	1.12 (0.840)	1.70 (0.015)
owned	25.0%	2.5%		1.51 (0.038)
rescue	18.0%	1.8%		
Overall	25.8%	2.1%		

[1] Standard Error.

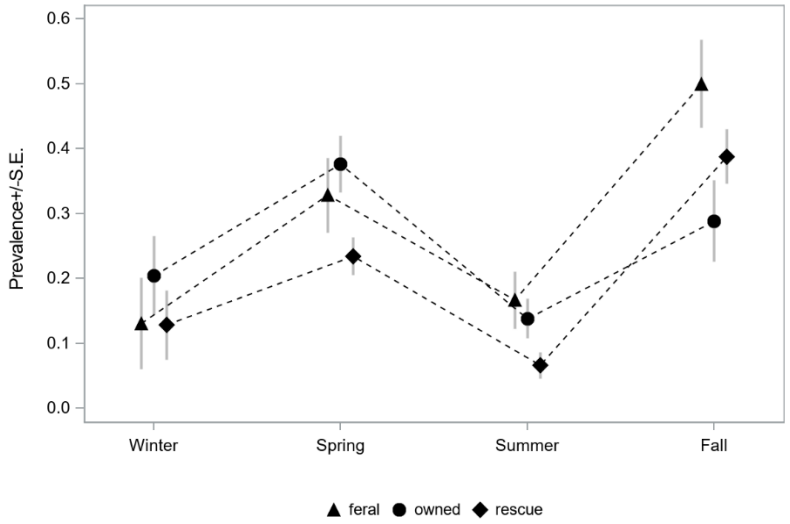


Figure 3.5: *Cytauxzoon felis* among different cat lifestyle populations and season. Lifestyle is indicated by symbol for feral cats (triangle), owned cats (circle), and rescue/rescinded (diamond) cats.

Discussion.

Acute cytauxzoonosis is one of the most fatal diseases in domestic cats, such that for a long time domestic cats were thought to have a minimal, if any, role in the enzootic maintenance of *C. felis* [11,18,32]. Over the past two decades, several prevalence studies have confirmed domestic cats as *C. felis* reservoirs [39-41] and experimentally demonstrated that domestic cats can serve as competent reservoirs for subsequent *C. felis* transmission by ticks, especially *A. americanum* [3,4,38]. *C. felis*-carrier surveys in states adjacent to Kansas have identified that 0.8–12.9% of domestic cats may be *C. felis*-carriers [39, 41]. Anecdotal reports of increasing cytauxzoonosis cases and an abundant population of *A. americanum* in eastern Kansas suggest that domestic cat carriers may be contributing to the increased attention for this disease. In the present study, we demonstrate: (1) there is a high prevalence of asymptomatic *C. felis*-domestic cat carriers in eastern Kansas, (2) *C. felis*-domestic cat carriers are more common during spring and fall months, and (3) feral and owned cats are more likely to be *C. felis*-carriers than rescue/rescinded cats.

The prevalence of active *C. felis* parasitemia identified in this study was significantly greater than that determined in previous surveys conducted in neighboring states. However, direct comparisons of our findings with those of other studies is difficult as different assay platforms (conventional versus quantitative PCR), gene targets (ITS-1 rRNA, 18S rRNA, Cox3 miDNA), and gene target copy numbers (single-copy, multi-copy) were used to detect *C. felis* infection [29, 39-41]. Although most *C. felis* prevalence studies involving domestic cats have used conventional or nested endpoint PCR assays targeting the 18S rRNA *C. felis* gene [13,40,41,43], a recent study demonstrated that a Cox3 miDNA real-time PCR assay performed with similar sensitivity to the commonly used 18S rRNA endpoint PCR assay in detecting *C. felis* in chronically infected cats [42]. For this study, we chose to use the published *C. felis* Cox3 qPCR assay [42] because it

targeted a highly conserved multi-copy gene, and greatly improved testing efficiency while retaining a level of sensitivity similar to previous endpoint PCR assays. A sensitive and specific assay was important as parasitemia levels are often low in reservoir hosts. The specific *cox3* copy number per *C. felis* parasite is unknown and could possibly vary in different *C. felis* developmental stages. Additionally, whether *C. felis* parasitemia levels remain stable, cyclically vary like other tick-borne pathogens, or slowly wane during persistent infection of reservoir hosts is unknown. Differences in molecular assay target copy number during different pathogen developmental stages or fluctuating parasitemia in carrier cats could affect identification of reservoir hosts.

Of note, our study and previous *C. felis* infection surveys used molecular assays to assess infection prevalence, compared to many other tick-borne pathogen surveys of companion animals which commonly use serologic-based diagnostic assays. This difference is notable because a positive molecular test result provides evidence of likely active infection (pathogen must be present in order to detect its genetic material) compared to serologic-based assays which evaluate the presence of a pathogen-specific host antibody (host may or may be not be actively infected). Currently, no serologic assays are commercially available to detect *C. felis* infection or exposure in domestic cats.

Once thought to play a minimal or no role in the enzootic maintenance of *C. felis*, domestic cats are now being recognized as likely important *C. felis* infection reservoirs [39, 40, 44]. Our findings support that domestic cats are not only a competent reservoir of *C. felis* but may play a prominent role in local enzootic maintenance, with 25% of surveyed cats positive for *C. felis* infection. This large percentage of *C. felis*-carrier cats suggests that the mortality rate for this disease is less than previously thought, or that multiple *C. felis* strains of varying virulence are circulating in eastern Kansas. *C. felis* strain may also impact treatment success as one study

evaluating the efficacy of atovaquone and azithromycin treatment observed greater treatment success and improved survival in cats infected with *C. felis* with a specific cytochrome b genotype [43]; however, other genetic loci were not predictive of treatment success [18]. While genetic variation among *C. felis* is described [45–48], how virulence or treatment success varies for different *C. felis* strains/isolates is largely unexplored. Additional studies evaluating genetic differences among cats that survive or succumb to *C. felis* infection will be needed. However, a significant roadblock to conducting studies that could address these questions is the lack of in vitro assay systems and isolate/strain repositories.

The significant seasonal variation pattern of detecting *C. felis*-carriers was surprising as we anticipated that as long-term or chronic infection carriers, there would be minimal to no seasonal influence (e.g., once infected, the cat stays infected). The observed seasonal fluctuation in carrier cats could represent a new influx of cats recently recovered from the schizogenous phase that correlates with seasonal tick vector activity or possibly a seasonal fluctuation in carrier cat parasitemia. Whether parasitemia remains consistent, cycles, or slowly wanes overtime in response to ongoing merogony cycles, seasonality, and/or host immune responses is unknown. Cats that have survived a schizogenous *C. felis* phase are thought to be immune to clinical disease associated with additional *C. felis* challenge [1, 49, 50]. Although these cats may not develop clinical signs of disease, whether re-exposure/infection, especially with a different *C. felis* strain, impacts parasitemia is also unknown. A study evaluating the prevalence of *C. felis* in southern Illinois wild-caught bobcats identified one individual that appeared to have been infected with a different strain (ITS-1 single nucleotide polymorphism) of *C. felis* between captures [28]. More recently, a domestic shorthair cat successfully treated for acute cytauxzoonosis seven years prior presented with a repeat acute *C. felis* infection confirmed on splenic histology [51]. Evidence of *C. felis*

seasonal variation among cat carriers, susceptibility to co- or sequential infections of different *C. felis* strains, and the possibility of recrudescent or newly acquired infection leading to additional clinical disease suggests that *C. felis*-carrier cats may both contribute to sustained pathogen transmission while still remaining vulnerable to additional disease events.

As expected, feral cats had the greatest prevalence of *C. felis* infection. Feral cats are 100% outdoors, likely encounter large numbers of ticks, and are least likely to be treated with any sort of acaricide product, all increasing the *C. felis* infection risk and reservoir potential. Interestingly, although slightly lower, owned cats were statistically as likely to be *C. felis*-carriers, likely highlighting how pervasive *A. americanum* populations are in eastern Kansas. Rescue cats were significantly the least likely to be *C. felis*-carriers. This may be due to rescue cats typically being younger, and a tendency for rescue facilities to keep them indoors. One of our study limitations was that we were unable to collect additional background details on our study subjects including age, sex, detailed lifestyle data (e.g., percent of time spent outdoors), type of outdoor environment (e.g., woods, pasture, manicured yard), and acaricide usage. The only population of subjects that we could be sure spent significant time outdoors was the feral catch-and-release cats. An unknown percentage of pet cats presented to the veterinary clinics and rescue/rescinded cats may have had an unreported indoor-exclusive lifestyle. If so, then our prevalence rate would be artificially decreased for those populations. Additional studies will be needed to more comprehensively evaluate specific risk factors associated with *C. felis* infection and reservoir risk.

Most of the blood samples in this study came from three counties, with far fewer from the remaining 23 counties from which samples were obtained. As such, analysis of whether *C. felis* reservoir cat populations were more or less common in specific counties could not be performed. Only one previous study identified location-based differences in *C. felis*-carrier cat prevalence.

Rizzi et al. determined that cats from eastern Oklahoma had a higher prevalence of *C. felis*-carriers than those from central Oklahoma and suggested this difference could be due to strain or virulence differences [39]. Meanwhile, Raghavan et al. concluded that the main risk factor for *Cytauxzoon* infection in cats, regardless of location, was land cover and humidity that favor the tick vector [52]. The prevalence of *C. felis*-carrier cats in specific areas is likely highly nuanced and dependent on several of the abovementioned variables. Further studies gathering samples using a standardized sampling method across counties are needed to more accurately assess if domestic cat *C. felis* reservoirs are more common in specific Kansas counties.

Conclusion.

We determined the prevalence of *C. felis*-carrier domestic cats in eastern Kansas to be 25.8% (95% CI 23.7–27.9%). These findings suggest *C. felis* infection in domestic cats is far more prevalent than previously thought with many cats surviving the acute phase of the disease. These persistently parasitemic individuals then act as a disease reservoir for other felids. Studying *C. felis* is challenging in many ways as complex factors affect each stage of the organism, the tick vector, the host felid, and the environment, not to mention the lack of an in vitro study system. As such, all cats living in *C. felis* endemic areas, including indoor-exclusive cats, should be aggressively treated with acaricides known to prevent or minimize tick transmission per manufacturer recommendations. More studies are needed to better understand factors affecting *C. felis* and other *Cytauxzoon* spp. infections within the U.S. and globally and their outcomes.

References

[1] Jalovecka, M.; Hajdusek, O.; Sojka, D.; Kopacek, P.; Malandrin, L. The Complexity of Piroplasms Life Cycles. *Front. Cell. Infect. Microbiol.* 2018, *8*, 248, doi:10.3389/fcimb.2018.00248.

[2] Reichard, M.V.; Edwards, A.C.; Meinkoth, J.H.; Snider, T.; Meinkoth, K.R.; Heinz, R.E.; Little, S.E. Confirmation of *Amblyomma americanum* (Acari: Ixodidae) as a vector for *Cytauxzoon felis* (Piroplasmorida: Theileriidae) to domestic cats. *J. Med. Èntomol.* 2010, *47*, 890–896, doi:10.1603/me10013.

[3] Thomas, E.J.; Ohmes, C.M.; Payton, E.M.; Hostetler, A.J.; Reichard, M.V. Minimum transmission time of *Cytauxzoon felis* by *Amblyomma americanum* to domestic cats in relation to duration of infestation, and investigation of ingestion of infected ticks as a potential route of transmission. *J. Feline Med. Surg.* 2017, *20*, 67–72, doi:10.1177/1098612x17691172.

[4] Allen, K.E.; Thomas, J.E.; Wohltjen, M.L.; Reichard, M.V. Transmission of *Cytauxzoon felis* to domestic cats by *Amblyomma americanum* nymphs. *Parasites Vectors* 2019, *12*, 28, doi:10.1186/s13071-018-3276-8.

[5] Little, S.E.; Barrett, A.W.; Nagamori, Y.; Herrin, B.H.; Normile, D.; Heaney, K.; Armstrong, R. Ticks from cats in the United States: Patterns of infestation and infection with pathogens. *Vet. Parasitol.* 2018, *257*, 15–20, doi:10.1016/j.vetpar.2018.05.002.

[6] Chan, W.-H.; Kaufman, P.E. Common Name: American Dog Tick Scientific Name: *Dermacentor variabilis* (Say) (Arachnida: Ixodida: Ixodidae). Available online: http://entnemdept.ufl.edu/creatures/urban/medical/american_dog_tick.htm (accessed on 24 March 2020).

[7] Minigan, J.N.; Gedalof, Z.; Peregrine, A.S.; Newman, J.A. Current and potential future distribution of the American dog tick (*Dermacentor variabilis*, Say) in North America. *Ticks Tick-Borne Dis.* 2018, *9*, 354–362, doi:10.1016/j.ttbdis.2017.11.012.

[8] Holderman, C.J.; Kaufman, P.E. Common Name: Lone Star Tick Scientific Name: *Amblyomma americanum* (Linnaeus) (Acari: Ixodidae). Available online: http://entnemdept.ufl.edu/creatures/urban/medical/lone_star_tick.htm (accessed on 15 March 2020).

[9] Monzón, J.D.; Atkinson, E.G.; Henn, B.M.; Benach, J.L. Population and Evolutionary Genomics of *Amblyomma americanum*, an Expanding Arthropod Disease Vector. *Genome Biol. Evol.* 2016, *8*, 1351–1360, doi:10.1093/gbe/evw080.

[10] Reichard, M.V.; Baum, K.A.; Cadenhead, S.C.; Snider, T. Temporal occurrence and environmental risk factors associated with cytauxzoonosis in domestic cats. *Vet. Parasitol.* 2008, *152*, 314–320, doi:10.1016/j.vetpar.2007.12.031.

[11] Ferris, D. A progress report on the status of a new disease of American cats: Cytauxzoonosis. *Comp. Immunol. Microbiol. Infect. Dis.* 1979, *1*, 269–276, doi:10.1016/0147-9571(79)90028-6.

[12] Meinkoth, J.; Kocan, A.A.; Whitworth, L.; Murphy, G.; Fox, J.C.; Woods, J.P. Cats surviving natural infection with *Cytauxzoon felis*: 18 cases (1997–1998). *J. Vet. Intern. Med.* 2000, *14*, 521–525.

[13] Birkenheuer, A.J.; Le, J.A.; Valenzisi, A.M.; Tucker, M.D.; Levy, M.G.; Breitschwerdt, E.B. *Cytauxzoon felis* infection in cats in the mid-Atlantic states: 34 cases (1998–2004). *J. Am. Vet. Med. Assoc.* 2006, *228*, 568–571, doi:10.2460/javma.228.4.568.

[14] Snider, T.; Confer, A.W.; Payton, M.E. Pulmonary Histopathology of *Cytauxzoon felis* Infections in the Cat. *Vet. Pathol.* 2010, *47*, 698–702, doi:10.1177/0300985810364527.

[15] Frontera-Acevedo, K. Feline Immune Response to Infection with *Cytauxzoon felis* and the Role of CD18 in the Pathogenesis of Cytauxzoonosis; University of Georgia: 2013. Athens, GA.

[16] Frontera-Acevedo, K.; Sakamoto, K. Local pulmonary immune responses in domestic cats naturally infected with *Cytauxzoon felis*. *Vet. Immunol. Immunopathol.* 2015, *163*, 1–7, doi:10.1016/j.vetimm.2014.10.012.

[17] Conner, B.J.; Hanel, R.M.; Brooks, M.B.; Cohn, L.A.; Birkenheuer, A.J. Coagulation abnormalities in 5 cats with naturally occurring cytauxzoonosis. *J. Vet. Emerg. Crit. Care* 2015, *25*, 538–545, doi:10.1111/vec.12326.

[18] A Cohn, L.; Birkenheuer, A.; Brunker, J.; Ratcliff, E.; Craig, A. Efficacy of Atovaquone and Azithromycin or Imidocarb Dipropionate in Cats with Acute Cytauxzoonosis. *J. Vet. Intern. Med.* 2010, *25*, 55–60, doi:10.1111/j.1939-1676.2010.0646.x.

[19] Wang, J.-L.; Li, T.-T.; Liu, G.-H.; Zhu, X.-Q.; Yao, C. Two Tales of *Cytauxzoon felis* Infections in Domestic Cats. *Clin. Microbiol. Rev.* 2017, *30*, 861–885, doi:10.1128/cmr.00010-17.

[20] Zou, F.-C.; Li, Z.; Yang, J.-F.; Chang, J.-Y.; Liu, G.-H.; Lv, Y.; Zhu, X.-Q. *Cytauxzoon felis* Infection in Domestic Cats, Yunnan Province, China, 2016. *Emerg. Infect. Dis.* 2019, *25*, 353–354, doi:10.3201/eid2502.181182.

[21] Furtado, M.M.; Taniwaki, S.A.; Metzger, B.; Paduan, K.D.S.; O'Dwyer, H.L.; Jácomo, A.T.D.A.; Porfirio, G.; Silveira, L.; Sollmann, R.; Torres, N.; et al. Is the free-ranging jaguar (*Panthera onca*) a reservoir for *Cytauxzoon felis* in Brazil? *Ticks Tick-Borne Dis.* 2017, *8*, 470–476, doi:10.1016/j.ttbdis.2017.02.005.

[22] Legroux, J.-P.; Halos, L.; René-Martellet, M.; Servonnet, M.; Pingret, J.-L.; Bourdoiseau, G.; Baneth, G.; Chabanne, L. First clinical case report of *Cytauxzoon* sp. infection in a domestic cat in France. *BMC Veter- Res.* 2017, *13*, 81, doi:10.1186/s12917-017-1009-4.

[23] Veronesi, F.; Ravagnan, S.; Cerquetella, M.; Carli, E.; Olivieri, E.; Santoro, A.; Pesaro, S.; Berardi, S.; Rossi, G.; Ragni, B.; et al. First detection of *Cytauxzoon* spp. infection in European wildcats (*Felis silvestris silvestris*) of Italy. *Ticks Tick-Borne Dis.* 2016, *7*, 853–858, doi:10.1016/j.ttbdis.2016.04.003.

[24] Glenn, B.L.; Kocan, A.A.; Blouin, E.F. Cytauxzoonosis in bobcats. *J. Am. Vet. Med. Assoc.* 1983;183(11):1155-8.

[25] Blouin, E.F.; Kocan, A.A.; Glenn, B.L.; Kocan, K.M.; Hair, J.A. Transmission of *Cytauxzoon felis* by Kier, 1979 from Bobcats, *Felis rufus* (Schreber), to Domestic Cats by *Dermacentor veriabilis* (Say). *J. Wildl*. 1984, *20*, 241–242.

[26] Birkenheuer, A.J.; Marr, H.S.; Warren, C.; Acton, A.E.; Mucker, E.M.; Humphreys, J.G.; Tucker, M.D. *Cytauxzoon felis* infections are present in bobcats (*Lynx rufus*) in a region where cytauxzoonosis is not recognized in domestic cats. *Vet. Parasitol.* 2008, *153*, 126–130, doi:10.1016/j.vetpar.2008.01.020.

[27] Zieman, E.A.; Jiménez, F.A.; Nielsen, C.K. Concurrent Examination of Bobcats and Ticks Reveals High Prevalence of *Cytauxzoon felis* in Southern Illinois. *J. Parasitol.* 2017, *103*, 343–348, doi:10.1645/16-133.

[28] Zieman, E.A.; Nielsen, C.K.; Jiménez, F.A. Chronic *Cytauxzoon felis* infections in wild-caught bobcats (*Lynx rufus*). *Vet. Parasitol.* 2018, *252*, 67–69, doi:10.1016/j.vetpar.2018.01.022.

[29] Shock, B.C.; Murphy, S.M.; Patton, L.L.; Shock, P.M.; Olfenbuttel, C.; Beringer, J.; Prange, S.; Grove, D.M.; Peek, M.; Butfiloski, J.W.; et al. Distribution and prevalence of *Cytauxzoon felis* in bobcats (*Lynx rufus*), the natural reservoir, and other wild felids in thirteen states. *Vet. Parasitol.* 2011, *175*, 325–330, doi:10.1016/j.vetpar.2010.10.009.

[30] Brown, H.M.; Lockhart, J.M.; Latimer, K.S.; Peterson, D.S. Identification and genetic characterization of *Cytauxzoon felis* in asymptomatic domestic cats and bobcats. *Vet. Parasitol.* 2010, *172*, 311–316, doi:10.1016/j.vetpar.2010.04.041.

[31] Lewis, K.; Cohn, A.L.; Marr, H.; Birkenheuer, A. Diminazene Diaceturate for Treatment of Chronic *Cytauxzoon felis* Parasitemia in Naturally Infected Cats. *J. Vet. Intern. Med.* 2012, *26*, 1490–1493, doi:10.1111/j.1939-1676.2012.01003.x.

[32] Butt, M.T.; Bowman, D.; Barr, M.C.; Roelke, M.E. Iatrogenic Transmission of *Cytauxzoon felis* from a Florida Panther (*Felix concolor coryi*) to a Domestic Cat. *J. Wildl. Dis*. 1991, *27*, 342–347.

[33] Rotstein, D.S.; Taylor, S.K.; Harvey, J.W.; Bean, J. Hematologic Effects of Cytauxzoonosis in Florida Panthers and Texas Cougars in Florida. *J. Wildl. Dis.* 1999, *35*, 613–617, doi:10.7589/0090-3558-35.3.613.

[34] Harvey, J.W.; Dunbar, M.R.; Norton, T.M.; Yabsley, M.J. Laboratory Findings in Acute *Cytauxzoon felis* Infection in Cougars (*Puma concolor couguar*) in Florida. *J. Zoo Wildl. Med.* 2007, *38*, 285–291, doi:10.1638/1042-7260(2007)038[0285:lfiacf]2.0.co;2.

[35] Lewis, K.M.; A Cohn, L.; Downey, M.E.; Whitney, M.S.; Birkenheuer, A.J. Evaluation of *Cytauxzoon felis* infection status in captive-born wild felids housed in an area endemic for the pathogen. *J. Am. Vet. Med. Assoc.* 2012, *241*, 1088–1092, doi:10.2460/javma.241.8.1088.

[36] E Wagner, J. A fatal cytauxzoonosis-like disease in cats. *J. Am. Veter- Med Assoc.* 1976, *168*, 585–588.

[37] Hoover, J.P.; Walker, D.B.; Hedges, J.D. Cytauxzoonosis in cats: Eight cases (1985–1992). *J. Am. Vet. Med Assoc.* 1994, *205*.

[38] Reichard, M.V.; Meinkoth, J.H.; Edwards, A.C.; Snider, T.; Kocan, K.M.; Blouin, E.F.; Little, S.E. Transmission of *Cytauxzoon felis* to a domestic cat by *Amblyomma americanum*. *Vet. Parasitol.* 2009, *161*, 110–115, doi:10.1016/j.vetpar.2008.12.016.

[39] E Rizzi, T.; Reichard, M.V.; A Cohn, L.; Birkenheuer, A.J.; Taylor, J.D.; Meinkoth, J.H. Prevalence of *Cytauxzoon felis* infection in healthy cats from enzootic areas in Arkansas, Missouri, and Oklahoma. *Parasites Vectors* 2015, *8*, 13, doi:10.1186/s13071-014-0618-z.

[40] Haber, M.D.; Tucker, M.D.; Marr, H.S.; Levy, J.K.; Burgess, J.; Lappin, M.R.; Birkenheuer, A.J. The detection of *Cytauxzoon felis* in apparently healthy free-roaming cats in the USA. *Vet. Parasitol.* 2007, *146*, 316–320, doi:10.1016/j.vetpar.2007.02.029.

[41] Nagamori, Y.; E Slovak, J.; Reichard, M.V. Prevalence of *Cytauxzoon felis* infection in healthy free-roaming cats in north-central Oklahoma and central Iowa. *J. Feline Med. Surg. Open Rep.* 2016, *2*, 1–4, doi:10.1177/2055116916655174.

[42] Schreeg, M.E.; Marr, H.S.; Griffith, E.H.; Tarigo, J.L.; Bird, D.M.; Reichard, M.V.; Cohn, L.A.; Levy, M.G.; Birkenheuer, A.J.; Schrîg, M.E. PCR amplification of a multi-copy mitochondrial gene (cox3) improves detection of *Cytauxzoon felis* infection as compared to a ribosomal gene (18S). *Vet. Parasitol.* 2016, *225*, 123–130, doi:10.1016/j.vetpar.2016.06.013.

[43] Schreeg, M.E.; Marr, H.S.; Tarigo, J.; Cohn, L.A.; Levy, M.G.; Birkenheuer, A.J. Pharmacogenomics of *Cytauxzoon felis* Cytochrome b: Implications for Atovaquone and Azithromycin Therapy in Domestic Cats with Cytauxzoonosis. *J. Clin. Microbiol.* 2013, *51*, 3066–3069, doi:10.1128/jcm.01407-13.

[44] Brown, H.M.; Latimer, K.S.; Erikson, L.E.; Cashwell, M.E.; Britt, J.O.; Peterson, D.S. Detection of persistent *Cytauxzoon felis* infection by polymerase chain reaction in three asymptomatic domestic cats. *J. Vet. Diagn. Investig.* 2008, *20*, 485–488, doi:10.1177/104063870802000411.

[45] Bondy, P.J.; Cohn, L.A.; Tyler, J.W.; Marsh, A.E. 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. *J. Parasitol.* 2005, *91*, 458–461, doi:10.1645/ge-374r.

[46] Moore Brown, H. *Cytauxzoon felis*: Assessing Genetic Variability in an Emerging Feline Pathogen; University of Georgia: 2010. Athens, GA.

[47] Pollard, D.A.; Reichard, M.V.; Cohn, L.A.; James, A.M.; Holman, P.J. Genetic variability of cloned *Cytauxzoon felis* ribosomal RNA ITS1 and ITS2 genomic regions from domestic cats with varied clinical outcomes from five states. *Vet. Parasitol.* 2017, *244*, 136–143, doi:10.1016/j.vetpar.2017.08.002.

[48] Shock, B.C.; Birkenheuer, A.J.; Patton, L.L.; Olfenbuttel, C.; Beringer, J.; Grove, D.M.; Peek, M.; Butfiloski, J.W.; Hughes, D.W.; Lockhart, J.M.; et al. Variation in the ITS-1 and ITS-2 rRNA genomic regions of *Cytauxzoon felis* from bobcats and pumas in the eastern United States and comparison with sequences from domestic cats. *Vet. Parasitol.* 2012, *190*, 29–35, doi:10.1016/j.vetpar.2012.06.010.

[49] Motzel, S.; Wagner, J. Treatment of experimentally induced cytauxzoonosis in cats with parvaquone and buparvaquone. *Vet. Parasitol.* 1990, *35*, 131–138, doi:10.1016/0304-4017(90)90122-r.

[50] Tarigo, J.L.; Scholl, E.H.; Bird, D.M.; Brown, C.C.; Cohn, L.A.; Dean, G.; Levy, M.G.; Rn, D.M.D.; Trieu, A.; Nordone, S.K.; et al. A Novel Candidate Vaccine for Cytauxzoonosis Inferred from Comparative Apicomplexan Genomics. *PLoS ONE* 2013, *8*, e71233, doi:10.1371/journal.pone.0071233.

[51] A Cohn, L.; Shaw, D.; Shoemake, C.; Birkenheuer, A.J. Second illness due to subsequent *Cytauxzoon felis* infection in a domestic cat. *J. Feline Med. Surg. Open Rep.* 2020, *6*, 1–5, doi:10.1177/2055116920908963.

[52] Raghavan, R.; Almes, K.; Goodin, D.G.; Harrington, J.A.; Stackhouse, P.W. Spatially Heterogeneous Land Cover/Land Use and Climatic Risk Factors of Tick-Borne Feline Cytauxzoonosis. *Vector Borne Zoonotic Dis.* 2014, *14*, 486–495, doi:10.1089/vbz.2013.1496.

[53] Pets by the Numbers: Data and Statistics on Pet Ownership, Community Cat, and Shelter Population in the United States. Available online: https://www.animalsheltering.org/page/pets-by-the-numbers (accessed on 10 August 2020).

[54] Pets by the Numbers: U.S. Pet Ownership, Community Cat and Shelter Population Estimates. Available online: https://www.humanesociety.org/resources/pets-numbers (accessed on 10 August 2020).

[55] Gedeon, J. Special Report: States with the Most and Least Cat Owners. Available online: https://247wallst.com/special-report/2017/07/19/states-with-the-most-and-least-cat-owners/ (accessed on 10 August 2020).

[56] Pet Statistics. Available online: https://www.aspca.org/animal-homelessness/shelter-intake-and-surrender/pet-statistics (accessed on 10 August 2020).

[57] History of National Feral Cat Day. Available online: https://nationaltoday.com/national-feral-cat-day/ (accessed on 10 August 2020).

Appendix A - Supplementary Figures and Tables

Table A2.1: Acute cytauxzoonosis case incidence by year and year block

Year block	Year	# of Incidence per year
2005-2009	2006	16
	2007	10
	2008	9
	2009	18
	Total	**53**
2010-2014	2010	13
	2011	12
	2012	18
	2013	11
	2014	4
	Total	**58**
2015-2019	2015	2
	2016	10
	2017	16
	2018	16
	2019	15
	Total	**59**

Table A2.2: Acute cytauxzoonosis case incidence by year block.

Year Block	Total incidence #	Average incidence # per year
2005~2009	53	13.3
2010~2014	58	11.6
2015~2019	59	11.8

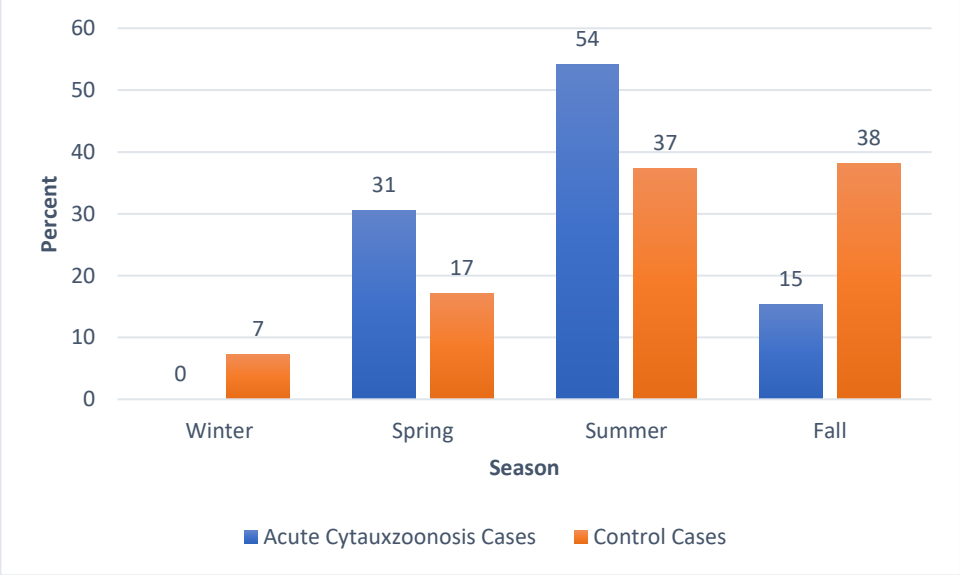


Figure A2.1: Acute cytauxzoonosis and control case percentages by season. The number above the bar represents the percent of cases by season.

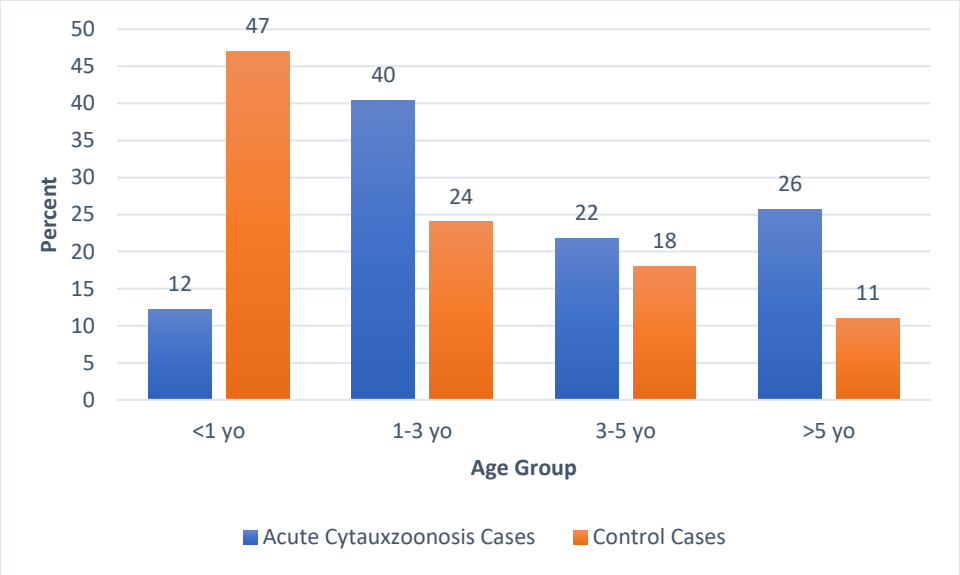


Figure A2.2: Acute cytauxzoonosis and control case percentage by age group (years old). The number above the bar represents the percent of cases within a given age group.

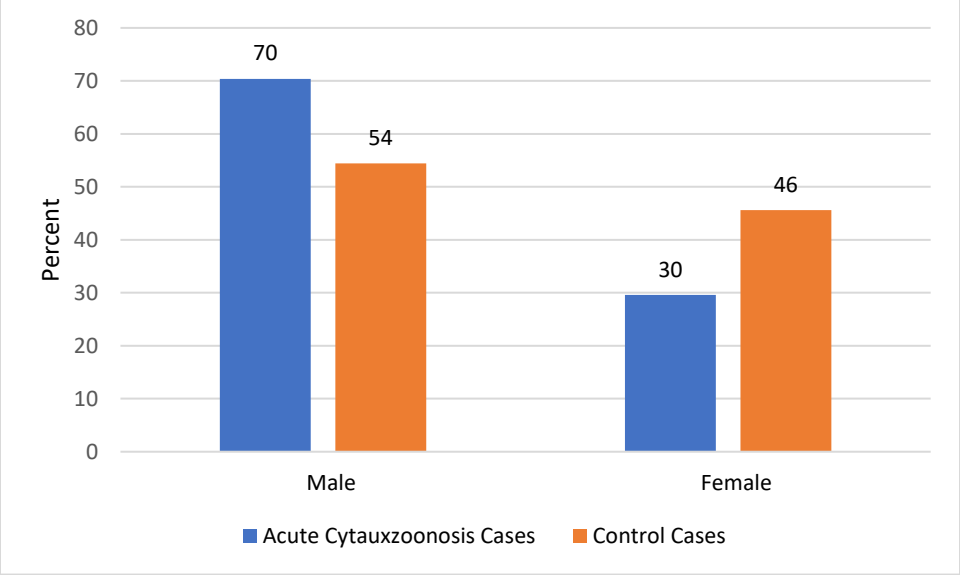


Figure A2.3: Acute cytauxzoonosis and control case percentage by sex. The number above the bar represents the percent of cases by sex.

Table A3.1: *Cytauxzoon felis* prevalence by month and lifestyle.

Month	feral			owned			rescue			Total		
	Prevalence	**Incidence**	**# of Cats**	**Prevalence**	**Incidence**	**# of Cats**	**Prevalence**	**Incidence**	**# of Cats**	**Prevalence**	**Incidence**	**# of Cats**
FEB	13.0%	3	23	12.8%	5	39	11.8%	2	17	12.7%	10	79
MAR	20.0%	2	10	40.6%	13	32	10.3%	8	78	19.2%	23	120
APR	31.0%	13	42	23.9%	11	46	28.8%	21	73	28.0%	45	161
MAY	46.7%	7	15	48.9%	23	47	33.9%	21	62	41.1%	51	124
JUN	34.4%	11	32	15.5%	9	58	15.9%	7	44	20.1%	27	134
JUL	9.1%	1	11	7.3%	3	41	2.6%	1	39	5.5%	5	91
AUG	0.0%	0	29	19.4%	6	31	2.9%	2	68	6.3%	8	128
SEP	13.0%	3	23	28.8%	15	52	22.6%	7	31	23.6%	25	106
OCT	75.0%	9	12	.	.	.	38.8%	31	80	43.5%	40	92
NOV	78.9%	15	19	.	.	.	60.9%	14	23	69.0%	29	42
JAN	.	.	.	80.0%	4	5	11.1%	2	18	26.1%	6	23
DEC	.	.	.	.	.	.	25.0%	1	4	25.0%	1	4
Total	29.6%	64	216	25.4%	89	351	21.8%	117	537	24.5%	270	1104

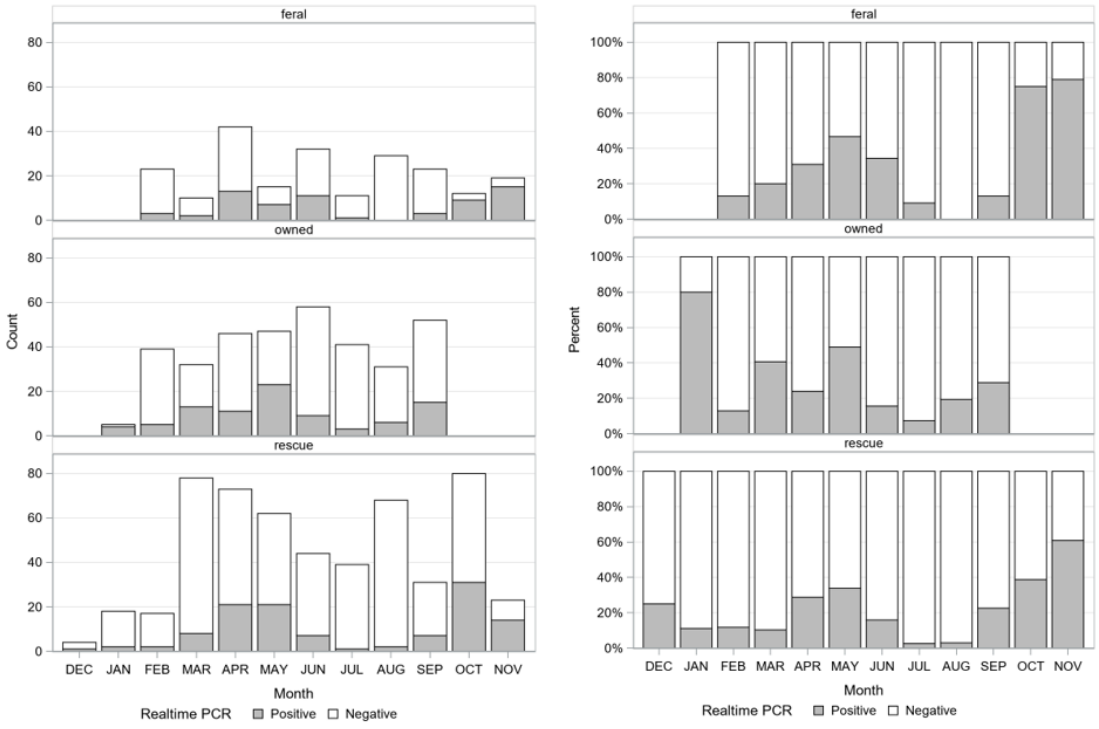
Table A3.2: *Cytauxzoon felis* prevalence by season and lifestyle.

Season	feral			owned			rescue			Total		
	Prevalence	**Incidence**	**# of Cats**	**Prevalence**	**Incidence**	**# of Cats**	**Prevalence**	**Incidence**	**# of Cats**	**Prevalence**	**Incidence**	**# of Cats**
Winter	13.0%	3	23	20.5%	9	44	12.8%	5	39	16.0%	17	106
Spring	32.8%	22	67	37.6%	47	125	23.5%	50	213	29.4%	119	405
Summer	16.7%	12	72	13.8%	18	130	6.6%	10	151	11.3%	40	353
Fall	50.0%	27	54	28.8%	15	52	38.8%	52	134	39.2%	94	240
Total	29.6%	64	216	25.4%	89	351	21.8%	117	537	24.5%	270	1104

Table A3.3: Cat blood samples collected per season and lifestyle.

Season	feral		owned		rescue		Total
	N[1]	Row Percent	N[1]	Row Percent	N[1]	Row Percent	N[1]
Winter	23	21.7%	44	41.5%	39	36.8%	**106**
Spring	67	16.5%	125	30.9%	213	52.6%	**405**
Summer	72	20.4%	130	36.8%	151	42.8%	**353**
Fall	54	22.5%	52	21.7%	134	55.8%	**240**
Total	**216**	**19.6%**	**351**	**31.8%**	**537**	**48.6%**	**1104**

[1] Number of cat blood samples.



(a) **(b)**

Figure A3.1: Number and percent of *C. felis* reservoir cats identified by collection month and lifestyle. (a) Raw counts of the number of *C. felis*-infected cats (gray bars) among total cats tested (white bars). (b) Percentage of cats infected with *C. felis* (gray bars) among total tested by month (white bars).

Table A3.4: Cat blood samples submitted by blood collector organization and lifestyle.

Sample Submitter	Predominant Location	County Location	Samples Submitted	Lifestyle
AD[1]	Junction City	Geary	5	pet
BSVC[2]	Sabetha	Bern	9	pet
KSVDL[3]	Manhattan	Riley	337	pet
LHS[4]	Lawrence	Douglas	33	rescue
HHHS[5]	Topeka	Shawnee	195	rescue
KSUSM[7]	Ottawa	Franklin	309	rescue
	Manhattan	Riley		
	Hutchinson	Reno		
KSUSM[7]	Topeka	Shawnee	216	feral
	Wichita	Sedgwick		

[1] Animal Doctor (Junction City, KS), [2] Bern-Sabetha Veterinary Clinic (Sabetha, KS), [3] Kansas State Diagnostic Clinical Pathology Laboratory (Manhattan, KS), [4] Lawrence Humane Society (Lawrence, KS), [5] Helping Hands Humane Society (Topeka, KS), and [7] KSU Shelter Medicine (Manhattan, KS).

Table A3.5: *Cytauxzoon felis*-infection prevalence by lifestyle in each season.

Season	Status	Prevalence	SE[1]
Winter	feral	13.0%	7.0%
	owned	20.5%	6.1%
	rescue	12.8%	5.4%
Spring	feral	32.8%	5.7%
	owned	37.6%	4.3%
	rescue	23.5%	2.9%
Summer	feral	16.7%	4.4%
	owned	13.8%	3.0%
	rescue	6.6%	2.0%
Fall	feral	50.0%	6.8%
	owned	28.8%	6.3%
	rescue	38.8%	4.2%

[1] Standard Error.

Table A3.6: *Cytauxzoon felis*-infection statistical tests of season, lifestyle, and season-by-lifestyle interaction.

Effect	P-value	
	Full Model	Reduced (Final) Model
season	<0.001	<0.001
lifestyle	0.079	0.007
season*lifestyle	0.130	--