ABSTRACT

YANG, TZUSHAN SHARON. Exploiting the Parasite-Vector Relationship: Tick-based Experimental Models for *Cytauxzoon felis* (Under the direction of Dr. Adam Birkenheuer).

Cytauxzoon felis is an important tick-borne hemoprotozoan parasite that causes life-threatening disease in domestic cats. Even with the best medical management, acute cytauxzoonosis is still associated with a mortality rate of 20–40%. The parasite is primarily transmitted by *Amblyomma americanum.* Due to the growing distribution of *A. americanum*, the geographic range of cytauxzoonosis in domestic cats has now reached at least 39% of the continental U.S. Tick avoidance and control remain the only preventive measures for the infection as a vaccine against cytauxzoonosis does not exist. Unlike many other related tick-borne pathogens, *C. felis* has never been successfully cultured *in vitro*, which severely limits our ability to study the parasite without experimentally infecting cats. Therefore, to minimize the use of live animals in *Cytauxzoon* research, this dissertation took an alternative approach and focused on the tick host as the main platform to study *C. felis.*

To establish a basis for these tick-based experimental models, we first optimized a technique to prepare paraffin-embedded whole tick histology sections for Ixodid ticks using *A. americanum* as an example species. With this protocol, we were able to have a comprehensive scope of the tick internal histoanatomy, facilitating other studies in this dissertation. By combining various microscopic and molecular techniques, we were not only able to detect and localize the *C. felis* RNA in tick salivary glands but visualized the organisms for the first time on light and electron microscopy in ticks.

Considering that the only established model to infect *A. americanum* with *C. felis* is through acquisition feeding on chronically infected cats, we attempted two *in vitro* infection techniques, direct injection and artificial membrane feeding. We were able to detect the parasite

RNA within the injected *A. americanum* adults, but these ticks failed to transmit the infection to naïve cats. We were able to feed *A. americanum* nymphs to repletion in the membrane feeding system, but the adults similarly failed to transmit the infection to a naïve cat. Nonetheless, these studies demonstrated the feasibility of both direct injection and *in vitro* membrane feeding for *A. americanum,* suggesting their potential for *Cytauxzoon* research.

The current experimental models for cytauxzoonosis rely on either tick transmission or direct injection of infected cat tissues. To investigate an additional route of infection and produce resources that could be shared more easily, we validated the feasibility of producing sporozoites-based inoculums. We collected salivary glands from *C. felis*-infected ticks and were able to induce cytauxzoonosis in a naïve cat via direct injection. Sporozoites-based inoculums not only represent an additional resource for experimental cytauxzoonosis but may also serve as a basis for *in vitro* cultivation and vaccine development.

In conclusion, we have shown that tick-based experimental models have great potential to advance *Cytauxzoon* research and should continue to be explored and optimized. We believe this work can lead to many new discoveries to improve the diagnosis, treatment, and prevention of cytauxzoonosis.

Exploiting the Parasite-Vector Relationship: Tick-based Experimental Models for *Cytauxzoon felis*

by
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BIOGRAPHY

Tzushan Sharon Yang was born in Starkville, Mississippi, and was raised in Taiwan until she returned to the United States for college in 2006. She graduated with a Bachelor of Science Degree in Wildlife Science in 2010 and completed veterinary school from Mississippi State University in 2015. After finishing a small animal rotating internship, she pursued her passion for the study of veterinary diseases and her love for the microscope by completing a residency in anatomic pathology and became a board-certified veterinary pathologist in 2019.

Throughout her education, Sharon has always been fascinated by infectious diseases and parasites, particularly vector-borne diseases. She decided to join Dr. Adam Birkenheuer's team to study one of the most fascinating and mysterious parasites, *Cytauxzoon felis.* Throughout her PhD experience, she enjoyed being challenged by the pilot nature of many of her projects and continues to be enthralled by the opportunities this research continues to offer. She hopes that her work can be built on in the small *Cytauxzoon* community, but also potentially benefit many other tick-borne disease investigators.

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All the work in this dissertation would not have been possible without a strong support network, which consists of amazing mentors, friends, and family.

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Most importantly, I would like to thank my husband Nick Hyer for being by my side every single day and enduring my seemingly endless pursuit for education. This process would not have been possible without his positivity and unrelenting faith in me. He has been my rock and the best partner every step of the way, and this work is more meaningful because of his incredible support. Special thanks to my little animal family, Rimmie and Gizmo, for the emotional support and the reminder of why our work matters.

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CHAPTER 1

Literature Review

INTRODUCTION

Cytauxzoonosis is a life-threatening infectious disease of domestic cats in the United States caused by the tick-borne protozoa *C. felis*. The *Cytauxzoon* genus is classified under the phylum Apicomplexa, order Piroplasmida, and family Theileriidae. Since the first report of fatal cytauxzoonosis in domestic cats in 1976 [1], this disease is now documented in 19 states [2–7] and may continue to extend further north and east as the range of its primary tick vector, *Amblyomma americanum*, expands [8]. Furthermore, infection of domestic cats with *C. felis* and other *Cytauxzoon* spp. are continuing to emerge throughout the world, including Asia, South America, and Europe [9–13]. Despite a new treatment regimen that has greatly improved survival rate in cats with acute cytauxzoonosis [14], tick avoidance and control remain the only means of prevention for this disease as a vaccine against this disease does not exist. This is largely due to the inability to establish an *in vitro* culture system for the parasite, which is undoubtedly the greatest barrier in *Cytauxzoon* research. Without an established culture system, many conventional techniques we use to study infectious agents or develop a vaccine difficult to achieve. As a result, there is a continuous effort among *Cytauxzoon* researchers to explore alternative means and platforms to study this parasite.

Our knowledge regarding the *C. felis* life cycle is currently limited to the feline host, leaving large gaps in life cycle of the parasite (Figure 1). In cats that are naturally infected with *C. felis*, the infectious stage of the parasite (sporozoites) are transmitted through tick saliva and introduced into the cat during a blood meal. The sporozoites then invade mononuclear leukocytes and undergo asexual replication as schizonts. At this stage, large numbers of schizont-laden cells

accumulate and occlude blood vessels in many vital organs (Figure 2), triggering a marked systemic inflammatory response that leads to the clinical illness and mortality in acute cytauxzoonosis [15–17]. If infected cats recover from acute infection, they become chronic carriers and remain asymptomatic and parasitemic for life. These chronic carriers are believed to play a similar role as their natural reservoir host, the bobcat (*Lynx rufus*), and become a source of infection for ticks [18,19].

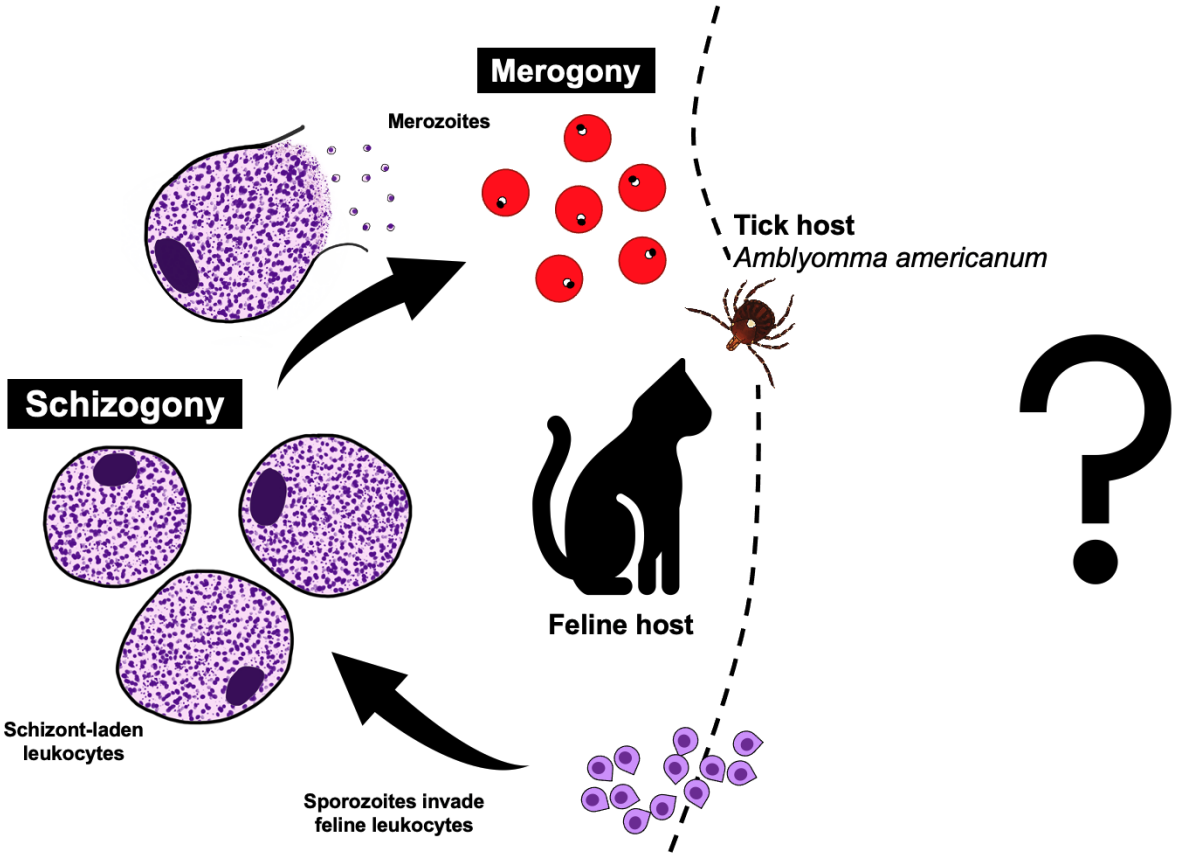

Figure 1. Current Knowledge of the *Cytauxzoon felis* Life Cycle.
Our current knowledge of the *C. felis* life cycle is limited to the feline host. Upon attachment and feeding by a *C. felis*-infected tick, sporozoites are introduced in the cat and undergo asexual replication (schizogony) within feline mononuclear leukocytes (presumed monocyte lineage cells). Schizont-laden leukocytes then rupture to release numerous merozoites that invade erythrocytes. These intraerythrocytic life stages are acquired by the tick host to continue parasite development within the vector. The vector life cycle of *C. felis* is unknown and is generally assumed to be comparable to *Theileria* spp.

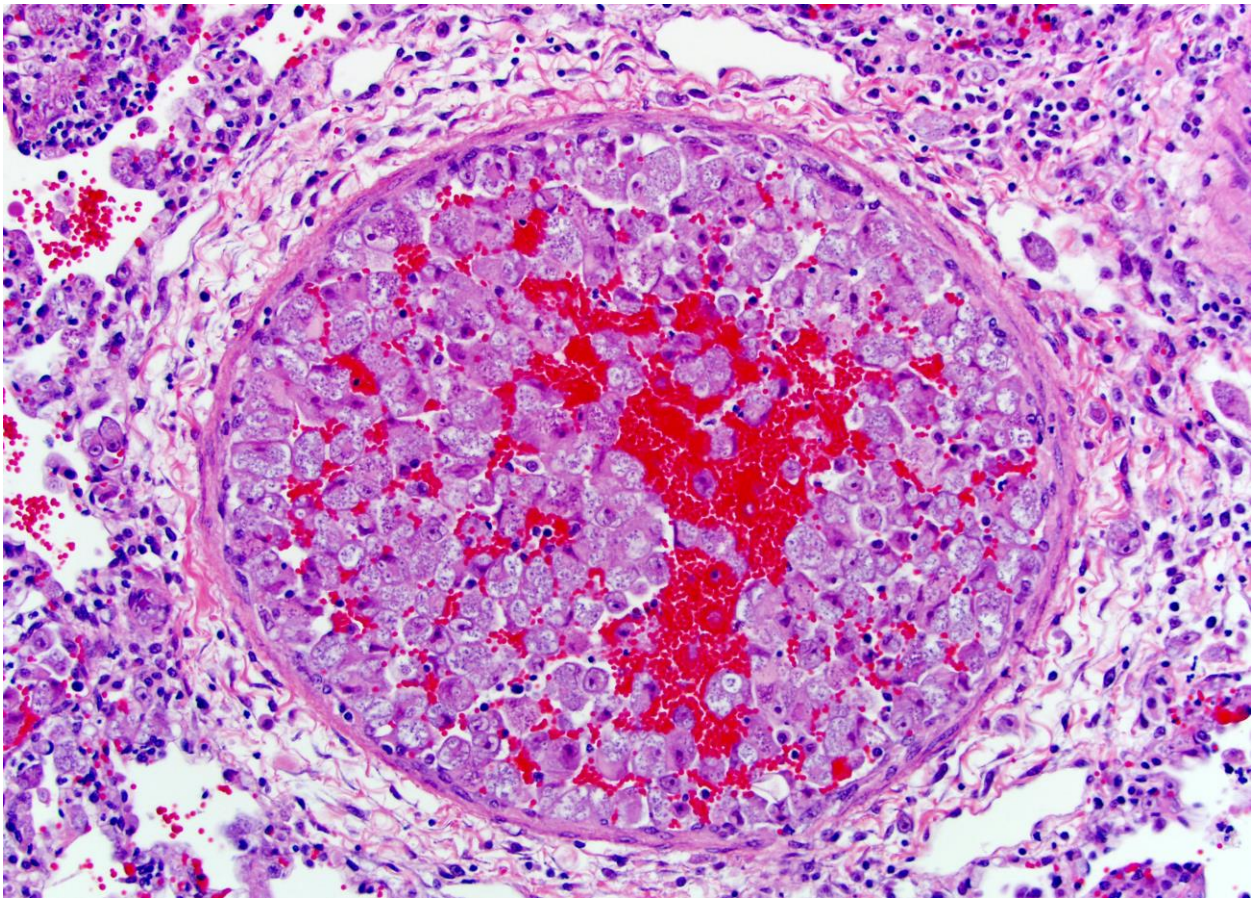

Figure 2. Pathognomonic histopathologic lesion for acute cytauxzoonosis in a cat. This pulmonary vessel is severely distended and largely occluded by numerous schizont-laden leukocytes. Many schizonts are also present in the adjacent alveolar capillaries. This lesion can be found in almost every organ in varying degrees, but is often most prominent in the lungs, spleen, and brain.

On the other hand, the detailed development within ticks has never been thoroughly investigated and remain uncharacterized for *C. felis*. In fact, it has long been presumed that this end of the life cycle is comparable to *Theileria* spp., one of their closest relatives among other piroplasmid parasites [17,20,21]. Cytauxzoonosis was first linked to a tick vector in 1984 by Blouin et al. [22] via experimental transmission to domestic cats by *Dermacentor variabilis*. However, the geographic and epidemiological trends of naturally occurring cytauxzoonosis were found to coincide more closely with that of *Amblyomma americanum*'s. The ability of *A.*

americanum to transmit *C. felis* was confirmed repeatedly in a laboratory setting [23–25]. As the attempts to transmit *C. felis* with other tick species, including *D. variabilis,* were unsuccessful, this has further confirmed that *A. americanum* is the primary tick vector of *C. felis* [24,26].

With the identification of the primary vector host for *C. felis*, *A. americanum* has been a crucial element in many experimental studies for cytauxzoonosis. However, besides being utilized as just a vector, this parasite-vector relationship has not been fully explored for *C. felis*. Therefore, this dissertation focuses on utilizing *A. ameircanum* as a platform to study *C. felis.* Majority of this work is done through a combination of light microscopy and various molecular detection techniques. To facilitate this process, **Chapter 1** of this thesis establishes a protocol to prepare whole tick histology for Ixodid ticks using *A. americanum* as an example species. This technique allows us to understand the internal histoanatomy of *A. americanum* and establishes a foundation for the tick-based experimental models for *C. felis* in this thesis. For instance, by combining various microscopy and molecular detection techniques, **Chapter 2** was able to showcase the first definitive detection and visualization of *C. felis* in *A. americanum* salivary glands, solidifying a foundation for future investigations regarding the parasite's development within the tick.

Acquisition feeding on *C. felis* carriers is currently the only reliable means to infect ticks with *C. felis*. However, this model requires access to a cat that is chronically infected with *C. felis* and the biocontainment facilities for tick feeding, which may not be feasible for all researchers. In order to minimize the involvement of live vertebrate animals for tick infection, **Chapter 3** and **chapter 4** of this thesis described our attempts in experimentally infecting *A. americanum* ticks with *C. felis* via direct injection and *in vitro* membrane feeding. Both studies

utilized leftover diagnostic blood samples collected from cats that were naturally infected with *C. felis* as inoculums to infect ticks and eliminated the process of tick feeding on cats.

There are only two experimental models that are currently available for inducing feline cytauxzoonosis. The most frequently utilized one is the tick transmission model, as it most closely mimics the natural infection of *C. felis* [23,25,27,28]. This usually starts with acquisition feeding of *A. americanum* nymphs on carrier cats that are chronically infected with *C. felis.* After nymph repletion and the completion of molting, the adults are subsequently used for transmission feeding on naïve cats. This model has been reported to have an 85–100% successful transmission rate but requires a minimum of two months to complete (1 week acquisition feeding, 4 weeks molting, 2 weeks waiting period after molting, and 1 week transmission). The other model for experimental cytauxzoonosis in cats involve inoculation of blood or tissue from *C. felis*-infected cats. Although the timeframe of infection is shorter, the route of infection is less natural, and the host immune response or clinical course of disease may deviate from typical cytauxzoonosis. In **Chapter 5**, we proposed an alternative feline infection model using sporozoite-based inoculums. The success of this technique will serve as a basis for future vaccine development and expand the availability of these resources to *Cytauxzoon* researchers in other institutions.

The first part of this literature review provides a brief outline of the developmental process of piroplasmids in their tick hosts, which could further lend support in understanding the incompletely characterized life cycle of *C. felis.* The second half discusses several experimental tick infection models that have been established for other tick-borne pathogens, with an emphasis on what has been established or attempted for other piroplasmid parasites.

PIROPLASMID LIFE CYCLES IN TICKS

The life cycles of many *Babesia* and *Theileria* species were studied in the late 1900's. As common molecular detection tools were not yet available then, researchers relied primarily on direct visualization of the parasites (e.g. light microscopy and electron microscopy) to characterize the parasite life stages. Most of this work also largely contributed to the taxonomic classifications of these parasites, which were later refined via molecular phylogenetic analyses [21,29].

- ***Theileria***

It is generally believed that all *Theileria* species follow a similar developmental process within their tick hosts, with some minor differences in their sizes, morphologies, and developmental timelines. The major differentiations among *Theileria* species that infect ruminants lie in their designated mammalian hosts and the features of the schizogenous phase. This further divide the genus into 2 major groups – Transforming *Theileria* (includes *T. parva, T. annulata,* and *lestoquardi*) and non-transforming *Theileria* (includes *T. orientalis* complex, *T. mutans, T. verlifera, T. cervi*) [30]. Transforming *Theileria* spp. is known for their capabilities of indefinite multiplication within the host leukocytes (lymphocytes or macrophages), resulting in neoplastic transformation of these host cells. In contrast, the schizogenous phase for non-transforming *Theileria* spp. is thought to only be transient prior to the erythrocytic life stages. Aside from these *Theileria* groups, *T. equi* is often discussed separately as its taxonomic classification has been heavily debated. However, it is now acknowledged that *T. equi*'s life cycle indeed bear more similarities to non-transforming *Theileria* species [31].

The vector life cycle of *Theileria* begins when the parasites are first ingested by the tick host during a blood meal, and they undergo sexual reproduction within the tick's midgut (Figure

3). It is generally believed that the only form that are capable of further development within the ticks are gametocytes (or gamonts in earlier studies) [32–34]. The gametocytes then differentiate into morphologically distinct sexual stages (macro- and microgametes or "ray bodies") within the tick midgut lumen within 24 hours after repletion. These two sexual forms fuse to form zygotes that invade the midgut epithelial cells where they further transform into mobile kinetes. Depending on the species of *Theileria,* the timeframe from repletion to zygote formation vary from 12–30 days. Kinetes then egressed from the midgut cells into the hemolymph by day 16–34 days after repletion. Differentiated and free kinetes are described as spindled teardrop shaped under light and electron microscopy and are capable of invading salivary gland cells as early as day 18 after repletion [35–39]. The kinetes' invasion of salivary glands often coincides with the completion of tick molting but has also been observed to occur prior to this timepoint [40,41].

Once the kinetes have successfully entered the salivary glands, they undergo asexual replication by first transforming into large polymorphous syncytial cells within the infected acinar cells (sporonts). The kinetes appear to have an affinity for D or E cells in type III salivary acini and the infected salivary acinar cells become swollen with an increased cytoplasmic mass that can be visualized under light microscopy [42–44]. The syncytium further differentiates into variably-sized cytomeres (sporoblasts) which then divide into individual sporozoites. The percentage of infected salivary glands can vary among ticks and a single infected acinar cell is estimated to contain as many as 50,000 sporozoites by the completion of sporogony. As sporozoites mature, they are released from the salivary glands into the mammalian host via tick saliva in a trickled fashion during a blood meal and capable of inducing clinical disease at this stage to complete their life cycle [40,45–49]. In most *Theileria* species, sporogony is only triggered by the onset of tick feeding, but mature sporozoites have been documented prior to this

timepoint in *T. equi* [45]. It is also important to note that *Theileria* development within ticks are strictly transstadial as there has been no evidence thus far of transovarial transmission from female ticks to the eggs or offspring [50].

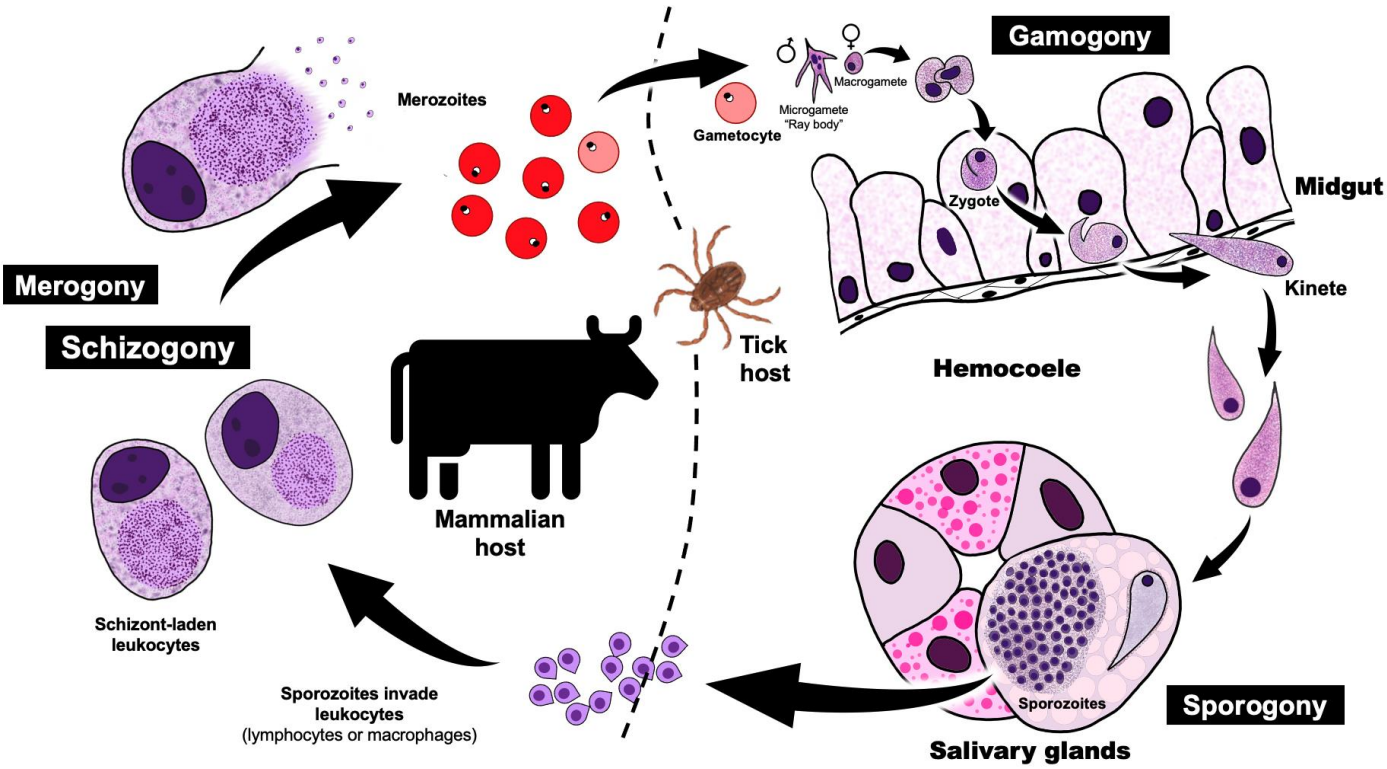

Figure 3. *Theileria* spp. Life Cycle.
Similar to *C. felis, Theileria* spp. also undergo schizogony within leukocytes (lymphocytes or macrophages) after invasion by sporozoites upon tick feeding. Depending on the species of *Theileria*, these schizont-laden cells may transform and undergo continuous proliferation. Merozoites released from ruptured schizonts then invade erythrocytes. Once ingested by the tick, gametocytes undergo gametogenesis within the tick midgut lumen and the gametes fuse into zygotes. Zygotes transform into kinetes that leave the midgut to invade the salivary glands and undergo sporogony to form sporozoites.

- ***Babaesia***

The life cycle of *Babesia* species mainly differ from other piroplasmids by the lack of schizongenous phase of development and that merogony occurs strictly within the erythrocytes in the mammalian hosts (Figure 4). The life cycle within the vector vary among *Babesia* species. *Babesia* sensu stricto group (e.g. *B. canis, B. bovis* and *B. bigemina*) is distinguished from *Babesia* sensu lato group based on the occurrence of transovarial transmission between generations [29,51,52].

The initial phases of life cycle progression for *Babesia* species mirror that of *Theileria,* where micro- and macrogametes fuse into zygotes within the midgut lumen and the zygote penetrates the peritrophic membrane to enter the midgut epithelial cells to develop into kinetes [53]. From this point on, the *Babesia* progression in the tick begin to differ from *Theileria* species. Instead of directly invading the salivary glands, the kinetes first invade other somatic tissues such as Malpighian tubules, nephrocytes, fat body to further form and multiply into secondary kinetes (sporokinetes in some literature) via asexual reproduction. This can occur as early as 2–6 days after repletion. At this stage, this process also occurs in the ovary in *Babesia* sensu stricto species, facilitating the transovarial transmission in eggs and subsequent offspring [54–57]. The secondary kinetes reenter the hemolymph to reach the salivary glands, where they undergo sporogony that is similar to *Theileria* to produce mature sporozoites. This process is also generally prompted by tick attachment like *Theileria* and happens between 3–16 days after the tick is attached and actively feeding. The numbers of sporozoites produced per cell are estimated to be lower than *Theileria* species, ranging from 5000–10,000 per cell [58–65].

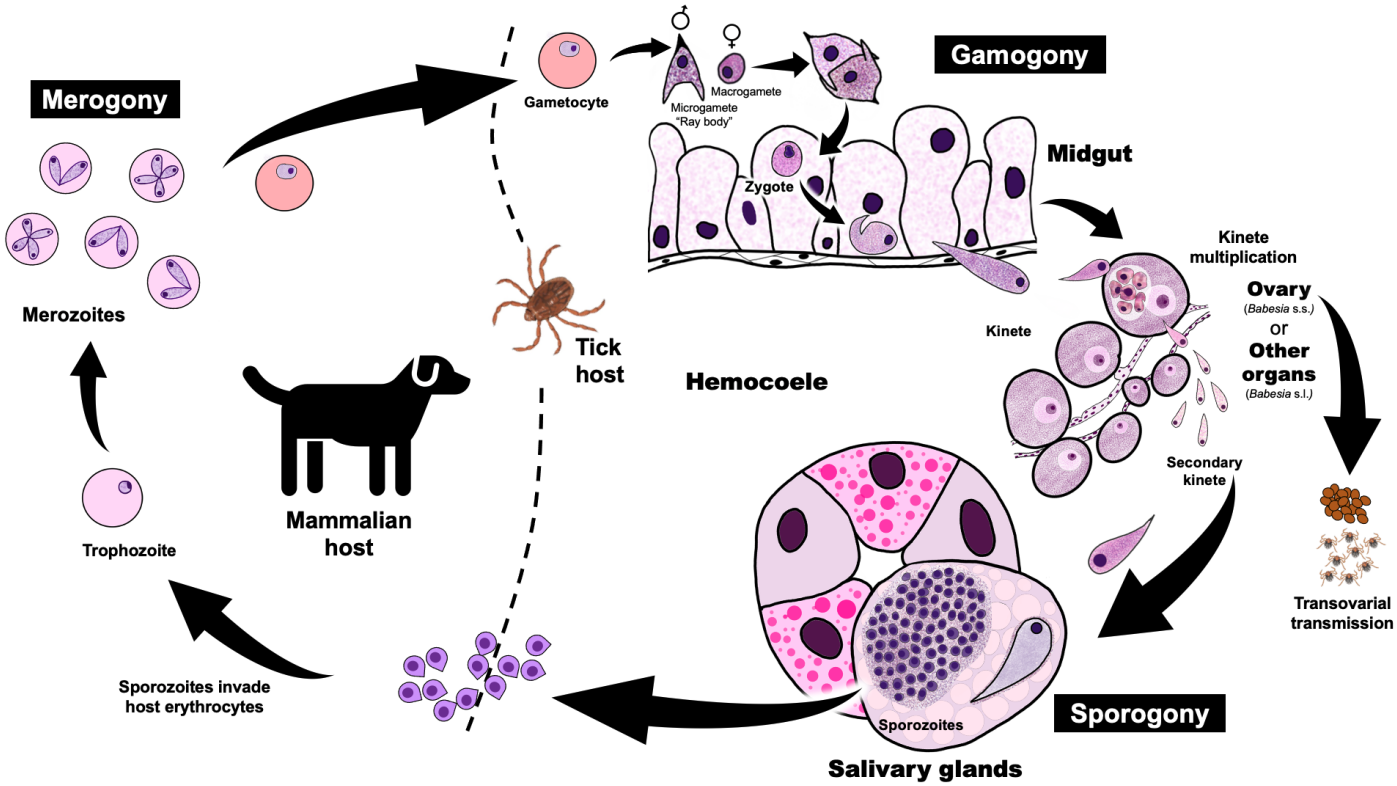

Figure 4. *Babesia* spp. Life Cycle.
Babesia spp. life cycle differs from *Theileria* spp. and *Cytauxzoon* spp. with the absence of schizont formation and merogony occurs solely within the erythrocytes. Parasite development within the vector mirrors *Theileria* except *Babesia* kinetes are capable of further dividing within other organs and form secondary kinetes (or sporokinetes). In *Babesia* sensu stricto (*Babesia* s.s.) species, kinetes also invade the ovary, facilitating transovarial transmission to eggs and offspring. This process does not occur for *Babesia* sensu lato (*Babesia* s.l.) species. Secondary kinetes then invade salivary glands to undergo sporogony and form sporozoites.

TICK INFECTION MODELS OF PIROPLASMIDS

The establishment of successful infection in ticks is typically the first hurdle in most tick-borne pathogen (TBP) research. In a natural setting, ticks typically acquire TBP through a blood meal when feeding on an infected animal. There are several alternative tick infection techniques that have been established for other tick-borne pathogens, although all are associated with varying reproducibility and practicality.

In vivo models

Feeding ticks on infected live animals is the most biologically natural route of infection and this process is often referred to as "acquisition feeding". Live animal feeding is therefore the most traditional practice to infect any stages of ticks with various classes of TBP using a wide range of animal species (**Figure 5**). *In vivo* models are also most commonly used to determine the competency of a potential vector by assessing the ticks' ability to acquire the pathogen and successfully transmit to a naïve animal [66–69].

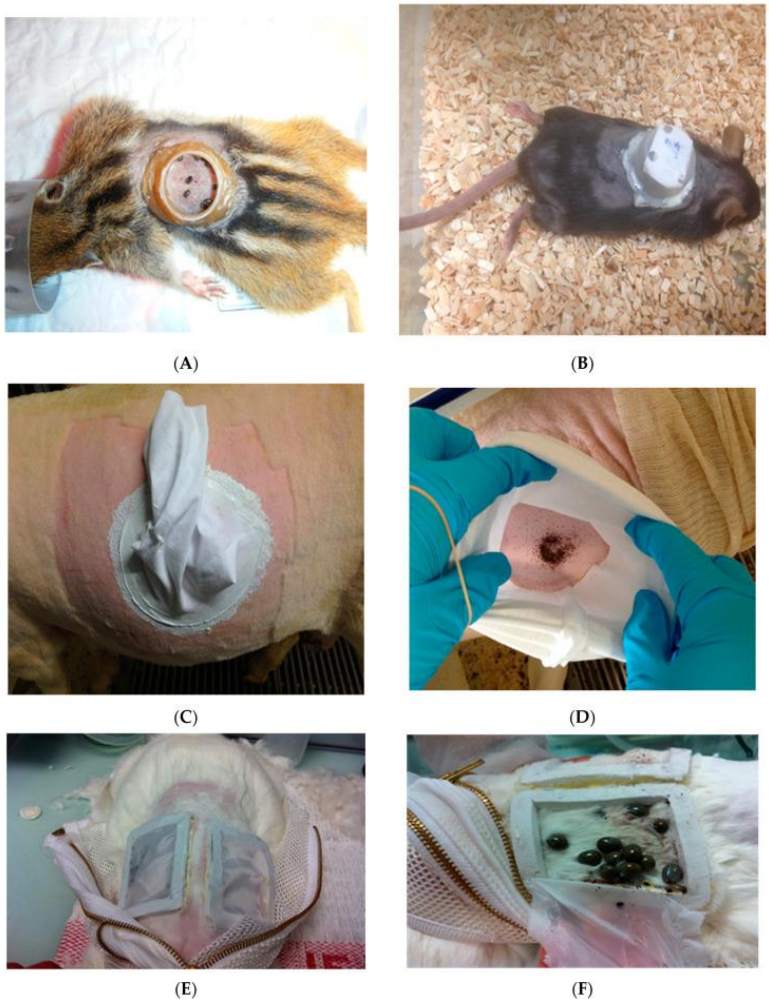

Figure 5. Various tick confinement devices for *in vivo* tick feeding (from Bonnet et. al 2021). (A–B) Small capsules on smaller animal models such as chipmunks and mouse. (C–D) Cloth patches that are glued directly on animal's skin on sheep. (E–F) Multiple feeding chambers composed of openable capsules on rabbits.

As some piroplasmid parasites organisms may be difficult to cultivate or maintain *in vitro*, chronically infected laboratory animals are often the most reliable source for tick infection. Experimental *in vivo* infections of vector ticks for tick-borne piroplasmids have been successfully accomplished for 11 Babesia species (*B. microti, B. divergens, B. venatorum, B. bovis, B. bigemina, B. bovis, B. major, B. ovata, B. caballi, B. ovis, B. canis,* and *B. rossi*) [70–93] and 6 *Theileria* species (*T. parva, T. annulate, T. lestoquardi, T. sargenti, T. orientalis,* and *T. equi*) [94–102]. Acquisition feeding is mostly done using their natural host species as sources, which include rodents, cattle, horses, sheep, and dogs. The setup for tick feeding is mainly focused on biocontainment of the ticks, especially when working with pathogens or tick species that are not native to where the experiments are being conducted. These involve various types of contraptions (bags, capsules, or cloths) to contain the ticks that are either taped or glued onto the animals (skin or ear). In some studies, ticks were allowed to freely roam on the animal and animal facilities are surrounded by moats to prevent accidental release of ticks.

Despite the common use and high success rate of *in vivo* infection and transmission, there are several considerations when using vertebrate animals for acquisition feeding. First, long-term maintenance for chronically infected animals in a laboratory setting is expensive. This is especially true for *Theileria* and *Babesia* spp. of veterinary importance since most of their mammalian hosts are large animals. Second, the process of acquisition feeding can be stressful, and the disease induced by TBP are inherently linked with some ethical concerns. For instance, in most *T. parva* studies, acutely infected calves used for acquisition feeding were not treated for Theileriosis and often euthanized at the end of the feeding period due to the severity of the clinical disease [49,94]. Third, some tick species have specificity or preference only for certain hosts, which may limit the design or resources for the experiments. For this reason, if the ticks'

natural host is wildlife or difficult to maintain in a laboratory setting, it may not be feasible to utilize these animals for tick infection [103]. Lastly, the host animal's parasitemia level may be difficult to predict at the time of feeding, limiting researchers' abilities to adjust or titrate dosages used for tick infection.

In part to maximize infection rate and parasite load in acquisition-fed ticks, most experimental studies for *Theileria* and *Babesia* perform acquisition feeding on animals that are acutely infected and when parasitemia is high. This was demonstrated in a few studies that the ticks fed on chronically infected animals generally had a lower infection rate compared to those fed on hosts with high parasitemia [104–106]. However, some studies suggested that the strains and biological differences among ticks may play of bigger role in influencing the ticks' ability to acquire pathogens than the parasitemia level of the host [107,108].

Besides utilizing the parasites' natural host species, some studies have demonstrated the feasibility of using smaller animal models like severe combined immunodeficient (SCID) mice to culture the parasites *in vivo*. This has been successfully done for several *Babesia* and *Theileria* species by transfusing the mutant mice with piroplasmid-infected mammalian blood [109–111]. While the parasites remain viable with evidence of multiplication, the parasitemia often vary among animals and parasite morphology also appeared atypical. Stemming from this concept, a mouse-tick infection model was similarly developed by transfusing the splenectomized SCID mice with *T. orientalis*-infected bovine blood [112]. Larval vector ticks that were fed on this model became successfully infected with *T. orientalis.* This tick infection model has not been utilized for other piroplasmids, and its efficacy and practicality remain to be determined.

In vitro models

To minimize the use of live animals for tick feeding process, several alternative methods have been developed to emulate or bypass tick feeding in a laboratory setting [103]. These methods consist of varied methods of injection and *in vitro* feeding systems (membrane vs. capillary feeding) and have been described to successfully infect ticks with both tick-borne bacteria, viruses and protozoal pathogens. Comparatively, there are far more studies documenting the successful use of artificial feeding systems to infect ticks than injections. It is unknown whether this is because artificial feeding systems have a higher success rate or researchers are simply more drawn to feeding systems as they are more biologically sustainable. The utilizations along with the strengths and drawbacks that are associated these systems are discussed below.

- **Capillary feeding**

This is the first artificial feeding technique described in TBP research [113]. To infect ticks via this method, a capillary tube is manually pulled and broken to match the size of ticks' mouthpart (hypostome). The capillary tube openings are nestled between the paired palps to stimulate salivation and feeding (**Figure 6**). The same method can also be used for tick saliva collection using an empty capillary tube together with a muscarinic receptor agonist (e.g. pilocarpine) [114,115]. The main advantage of capillary feeding lies in the adjustable quantity and the ability to designate the type of inoculum used. However, the set-up is laborious and require precision under a dissection microscope [103]. Although this technique has been widely used to infect ticks with TBP of all classes [116–118], it is noted to be the least efficient among other means for artificial tick feeding and infection due to the inability to facilitate a large-scale

study. Additionally, this technique is unable to sustain the tick through the entire feeding period to repletion and often still requires pre-feeding on live animals prior to capillary tube feeding. This negates the goal of minimizing live animal usage in tick-borne disease research [103,119].

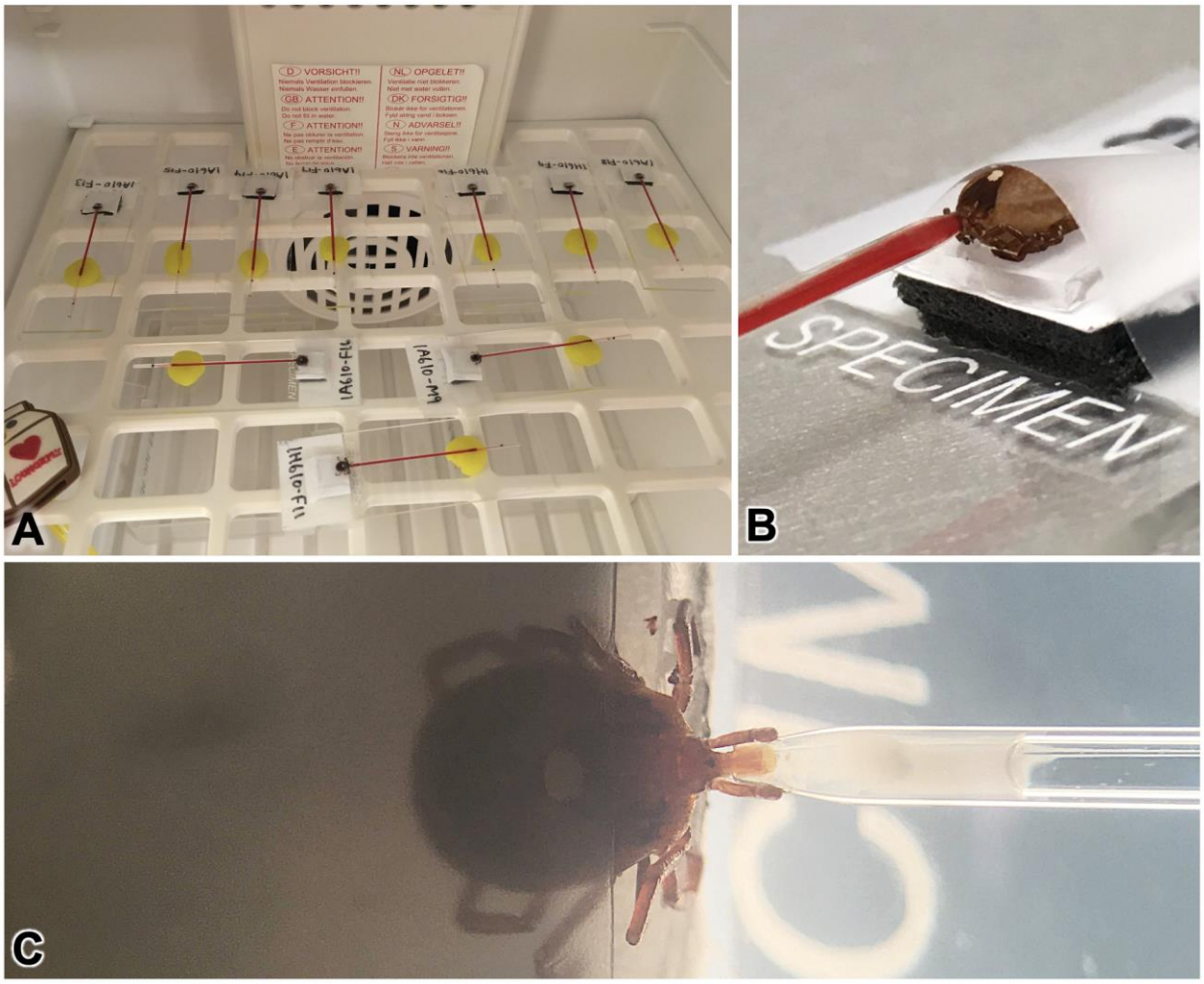

Figure 6. Capillary feeding for ticks. (A) Typical setup for capillary feeding comprised of ticks taped on a glass slide, pulled capillary tube loaded with blood on a slightly raised platform in a 37ºC incubator. (B) A partially fed *Amblyomma americanum* adult female demonstrating the capillary tube has a narrowed base and nestled between the palps over the hypostome. (C) Saliva collection from a partially fed *A. americanum* female using the capillary feeding system.

Capillary feeding was first used for *Theileria parva* with the objective to stimulate sporozoite formation. After a 4-day feeding period on either rabbit or cattle, *T. parva*-infected *Rhipicephalus appendiculatus* adults were able to secrete infectious sporozoites into capillary

tubes filled with uninfected bovine blood or serum. The remaining blood or serum within the capillary tube was found to be infectious to naïve cattle [120]. The efficacy of capillary feeding was also compared to direct injection by Walker et al, and ticks infected by either method were capable of inducing fulminant theileriosis in naïve cattle [121].

Due to the small and easily adjustable feeding volume of a capillary tube, this technique was specifically used to test newly recognized anti-*Babesia* proteins in two recent studies. Antibodies against these novel proteins were fed to the ticks via the capillary tubes to assess their impacts on parasite acquisition [122,123]. These studies did highlight the low throughput aspect of this feeding system, which is often compounded by biological differences between ticks. Therefore, this may not be the most suitable setup for acquisition feeding which typically requires larger quantities of ticks to maximize the number of infected ticks.

- **Membrane feeding**

Membrane feeding is the most frequently utilized technique to artificially infect ticks as it is thought to be the most biologically natural with the closest simulation to the tick natural feeding process. The technique is known to be customizable for all tick life stages and species. The process of tick feeding is extremely complex and generally involve several elements in the macro- and microenvironment. Some factors that can influence tick behavior during feeding may include ambient temperature, humidity, diurnal rhythms, tick age, and appropriate sensory stimuli, etc [124]. The set-up of an *in vitro* feeding system typically involves a feeding interface (synthetic or animal-derived membrane) that is fitted to a feeding unit and submerged in a feeding source (usually animal blood or pathogens suspended in culture medium) [125–128].

These models are versatile and have been used for tick infection, evaluate pathogen transmission, and *in vitro* acaricide testing [126,129].

The feeding systems that have been reported in the literature widely vary in their setups, ranging from simple assemblies using culture plates and clear PVC pipes [126,130], to specially manufactured apparatuses (Figure 7) [131–133]. Evidently the biggest obstacle for this infection technique is low tick attachment rates. Therefore, the process of optimizing the system for tick species of interest is extremely critical to promote tick attachment. For instance, in addition to assembling the feeding units, external stimuli to prompt tick attachment and feeding should be thoroughly considered and tested for the tick species of interest [125,129]. Such stimuli may include specific animal hair scent or extract, textural stimuli, oxygenation and carbon dioxide level, and temperature of the attachment surface. Depending on the system set-ups, membrane feeding may require larger feeding volumes compared to other infection techniques, as well as frequent daily blood exchanges for several days to weeks [130,134]. Some well-established systems feature fully automated attachments to facilitate continuous blood circulation during tick feeding, allowing a feeding period to last as long as 24 hours without changing blood or membrane wash [57,133]. The types of membranes that had been documented also vary, from silicone-based membranes used in most studies to membranes that are made from animal skins [43,57,135].

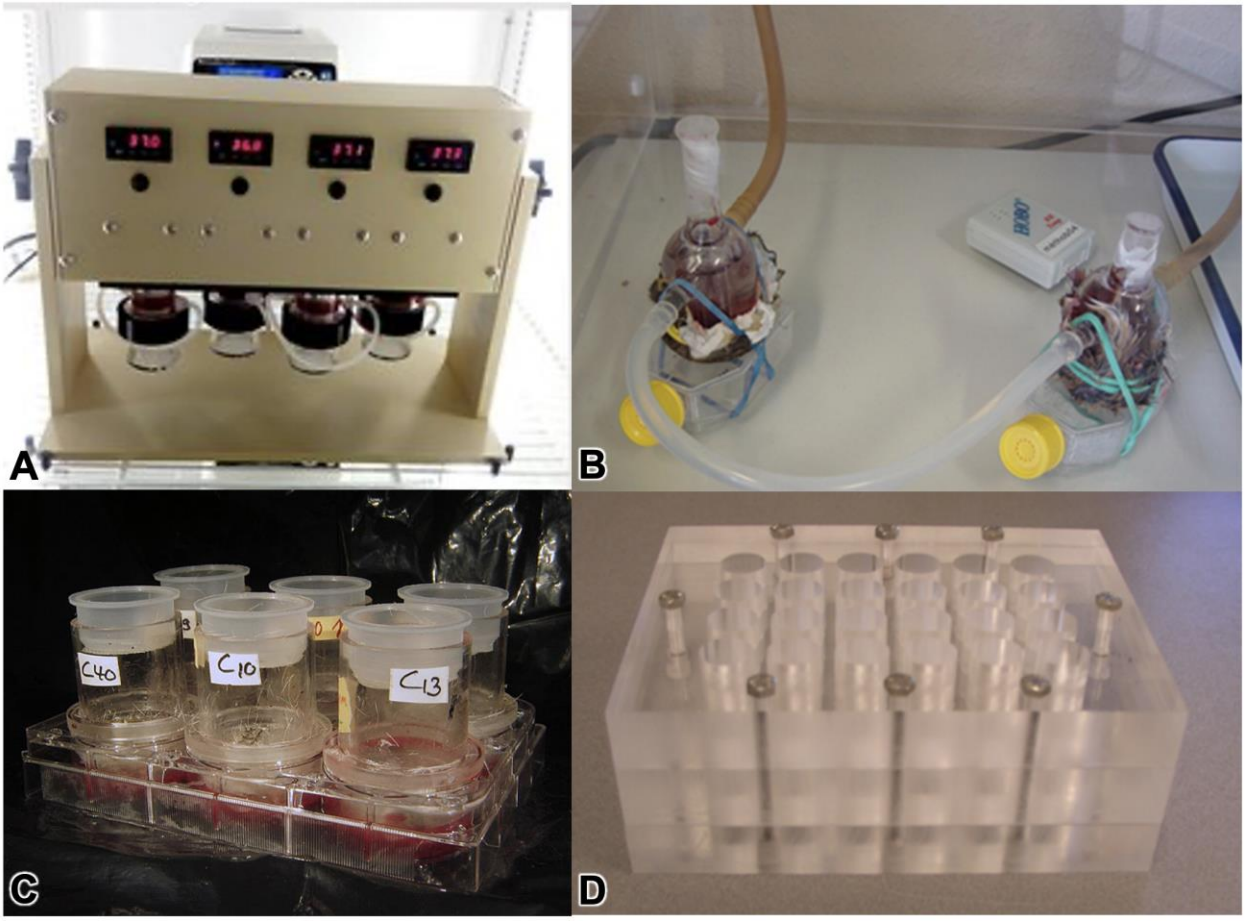

Figure 7. *In vitro* membrane feeding systems for Ixodid ticks. (A) An automated feeding system from Vimonish et al. (2021) that are specially manufactured with a base heating unit and blood connected to an automatic pump to facilitate continuous blood circulation throughout the feeding period. (B) A semi-automated feeding system from Bonnet et al. (2007) used to infect ticks with *Babesia*-infected bovine blood. The system is connected to continuous source of warm water to keep blood at a constant temperature during feeding. The ticks are confined within the culture flasks that is glued to a mouse-derived membrane. (C) Basic setup for *in vitro* membrane feeding from Krober and Guerin (2009) that is comprised of acrylic feeding chambers submerged in a 6 well culture filled with blood in a 37ºC incubator. (D) Manufactured perforated stacked Perspex plates layered with membrane, ticks, and netting in between (Trentelman et al. 2017)

Membrane feeding has been documented in at least 3 *Theileria* species: *T. parva, T. mutans,* and *T. lestoquardi* [43,95,136,137]. For *T. parva,* when membrane acquisition feeding was compared directly with the *in vivo* model using cattle, the tick molting rate and infection rate was comparable between the two methods. These membrane-fed ticks were also capable to transmit the infection to naïve cattle when challenged [138]. Interestingly, in another report,

sporogony appeared to be delayed with *in vitro*-fed ticks when compared to ticks that were stimulation-fed on rabbits based on light microscopic examination [139]. In a recent study for *T. lestoquardi,* even though the membrane-fed ticks were capable of transmitting the infection to naïve sheep, these infected animals only showed mild clinical signs and low parasitemia. This contrasts with the typical clinical picture of the highly fatal and fulminant disease that is associated with *T. lestoquardi* infection in sheep and goats [140,141]. This could be an indication that this system is less efficient at times for acquisition feeding depending on the tick species and pathogens.

Successful tick infection via *in vitro* membrane feeding for *Babesia* spp. had only been documented for *B. divergens* and *B. ovata.* All of these studies described a semi-artificial feeding system where mouse skin was used as the membrane. The feeding system described in Bonnet et al. was set up where continuous warm blood circulation apparatus was set atop the feeding chamber [57]. The *B. ovata* studies adapted a stationary system that was adapted from Hatta et al. [142]. Through this feeding system, the authors were able to investigate the tick-*Babesia* relationship within *Haemaphysalis longicornis* ticks as well as confirm the occurrence of transovarial transmission for *B. ovata* [116,143].

- **Direct injection**

Direct injection (figure 8) is an established infection method for all classes of TBP, including *Anaplasma, Hepatozoon, Rickettsia, Borrelia, Babesia,* and *Theileria* [144–149]. This method is likely the simplest of most *in vitro* infection techniques as it bypasses tick feeding and molting process, and only requires a microinjection device for injection. Other than the ability to determine the type and quantity of the inoculum, this technique also allows for a tighter control

of experimental conditions. However, this injection process is labor-intensive and may result in higher tick mortality. Most importantly, as injection is a physiologically unnatural infection process, the injected microorganisms may not be able to further progress naturally and fully develop within the tick [103,150]. Therefore, it is imperative to consider or incorporate these potential physiologic stimuli in the experimental design especially when working with pathogens that have complex life cycles within the ticks, such as tick-borne hemoprotozoa [50,151].

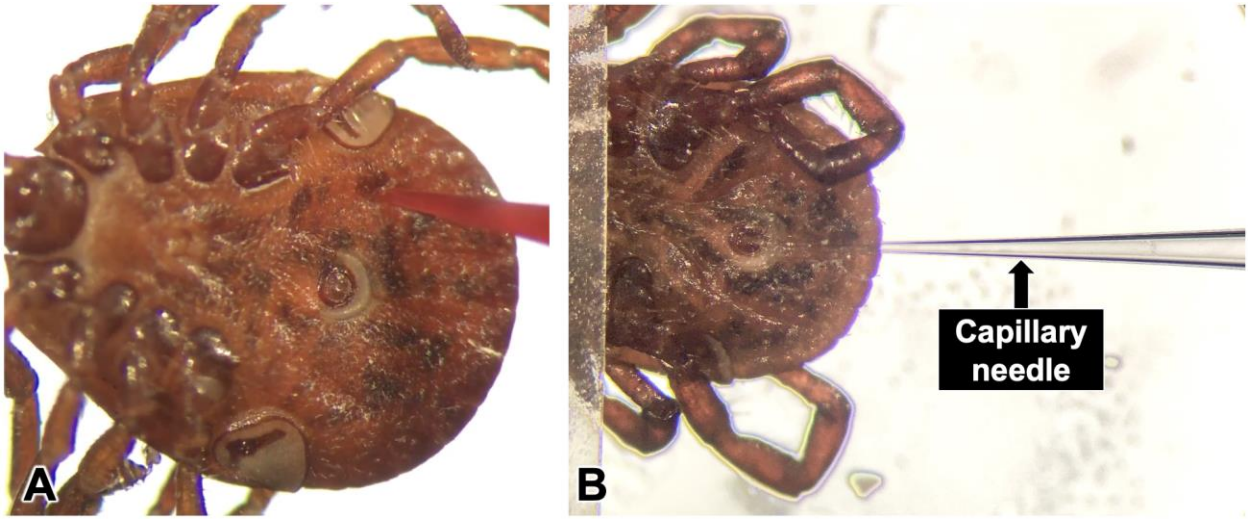

Figure 8. Tick infection via direct injection. (A) An *A. americanum* tick injected directly in the hemocoele via a custom-pulled capillary needle loaded with inoculum. (B) An *Rhipicephalus sanguineus* injected in the anal pore into the midgut.

Specifically for piroplasmids, direct injection as a mean for tick infection had only been documented in a few studies. The first attempts in 1979 and 1980 were both for *T. parva.* In these studies, repleted *Rhipicephalus appendiculatus* nymphs were injected with infected bovine blood that was collected from an experimentally infected calf with acute theileriosis. The inoculums were directly injected into the midgut or hemocoele using tuberculin syringes and hypodermic needles. Using either freshly collected infected blood or cryopreserved inoculums, the injected ticks were able to successfully transmit the infection when challenged on naïve

calves [121,148]. Despite these successes, it is unclear how widely utilized this technique is among *Theileria* researchers as there has not been additional related publications citing this as their infection technique for ticks.

Successful tick infection via injection was only demonstrated for two *Babesia* spp. This method was attempted for *Babesia gibsoni* in a non-vector soft tick *Ornithodoros moubata.* The ticks that were injected with *B. gibsoni*-infected dog blood were not only detectable in their ovaries after injection, but the injected ticks were also capable of transmitting the infection to naïve dogs [147]. More recently, injection was also used for *B. ovis* to compare differentially expressed genes in the salivary glands that are associated with feeding and infection. The injection method allowed the investigators to control the infection status of the ticks, but these ticks were not further challenged for their abilities to transmit the injected parasites [152]. Similar to the aforementioned *Theileria* injection studies, it is unclear if this model is still being utilized since the publication of this study and the practicality of this method remains questionable.

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CHAPTER 2

Establishing a Practical Protocol to Prepare Paraffin-embedded Whole Tick Histology Sections for Ixodid Ticks

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Establishing a Practical Protocol to Prepare Paraffin-embedded Whole Tick Histology for Ixodid ticks

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ABSTRACT

Ticks are important ectoparasites that are capable of transmitting multiple classes of pathogens and are currently linked with many emerging tick-borne diseases worldwide. With increasing occurrences of tick-borne diseases in both humans and veterinary species, there is a continuous need to further our understanding of ticks and the pathogens they transmit. Whole tick histology provides a full scope of the tick internal anatomy, allowing researchers to examine multiple organs of interest in a single section. This is in contrast to other techniques that are more commonly utilized in tick-borne disease research, such as electron microscopy and light microscopy of individual organs. There is a lack of literature describing a practical technique to process whole tick histologic sections. Therefore, the current study aims to provide researchers with a workable protocol to prepare high quality paraffin-embedded whole tick histology sections. *Amblyomma americanum* adults were used as an example species for this study. After a series of pilot experiments using a combination of various fixatives, softening agents and processing techniques, we elected to compare two common fixatives, 10% neutral-buffered formalin (NBF) and Bouin's solution. Equal numbers of *A. americanum* adults (n = 10/fixative) were processed identically and their whole tick histology sections were individually scored. Higher scores were assigned to whole tick sections that contained more internal organs that are crucial for tick-borne disease research (e.g. salivary glands and midgut), high integrity of tissues

and exoskeleton on the section, and good fixation and staining quality of the tissues. The mean total scores for Bouin's-fixed ticks were significantly higher compared to NBF-fixed ticks ($p = 0.001$). To further assess our preferred technique, we also demonstrated the feasibility of producing high quality whole tick sections for three other common tick species of medical importance (*Rhipicephalus sanguineus, Ixodes scapularis,* and *Dermacentor variabilis*) using Bouin's solution. While this technique may require further optimization for other tick species, we described a feasible protocol that uses commonly available tools, reagents and standard histologic equipment. This should allow any investigator to easily make adjustments to this protocol as needed based on their experimental goals.

1. INTRODUCTION

Ticks are ectoparasites and important arthropod vectors that affect humans, livestock, wildlife, and companion animals worldwide. Tick's ability to transmit a wide range of pathogens, which include a variety of bacteria, viruses, and protozoa, make them a major public health concern [1–3]. Due to the illnesses that they cause, ticks also contribute to significant economic losses in the livestock industry [4,5]. With new pathogens emerging and the increasing occurrences for tick-borne diseases in both humans and animals, there is a continuous need to further our understanding of ticks and the pathogens they transmit. As many research groups rely on molecular techniques to study these tick-borne pathogens, the associated tick pathology is often overlooked and infrequently highlighted.

Even though light microscopy is considered one of the most utilized techniques in biological research [6–8], tick histopathology is an uncommonly used tool to study tick-borne diseases. With significant advances in *in situ* techniques, such as immunohistochemistry (IHC) and *in situ* hybridization (ISH) in recent years [9–11], there is growing potential to apply these methods and contextualize the interaction between pathogens and the tick hosts.

Historically, due to the small size and intracellular nature of most tick-borne pathogens, scanning and transmission electron microscopy (EM) have been instrumental in characterizing the relationship between the pathogens and their tick vectors [12–15]. When light microscopy was used, many researchers dissected out the tick tissue of interest to prepare for wet mounts or histological sectioning [16–20]. While EM provides ultrastructural details and dissection ensures precision in tissue identification, these methods do not allow for appreciation of the anatomical relationship and overall dissemination of pathogens within the ticks. Additionally, these

techniques often require proficient technical expertise, and the preparation process can be labor-intensive [21,22].

In contrast to these traditional techniques, whole tick histology provides a full scope of the tick internal anatomy, allowing researchers to examine multiple organs of interest in a single section. However, the preparation of whole tick histology is often challenging due to the rigid and impermeable nature of the tick's chitinous exoskeleton [23,24]. While this problem can be overcome by embedding the specimens in hard media like epoxy resin [25–27], the sectioning process calls for specialized equipment and technique that is not routinely available in many standard histology laboratories. There is also a lack of literature in describing a working technique to process whole tick histologic sections. Therefore, the current study aims to provide researchers with an optimized protocol to prepare high quality paraffin-embedded whole tick histology sections.

Amblyomma americanum was used as an example species for this study due to their medical importance for both animal and human diseases. A series of pilot experiments were conducted using a combination of various fixatives, softening agents and processing techniques. Based on these results, we elected to compare two common fixatives, formalin and Bouin's solution. The whole tick sections were scored based on integrity of the section, presence of organs that are crucial for tick-borne disease research (e.g. salivary glands and midgut), and tissue staining quality. We also assessed the feasibility of whole tick sectioning for three other common tick species of medical importance (*Rhipicephalus sanguineus, Ixodes scapularis,* and *Dermacentor variabilis*) using Bouin's solution (our preferred technique based on the results).

2. MATERIAL AND METHODS

2.1 Tick maintenance

All ticks used were purchased from Oklahoma State University tick-rearing and maintained in conditions that were recommended by the rearing facility (12 hour light/12 hour dark cycle period, 90% humidity, and 25ºC).

2.2 Pilot experiments

Five different fixatives (70% ethanol, PenFix, 10% neutral-buffered formalin, Bouin's solution, and Davidson's solution) with or without softening agents (5% nitric acid, RapidCal, sodium thioglycolate, and 10% potassium hydroxide) were evaluated during the preparation of whole tick sections. Fixation times from 12 to 48 hours and the addition of a vacuum chamber were also assessed. Pilot experiments are outlined in supplemental material.

2.3 Optimized preparation of whole tick histologic sections

The optimized protocols for whole tick histologic sections for either Bouin's solution or 10% neutral-buffered formalin (NBF) are presented in Figure 1. Briefly, ticks were first placed in 100% methanol for 24 hours then placed in fixative (either 10% NBF or Bouin's solutions) for 48 hours. After fixation, the mouthpiece and posterior tip of the scutum were removed using a scalpel blade to facilitate paraffin infiltration during processing. Ticks were embedded dorsal side down in Histogel in a small Cryomold (10×10×5 mm) to facilitate tissue orientation prior to histology processing and sectioning. Histotechnicians made coronal sections (sectioning direction: dorsal to ventral) of embedded ticks using standard microtomes. We selected this sectioning orientation with the consideration that that a tick has a dorsoventrally flattened body, and coronal sections would allow us to visualize the most organs in one view as compared to

sagittal sections. Three 5 µm thick sections were produced per tick on positively charged slides, then stained with hematoxylin and eosin stain (H&E).

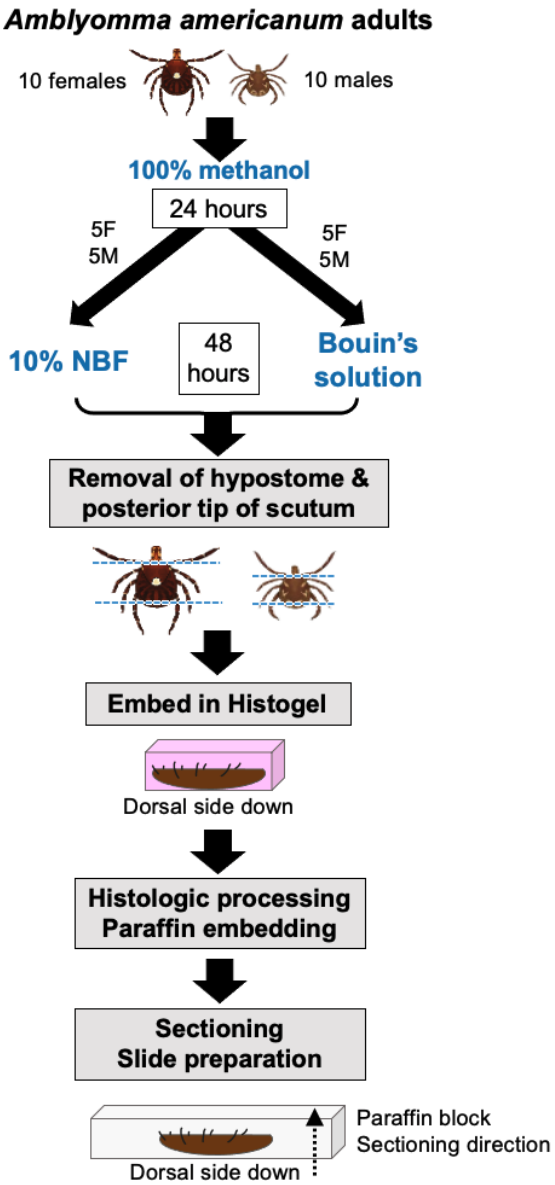

Figure 1. Preparation of *Amblyomma americanum* whole tick histology using 10% neutral-buffered formalin vs. Bouin's solution.

2.4 Comparison of Bouin's solution and 10% NBF for whole tick sectioning

We selected Bouin's solution and 10% NBF for a head-to-head comparison using flat (unfed) *A. americanum* adults. These two fixatives were selected since they are two of the most commonly used fixatives in histology laboratories and the staining quality from both fixatives were subjectively superior to others that were tested in the pilot experiments. Five female and 5 male ticks were subjected to either fixative. To compare the quality of sections produced, a scoring system was established. This system scored six criteria: A) Presence of salivary glands; B) Presence and distribution of midgut; C) Presence of other organs; D) Tissue integrity after sectioning; E) Quality of fixation/staining; and F) Exoskeleton integrity within the sections. Three sections were scored for each tick and reported as their mean score for each criterion. The sum of these mean scores was reported as the total score for each tick. The individual score assignments for whole tick sections are outlined and defined inTable 1. Basic guides of the internal organs of unfed *A. americanum* adults are demonstrated in Figures 2 (female) and 3 (male). All sections were scored independently by a board-certified veterinary pathologist (TSY).

Table 1. Scoring criteria for whole tick histology sections

	Scoring criteria	Scores			
		0	**1**	**2**	**3**
A	**Presence of salivary gland (SG)**	SG is not present	< 30 SG acini are present	30-80 SG acini are present	>80 SG acini are present
B	**Distribution of midgut (MG)**	MG is not present	MG is present in only one of 4 quadrants	MG is present in 2-3 of 4 quadrants	MG is present in all 4 quadrants
C	**Presence of other organs** ▪ **Synganglion** ▪ **Ovary (F) or testes (M)** ▪ **Malpighian tubules** ▪ **Rectal sac**	None of the other organs are present	1 of other organs are present	2 of other organs are present	>2 of other organs are present
D	**Tissue integrity**	No internal organs present in section	<30% of internal organs present in section	30–80% of internal organs present in section	>80% of whole tick tissue present in section
E	**Quality of fixation/staining**	Smudgy cellular borders and nuclear membranes	–	Well-defined cellular borders and nuclear membranes	–
F	**Exoskeleton integrity**	<50% of exoskeleton present	≥50% of exoskeleton present	–	–

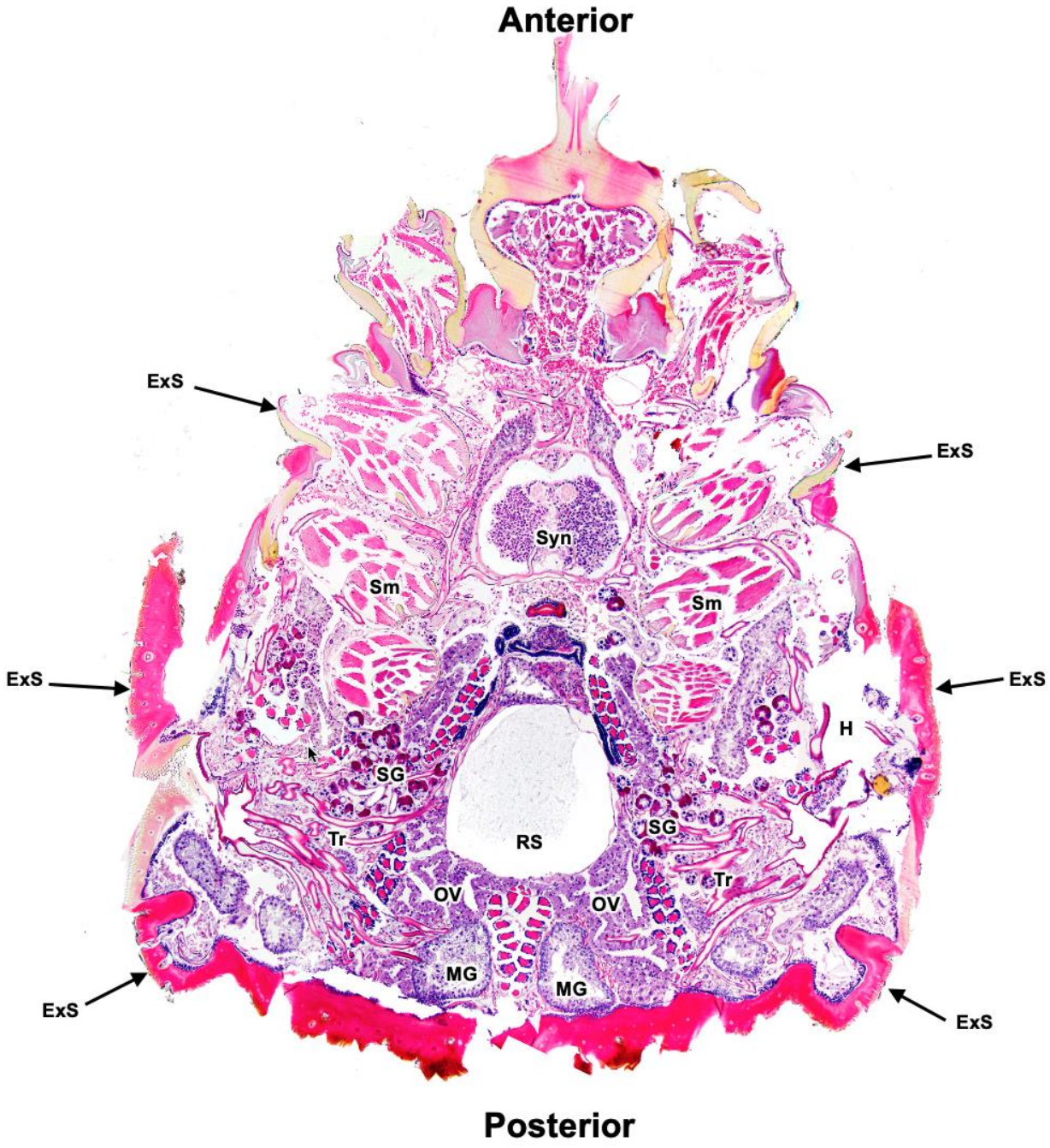

Figure 2. Female *A. americanum* internal histoanatomy (coronal section, H&E). Exs: Exoskeleton; H: Hemocoele; MG: Midgut; OV: Oviduct/ovary; RS: Rectal sac; SG: Salivary glands; Sm: Skeletal muscle; Syn: Synganglion; Tr: Tracheae.

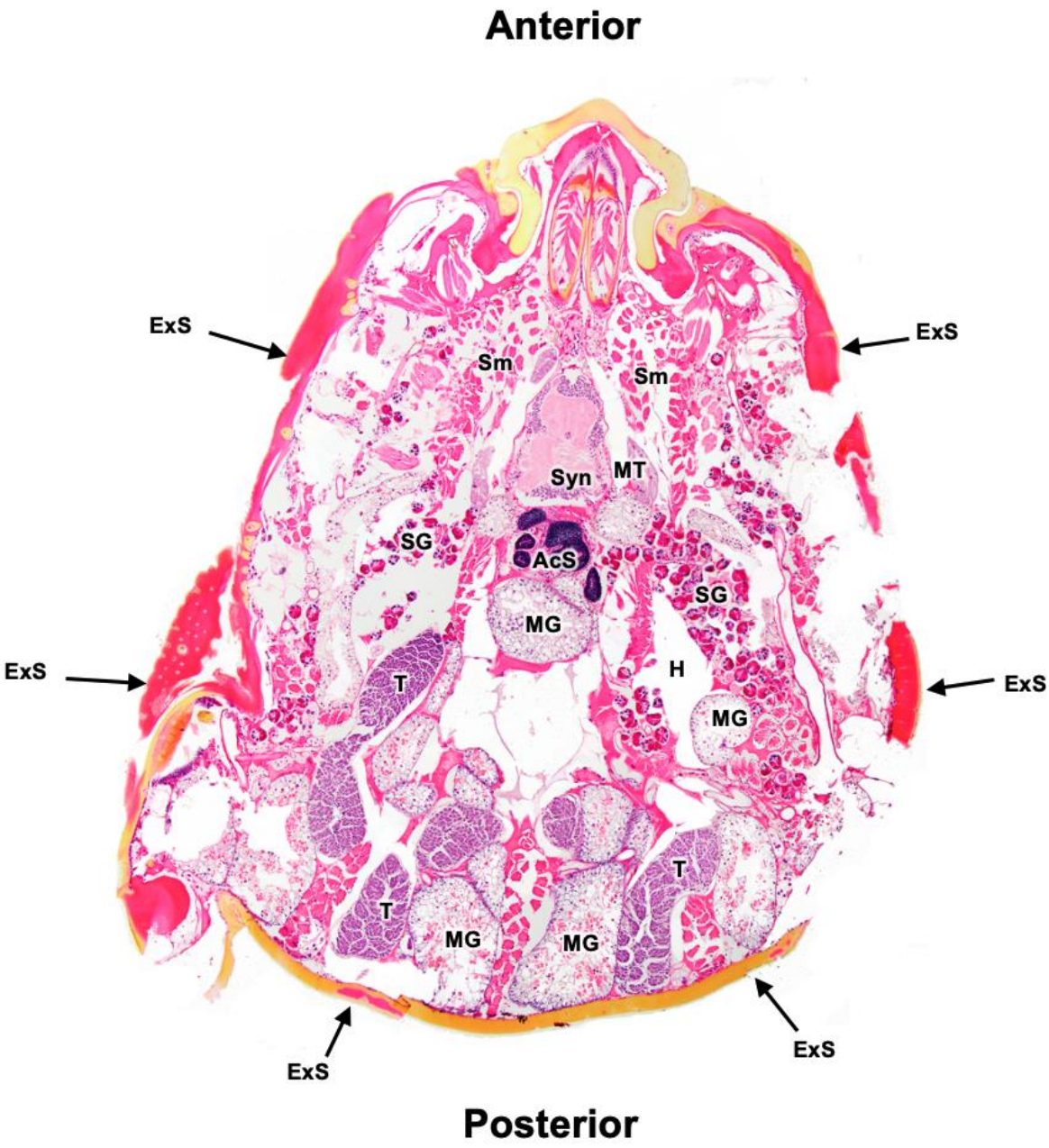

Figure 3. **Male *A. americanum* internal histoanatomy** (coronal section, H&E). Exs: Exoskeleton; H: Hemocoele; MT: Malpighian tubules; MG: Midgut; RS: Rectal sac; SG: Salivary glands; Sm: Skeletal muscle; Syn: Synganglion; T: Testes; Tr: Tracheae.

2.4.1 Statistical analysis

Based on a Shapiro-Wilk test, there was no significant departure from normality detected in the distribution of the scores. The comparison of total scores between the two fixation groups was done using the Welch-corrected t test. The significance level is set at 0.05.

Based on a Shapiro-Wilk test, scores in criteria A to E were not normally distributed. Therefore, the comparisons of these scoring criteria were done by evaluating the mean scores for each tick using a Wilcoxon rank sum test with a Bonferroni correction. The resulting p value was adjusted directly (multiplied by 5) and significance level was set at 0.05.

The comparison of scoring criterion F between the two fixation groups was done with a Fisher's exact test. For each fixation group, all individually scored tick sections (n = 30 per group) were used in this analysis. The significance level was set at 0.05.

2.5 Whole tick sectioning of additional tick species

Rhipicephalus sanguineus, Ixodes scapularis, and *Dermacentor variabilis* were used to evaluate the feasibility of our proposed processing technique in other species. Four ticks per species (2 females and 2 males) were processed identically as described in section 2.3 using Bouin's solution for fixation with a dorsal to ventral sectioning direction. Additionally, two ticks per species (one female and one male) were processed as described in 2.3 except they were embedded ventral side down in Histogel with a ventral to dorsal sectioning direction. All sections produced were scored as described in 2.4. The scores of these whole tick sections were deemed as either acceptable (score of 8 or above) or unacceptable (score of 7 or less).

3. RESULTS

3.1 Pilot experiments

Our initial experiments were performed using ticks that were stored in alcohol-based solution for 1 to 10 years (n = 8). The ticks were placed in Penfix solution, Bouin's solution, 10% KOH, or 5% nitric acid. Only one of these resulted in acceptable sections (Figure 2). This particular tick was stored for 10 years then fixed in Bouin's solution for 24 hours prior to sectioning. This timeline was not considered feasible and was not further evaluated.

Using fresh specimens (n = 109), we tested a combination of different fixatives (Davidson's solution, 70% ethanol, 10% NBF, and Bouin's solution), softening agents (5 or 10% potassium hydroxide, 5% nitric acid, commercial hair conditioner), and various preparation procedures (different orders of fixatives and reagents, usage of vacuum during fixation, partial dissections of the ticks to facilitate paraffin infiltration, and pre-embedding of ticks in Histogel prior to histologic processing). The results of these experiments were evaluated subjectively.

When compared to 70% ethanol, 10% NBF and Bouin's solution were found to have the best fixation and H&E staining quality after 48 hours (Figure 4a, b, c). This was evidenced by apparent intercellular and nuclear borders, distinct cytoplasmic details, and well-differentiated staining coloration within tissues. The duration of fixation was especially critical for Bouin's solution, as there was mild loss in cellular detail starting at 72 hours of fixation, and the tissues became brittle and friable after 96 hours of fixation. Our attempt to use Davidson's solution failed to produce acceptable sections and was not further tested.

The addition of softening agents was attempted to facilitate ease of sectioning through the rigid exoskeleton of ticks. However, we did not observe substantial improvement using softening agents (KOH, nitric acid, hair conditioner). In fact, KOH resulted in over digestion of internal organs (Figure 4d) resulting in poor cellular and tissue details. Nitric acid and commercial hair conditioner did not appear to effectively soften the exoskeleton, as whole ticks shelled out of the paraffin block when they encountered the microtome blade, or the blades became damaged due to the rigidity of the exoskeleton.

Placing ticks directly into either Bouin's solution or 10% NBF caused distortion of the tick size and shape due to tissue shrinkage or swelling during the fixation process. This resulted in absence of tissues in large proportions of some tick sections (Figure 4e, f). This was remedied by soaking ticks in an alcohol-based solution (e.g. 70% ethanol or methanol) for a short period of time (~12–24 hours) prior to fixation in Bouin's solution or 10% NBF. The use of a vacuum chamber during the first hour of fixation was attempted to assist in infiltrating fixatives through the tick exoskeleton into the hemocoele. However, we found that there was no difference in quality of fixation and staining and this step was therefore removed from our protocol.

We also discovered that the tick exoskeleton was not consistently permeable to molten paraffin during tissue processing and openings to the hemocoele would need to be created to allow paraffin access to internal organs. Incomplete paraffin infiltration led to a difference in tissue texture and was often associated with sectioning artefacts (e.g. chattering, tissue dropout) [28]. Simply removing legs or creating small slits along the scutum also resulted in poor section quality (Figures 4g, h), likely due to the small size of the openings. To overcome this problem,

we attempted several techniques ranging from the complete removal of the carapace to creating larger openings using needles or blades. All these techniques resulted in considerably improved sections, but some were more labor-intensive (carapace removal and slits created with needles) (Figure 4i). We developed a practical approach to remove the anterior and posterior end of the tick using a razor blade or scalpel blade, which resulted in complete paraffin infiltration and enhanced the consistency in quality between tick sections (Figure 5).

During the embedding process, proper orientation of the ticks within the paraffin block for coronal sectioning required considerable time, effort and training for the histotechnicians. In order to develop a process in which ticks could be sectioned by any histotechnician without any additional training and time, we elected to pre-embed the ticks in Histogel prior to submission for tissue processing and paraffin embedding. This created a material consistent in size that any histotechnician can embed in paraffin. The addition of Histogel in the protocol considerably shortened the turnaround time between sample submission and slide production by the histology lab, as the histotechnicians found Histogel blocks easier to handle and embed into paraffin blocks than to handle ticks alone.

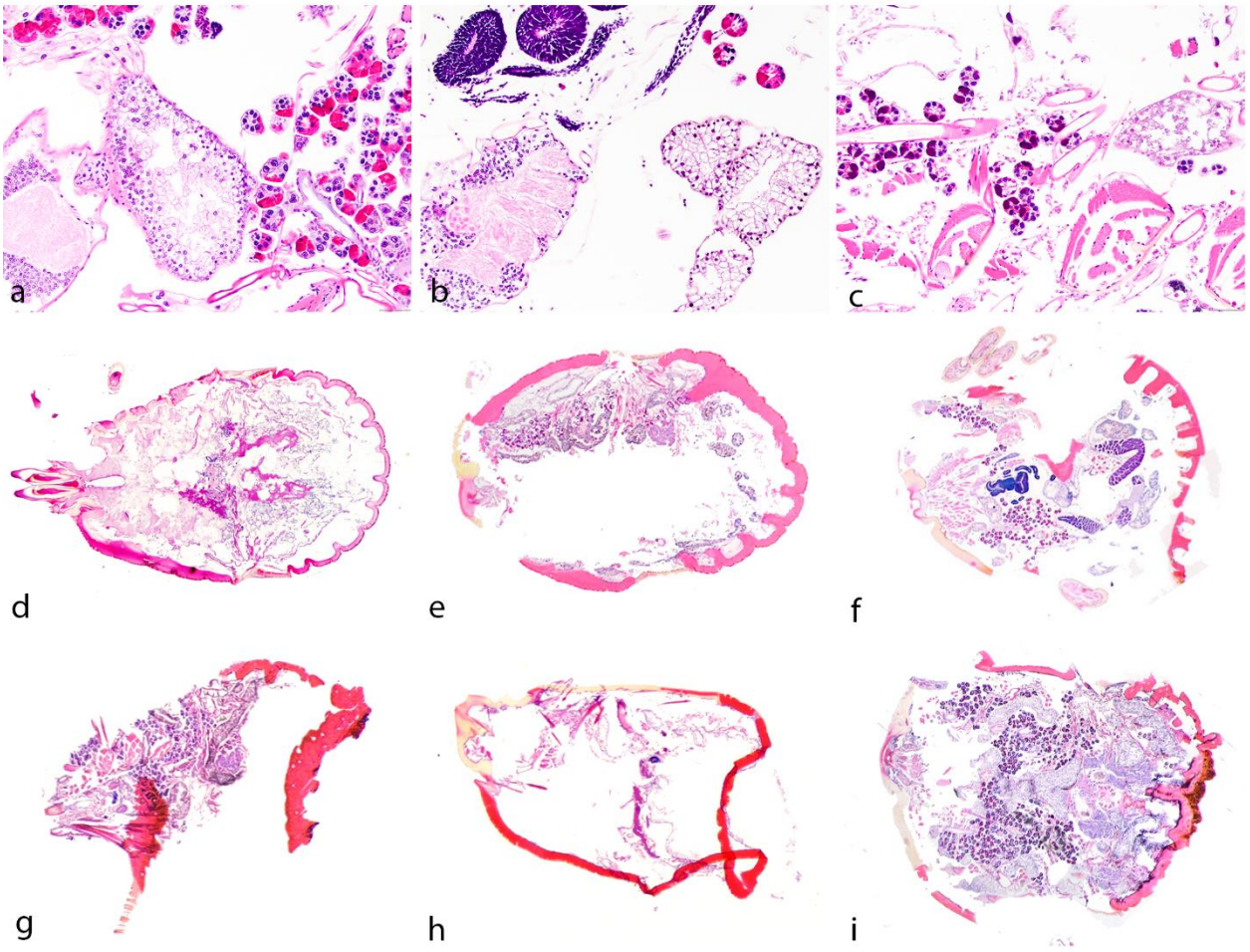

Figure 4. Representative photomicrographs and whole tick sections of *Amblyomma americanum* adults from pilot experiment. Comparison of fixation and staining quality of tick tissues using (a) Bouin's solution, (b) 10% neutral buffered formalin (NBF), and (c) 70% ethanol. (d) Overdigestion of internal organs when KOH was used as a softening agent. (e) Tissue dropout in whole tick sections due to tissue distortion from fixation when ticks were directly placed in Bouin's solution. (f) Tissue dropout in whole tick sections due to tissue distortion from fixation when ticks were directly placed in 10% NBF. (g-h) Incomplete paraffin infiltration resulted in poor tissue integrity when only legs are removed or small holes been made in the exoskeleton prior to tissue processing. (i) Improved tissue integrity in whole tick sections when carapace was removed prior to fixation and sectioning.

3.2 Comparison of Bouin's solution and 10% NBF for whole tick sectioning

For each fixation method, thirty sections (10 ticks, 3 sections per tick) were evaluated and scored based on the 6 scoring criteria. Mean scores for each criterion and mean total scores are presented in Table 2. Representative whole tick sections (highest total score) from each tick are demonstrated in Figure 5.

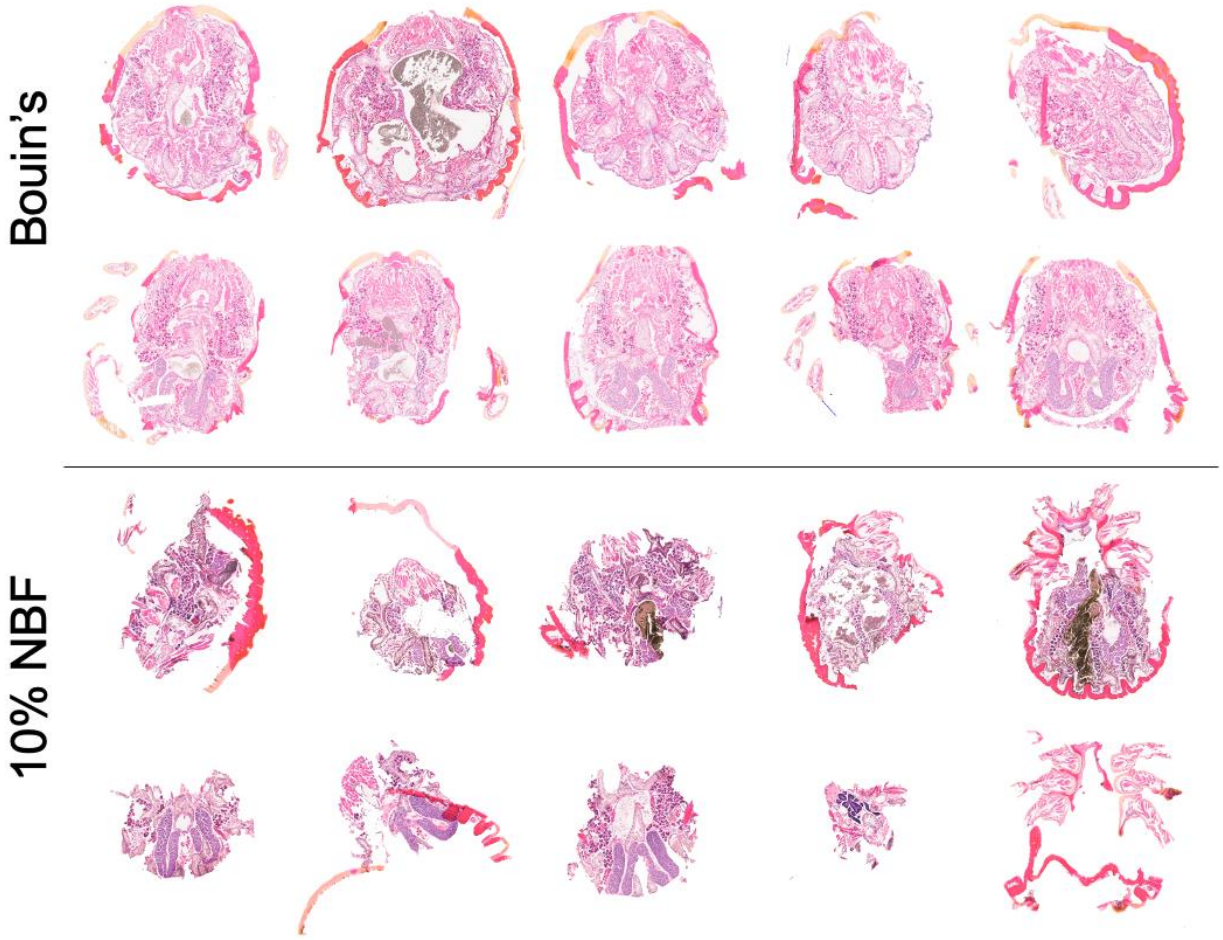

Figure 5. Comparison of Bouin's solution and 10% neutral-buffered formalin for whole tick sectioning – representative whole tick sections.

Table 2. Scoring of whole tick sections using Bouin's solution or 10% NBF

		Scoring Criteria: Mean scores (standard deviation) of 3 sections/tick						
	Tick	A Presence of SG	B Distribution of MG	C Presence of other organs	D Tissue integrity	E Staining and fixation quality	F Exoskeleton integrity	Total score
Bouin's solution	1	3 (0)	3 (0)	2 (0)	3 (0)	2 (0)	1 (0)	**14** (0)
	2	3 (0)	3 (0)	1 (0)	3 (0)	2 (0)	1 (0)	**13** (0)
	3	3 (0)	3 (0)	2 (0)	3 (0)	2 (0)	1 (0)	**14** (0)
	4	3 (0)	2.3 (0.6)	1 (0)	3 (0)	2 (0)	1 (0)	**12.3** (0.6)
	5	3 (0)	2 (0)	1.7 (0.6)	3 (0)	2 (0)	1 (0)	**12.7** (0.6)
	6	3 (0)	3 (0)	2 (0)	3 (0)	2 (0)	1 (0)	**14** (0)
	7	3 (0)	3 (0)	2 (0)	3 (0)	2 (0)	1 (0)	**14** (0)
	8	3 (0)	3 (0)	2 (0)	3 (0)	2 (0)	1 (0)	**14** (0)
	9	3 (0)	2 (0)	1.7 (0.6)	2 (0)	2 (0)	1 (0)	**11.7** (0.6)
	10	3 (0)	3 (0)	3 (0)	3 (0)	2 (0)	1 (0)	**15** (0)
Group mean scores		3 (0)	2.7 (0.4)	1.8 (0.6)	2.9 (0.3)	2 (0)	1 (0)	**13.5** (1)
10% NBF	11	3 (0)	1 (0)	1 (0)	1.3 (0.6)	0 (0)	0.6 (0.5)	**7** (0)
	12	3 (0)	2.3 (0.6)	2 (0)	2 (0)	2 (0)	0.6 (0.5)	**12** (1)
	13	3 (0)	3 (0)	2 (0)	2 (0)	0 (0)	0 (0)	**10** (0)
	14	1 (0)	2 (0)	2 (0)	2 (0)	0 (0)	0.3 (0.3)	**7.3** (0.6)
	15	2 (0)	2.3 (0.6)	2 (0)	2.3 (0.6)	0 (0)	1 (0)	**9.7** (1.2)
	16	2.3 (0.6)	2.7 (0.6)	1.3 (0.6)	2 (0)	2 (0)	0 (0)	**10.3** (0.6)
	17	1.7 (0.6)	2 (0)	1 (0)	2 (0)	2 (0)	0.6 (0.6)	**9.3** (1.2)
	18	3 (0)	3 (0)	2 (0)	3 (0)	2 (0)	0.6 (0.6)	**13.7** (0.6)
	19	1 (0)	1.3 (0.6)	0.7 (0.6)	1.7 (0.6)	2 (0)	0.6 (0.6)	**7.3** (1.5)
	20	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	1 (0)	**2** (0)
Group mean scores		2 (1.1)	2 (1)	1.4 (0.7)	1.9 (0.6)	1 (1)	0.6 (0.5)	**8.9** (3.2)

The mean total scores for Bouin's-fixed ticks were significantly higher compared to NBF-fixed ticks ($p = 0.001$). When comparing the 6 scoring criteria separately between the two fixative groups, Bouin's-fixed ticks had significantly higher scores than NBF-fixed ticks in the following 3 criteria: presence of SG (criterion A, $p = 0.03$), tissue integrity (criterion D, $p = 0.004$), and exoskeleton integrity (criterion F, $p<0.0001$). Representative sections for each of these criteria are demonstrated in Figure 6. The scores were not significantly different between the two fixative groups in the remaining 3 criteria: distribution of midgut (criterion B, $p = 0.173$), the presence of other organs in the section (criterion C, $p = 1$), and fixation and staining quality (criterion E, $p = 0.08$). Representative results from these criteria are demonstrated in Supplemental Figures 1 and 2.

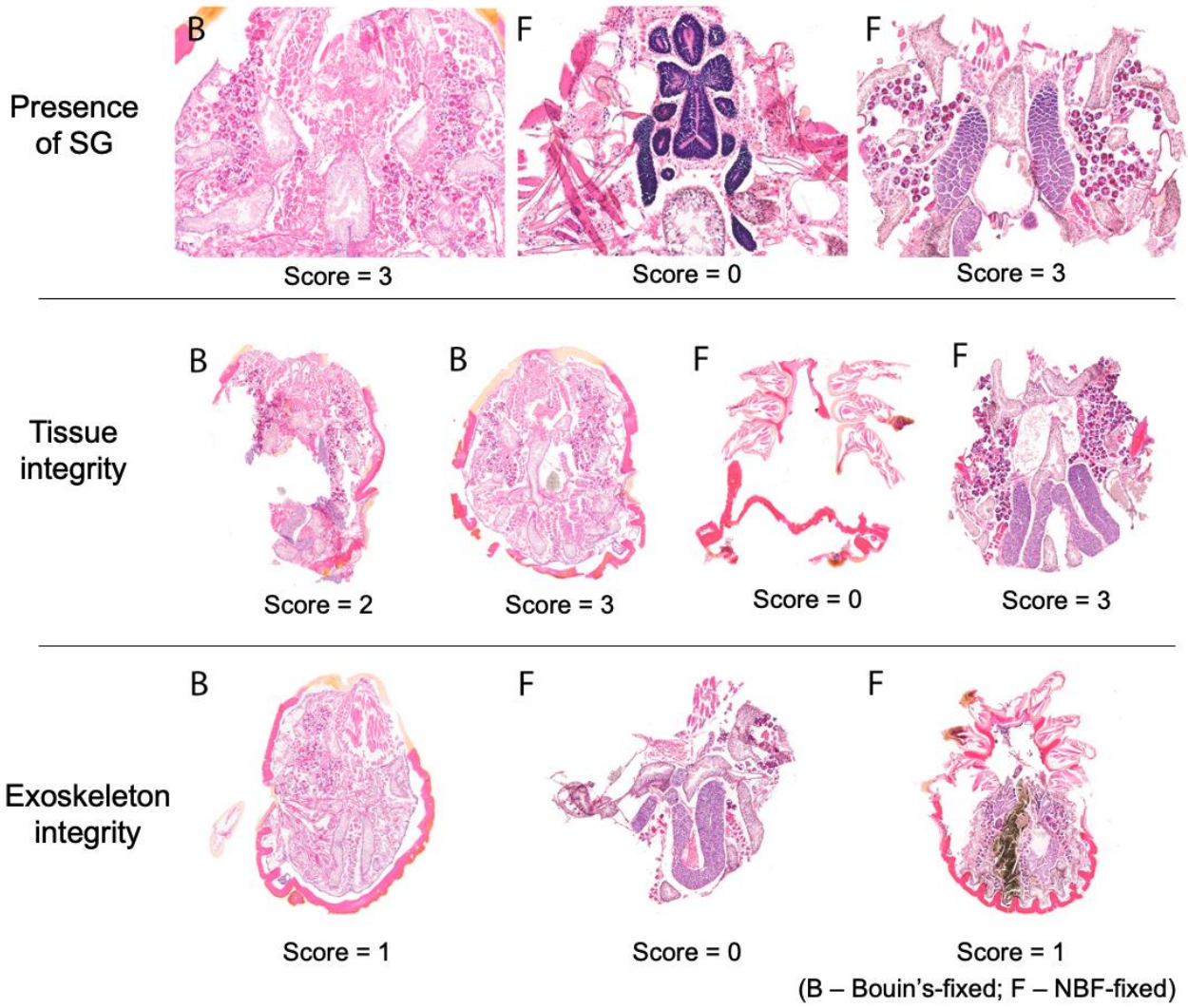

Figure 6. Comparison of Bouin's solution and 10% neutral-buffered formalin (NBF) for whole tick sectioning. Representative sections from the 3 criteria with scores that were statistically different. A low score (if applicable) and a high scored section from each fixation method is shown.

3.2.1 Other subjective observations

In NBF-fixed ticks, there were occasional acid-hematin pigments present in the midgut epithelium, which partially obscured cellular details in these sections. Additionally, in NBF-fixed ticks the exoskeleton was often folded or distorted when present on the slide (Figure 5). In contrast, the exoskeleton was frequently intact, and appeared to surround the hemocele and encompass the internal organs in Bouin's-fixed ticks.

3.3 Whole tick sectioning of additional tick species

Sections produced from all tick species received acceptable scores (range of total scores: 8–15). These results are shown in Supplemental Table and representative whole tick sections are demonstrated in Figure 7. We initially only processed ticks using our proposed protocol for *A. americanum* where all ticks were embedded dorsal side down with dorsal to ventral sectioning direction. For *D. variabilis, R. sanguineus,* and the *Ixodes scapularis* females, the sections were consisted of mainly SG and MG. In *I. scapularis* males, 80% of the sections were comprised of testes filled with spermatids. As the other organs were less frequently noted in these sections, these ticks received lower scores in criterion C. We suspected this was due to differences in the internal anatomy among tick species and sexes. For this reason, we processed 2 additional ticks per species (one male and one female), embedded them ventral side down, and used a ventral to dorsal sectioning direction with hopes to capture the organs that were more ventrally located within the ticks. For all three tick species, this technique successfully captured additional organs including the synganglion, uterus and oviduct/ovary in females, and accessory sex glands in males. Even though ventrally embedded ticks with a ventral to dorsal sectioning direction allowed visualization of more organs in a single section, we were only able to produce 1 to 2

sections from the smaller tick species (*R. sanguineus* and *I. scapularis*) due to the remaining tick carcass shelling out of the paraffin block.

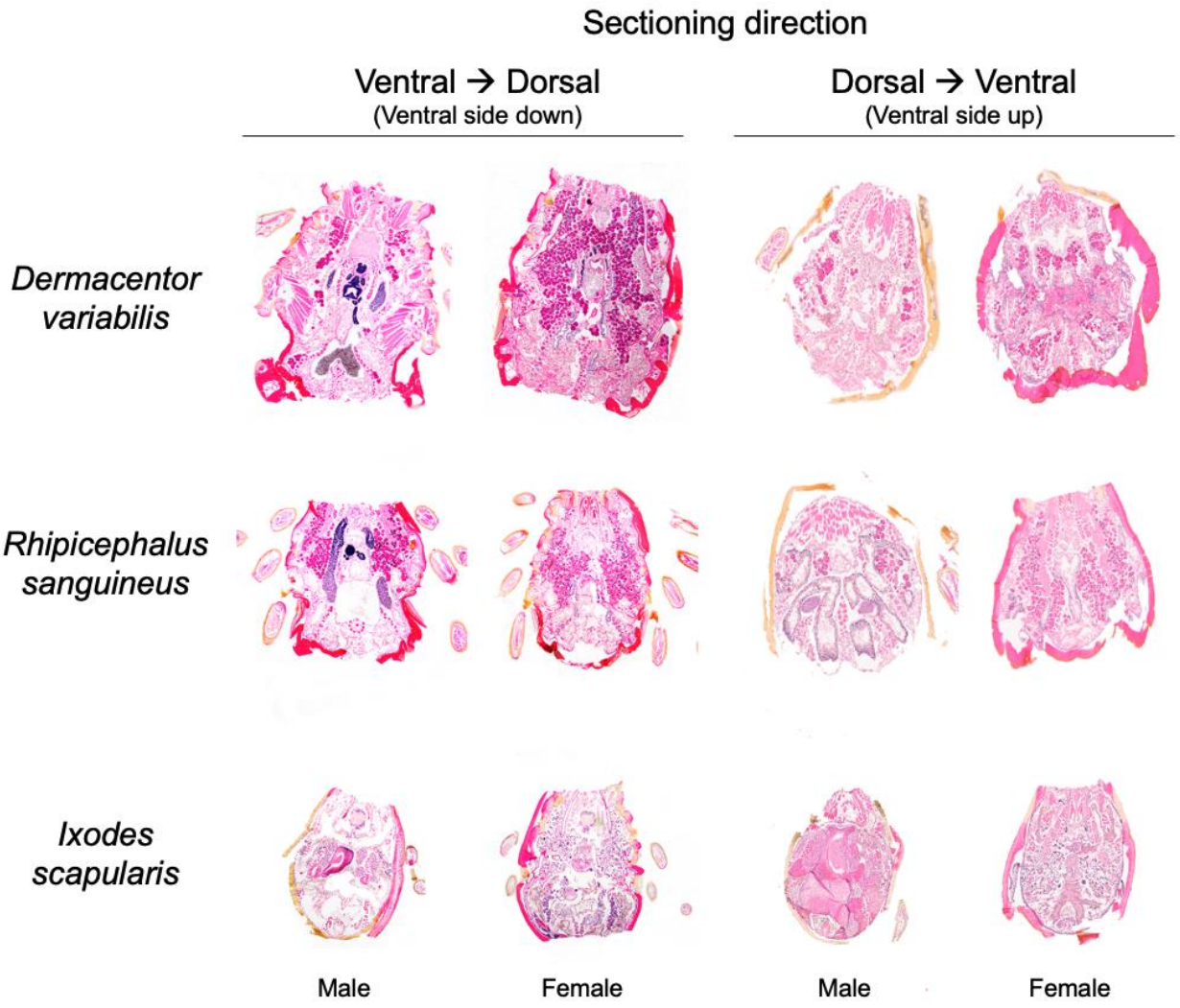

Figure 7. Representative whole tick sections from 3 additional tick species with 2 sectioning directions.

4. DISCUSSION

This current study aimed to establish a practical technique for producing histology sections for whole ticks with a focus on organs that are important in tick-borne disease research. Our pilot experiments provided us with valuable insights on what reagents, fixatives, and techniques were useful when preparing whole ticks for histology. When compared directly, Bouin's solution was found to be superior to NBF for consistently producing high quality sections. This technique also resulted in adequate whole tick sections for 3 other tick species.

In our study, Bouin's fixation was most useful in producing whole tick histologic sections that were consistent in quality. We believe this is likely due to the acidic content of Bouin's solution (contains picric acid and glacier acidic acid), which created a softening or decalcifying effect for the tick exoskeleton [29]. In contrast, it appeared that the exoskeleton remained more rigid and brittle in NBF-fixed ticks, and these sections were often associated with tissue dropout and sectioning artifacts secondary to a chipped microtome blade or jagged texture along the sectioning plane. While our protocols were successful in producing H&E-stained histology sections, further optimization may be required for tissue sections that are intended for other *in situ* molecular techniques (e.g. IHC, IF or ISH). For instance, in a previous study from our laboratory, we found that RNAscope *in situ* hybridization assays were associated with more non-specific chromogen staining in Bouin's-fixed tick tissues compared to NBF [30]. Therefore, should investigators need to use NBF as their fixative for whole ticks, further efforts to soften the exoskeleton may be necessary. This may be challenging as the softening agent we tested in the pilot experiments (KOH) frequently resulted in over digestion of the internal organs. However, we only evaluated 2 concentrations with 3 different durations. Lower concentrations or shorter durations might overcome these problems. Additionally, other reagents (such as EDTA, ImmunoCal™) are

available and could be evaluated for this purpose. Alternatively, investigators may choose to perform dissections of individual tick tissues or remove the entire carapace prior to NBF fixation.

Our protocol was established to specifically capture organs that are involved in the development and transmission of most tick-borne pathogens. This protocol worked well for *A. americanum*, but occasionally failed to capture these important organs in other tick species. For instance, when ticks were sectioned with a dorsal to ventral direction, all Bouin's-fixed *A. americanum* sections received the highest scores for the presence of salivary glands while the numbers of salivary glands in the other tick species varied. We suspected that this was likely dependent on the differences in the internal anatomies of tick species as well as the sectioning directions (dorsal to ventral vs. ventral to dorsal) of the whole ticks. These findings were further confirmed when we processed the additional tick species with a ventral to dorsal sectioning direction. In the ticks that were sectioned with a ventral to dorsal direction, we were able to capture more organs that were ventrally located within the ticks and were missing from the opposite sectioning direction. One of these organs was the synganglion, which was largely absent in the sections from all four tick species that were sectioned with a dorsal to ventral direction. Taking this into consideration, investigators are encouraged to perform pilot experiments with different embedding orientations and sectioning directions based on organs of interests.

Although ventrally embedded ticks with a ventral to dorsal sectioning direction allowed us to visualize more organs in a single section, the remaining tick carcasses of the smaller tick species appeared less secured in the paraffin block, which led to them shelling out of the block and prevented further sectioning. This was not a problem with dorsally embedded ticks that were

sectioned in a dorsal to ventral direction. We suspect that these tick carcasses were able to be anchored within the paraffin block by the tick extremities as they faced towards the top of the block during sectioning. This is in contrast to the ventrally embedded ticks as the extremities were cut off first during sectioning, the remaining tick carcasses were more likely to fall out of the paraffin block as the microtome encounter them. Knowing these potential challenges, investigators may need to further optimize sectioning techniques if a ventral to dorsal sectioning direction is desired when working with smaller ticks.

One interesting finding that we made during our pilot experiment was that one of the ticks that we were able to produce excellent histologic sections from had been stored in alcohol-based solution for at least 10 years. We are aware this is a common method of storage for ticks collected off of companion animals or wildlife by veterinary clinicians or biologists. Therefore, these archived ticks could serve as a useful resource and could still be considered for morphologic studies.

There were a few limitations in this study. First, our pilot experiments only evaluated 5 fixatives and the fixatives eliminated from early stages of the study were not retested after the protocol had been optimized. It is possible that these fixatives would work better if additional efforts were taken to allow full paraffin infiltration (i.e. removal of the anterior and posterior end of the tick) or to embed ticks in Histogel prior to histologic processing. Second, as removal of parts of the exoskeleton was an essential step in facilitating full paraffin infiltration, some hemolymph is usually lost during this preparation step. Therefore, if the examination of hemolymph is required, collection of hemolymph for cytologic, molecular or cultivation analyses should be implemented prior to or during exoskeleton removal. Lastly, even though we were able to produce acceptable

whole tick sections for 3 additional tick species, fixation length and processing technique may require further optimization for other species.

We believe that whole tick histology is an underutilized tool in tick-borne disease research. We described a practical protocol that uses commonly available tools, reagents and standard histologic equipment. This should allow any investigator to easily make adjustments to this protocol as needed based on their experimental goals.

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Authorship:

T. Yang and A. Birkenheuer contributed to study conception, execution, and design. L. Miller and N. White Hurst contributed to optimization of experimental techniques and procedures. All authors contributed to editing and approved the final manuscript.

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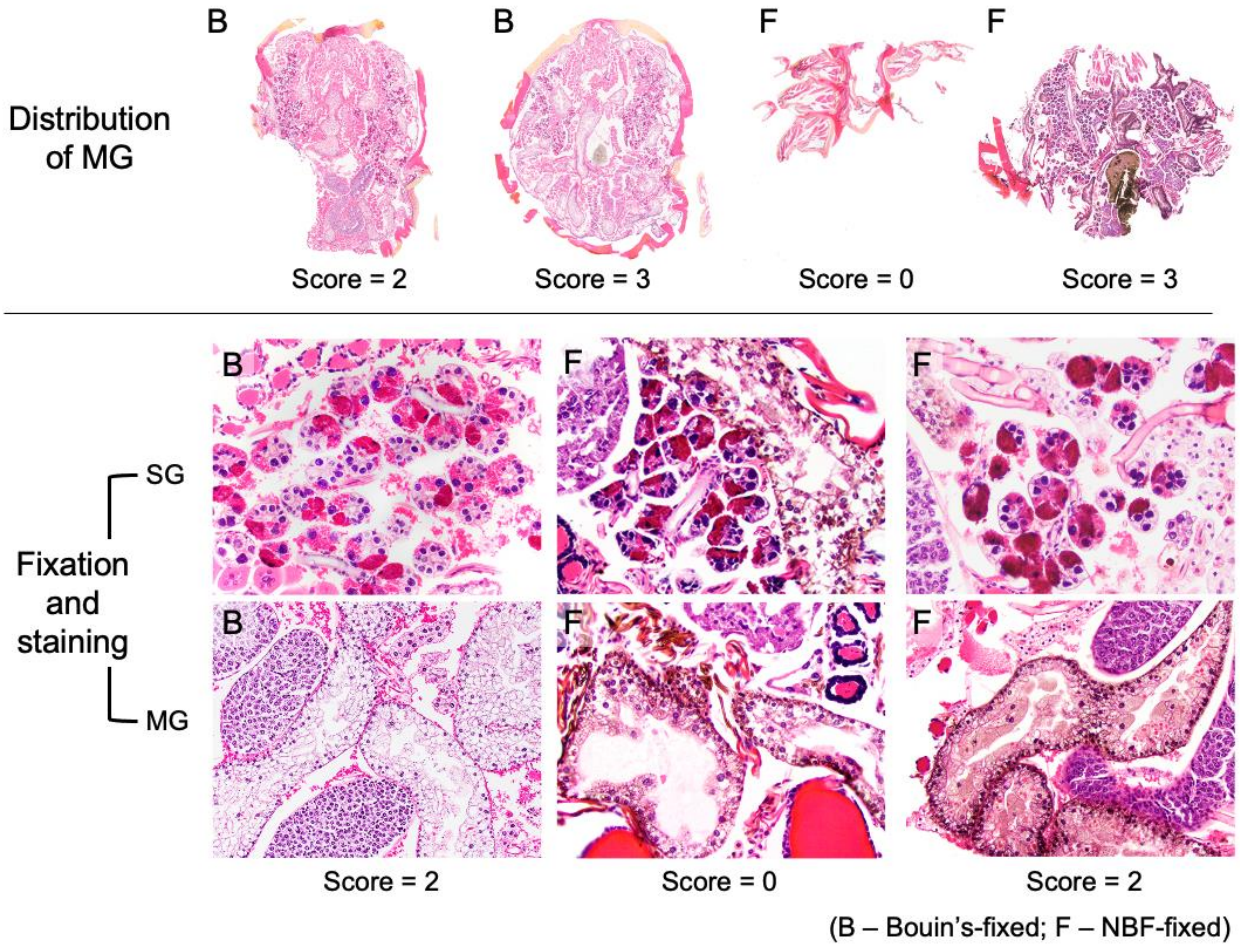
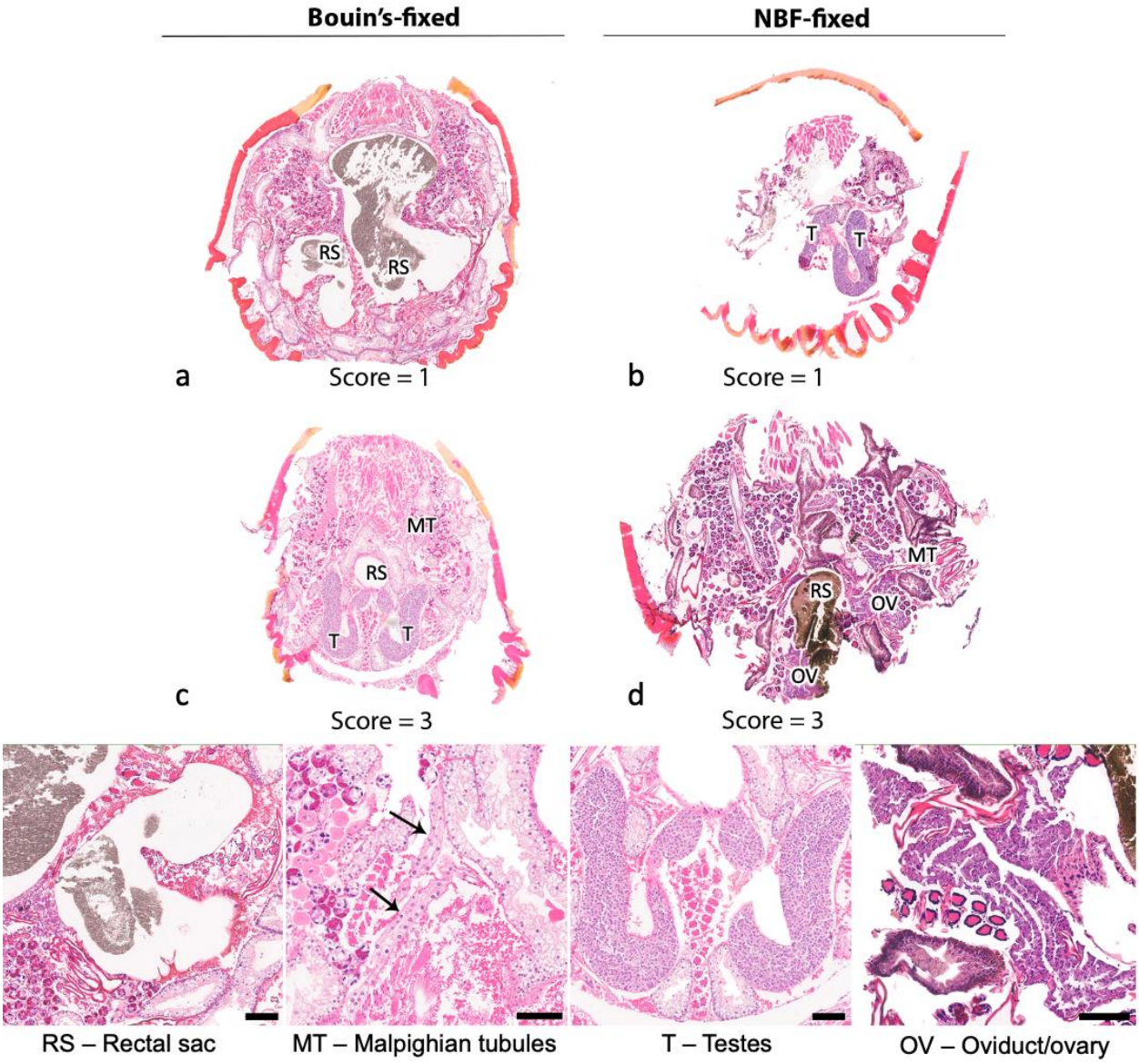
SUPPLEMENTAL MATERIAL



Supplemental Figure 1. Representative sections from criterion B (distribution of midgut) and criterion E (fixation and staining quality). Top panel – Distribution of midgut (MG) (left to right): Low-scored Bouin's-fixed tick with MG present in only 3 quadrants (score = 2), and high-scored Bouin's-fixed tick with MG present in all 4 quadrants (score = 3). Low-scored neutral-buffered formalin (NBF)-fixed ticks with no MG present in section (score = 0), and high-scored NBF-fixed tick with MG present in all 4 quadrants (score = 3). Bottom panel – Fixation and staining quality (left to right). Bouin's-fixed tick with clearly delineated cellular borders and tissue details in both SG and MG (score = 2). A low-scored NBF-fixed tick with poor tissue detail and smudgy cellular borders (score = 0). A high-scored NBF-fixed tick with clear cellular borders and tissue detail (score = 2).



Supplemental figure 2. Representative sections from scoring criterion C (presence of other organs). Top 2 panels demonstrates a subgross view of whole tick sections from each fixative group (a, c – Bouin's-fixed; b, d – neutral-buffered formalin (NBF)-fixed) with organs labeled. The bottom panel shows representative photomicrographs of labeled organs in higher magnification (bar = 100 μm). (a) Low-scored Bouin's-fixed tick: only rectal sac (RS) was noted in this section in addition to salivary glands (SG) and midgut (MG). This section received a score of 1. (b) Low-scored NBF-fixed tick: only testes (T) were noted in this section in addition to SG and MG. This section received a score of 1. (c) High-scored Bouin's-fixed tick: Rectal sac (RS), Malpighian tubules (MT), and testes (T) were noted in this section in addition to SG and MG. This section received a score of 3. (d) High-scored NBF-fixed tick: Rectal sac (RS), Malpighian tubules (MT), and oviduct/ovary (OV) were noted in this section in addition to SG and MG. This section received a score of 3. (SG: Salivary glands; MG: Midgut).

Supplemental table 1. Scoring whole tick sections of 3 additional tick species – *Rhipicephalus sanguineus*, *Dermacentor variabilis*, and *Ixodes scapularis*.

			Scoring Criteria Mean scores (standard deviation) of 3 sections/tick						
	Tick	**Sectioning direction**	**A Presence of SG**	**B Distribution of MG**	**C Presence of other organs**	**D Tissue integrity**	**E Staining and fixation**	**F Exoskeleton integrity**	**Total score**
Rhipicephalus sanguineus	**1**	D→V	2 (0)	3 (0)	1 (0)	3 (0)	2 (0)	1 (0)	**12** (0)
	2	D→V	1 (0)	2 (0)	1 (0)	2 (0)	2 (0)	1 (0)	**9** (0)
	3	D→V	3 (0)	2 (0)	1 (0)	3 (0)	2 (0)	1 (0)	**12** (0)
	4	D→V	2 (0)	3 (0)	1 (0)	2 (0)	2 (0)	1 (0)	**11** (0)
	5*	V→D	3 (NA)	2 (NA)	3 (NA)	3 (NA)	2 (NA)	1 (NA)	**14** (NA)
	6	V→D	3 (0)	2 (0)	3 (0)	3 (0)	2 (0)	1 (0)	**14** (0)
Dermacentor variabilis	**1**	D→V	2 (0)	3 (0)	1 (0)	2 (0)	2 (0)	1 (0)	**11** (0)
	2	D→V	1 (0)	3 (0)	1 (0)	2 (0)	2 (0)	1 (0)	**10** (0)
	3	D→V	2 (0)	3 (0)	1 (0)	3 (0)	2 (0)	1 (0)	**12** (0)
	4	D→V	2 (0)	2 (0)	1 (0)	3 (0)	2 (0)	1 (0)	**11** (0)
	5	V→D	3 (0)	2.3 (0.6)	2.3 (1.2)	3 (0)	2 (0)	1 (0)	**13.7** (0.6)
	6	V→D	2.7 (0.6)	2.3 (0.6)	3 (0)	3 (0)	2 (0)	1 (0)	**14** (0)
Ixodes scapularis	**1**	D→V	2 (0)	3 (0)	2 (0)	3 (0)	2 (0)	1 (0)	**13** (0)
	2	D→V	1 (0)	1 (0)	1 (0)	3 (0)	2 (0)	1 (0)	**9** (0)
	3	D→V	1 (0)	2 (0)	2 (0)	3 (0)	2 (0)	1 (0)	**11** (0)
	4	D→V	1 (0)	2 (0)	2 (0)	3 (0)	2 (0)	1 (0)	**11** (0)
	5#	V→D	2.5 (0.7)	2 (0)	2.5 (0.7)	3 (0)	2 (0)	1 (0)	**13** (0.7)
	6*	V→D	1 (NA)	3 (NA)	2 (NA)	3 (NA)	2 (NA)	1 (NA)	**12** (NA)

*Only one section was produced for scoring
#Only two sections were produced for scoring
D→V: Dorsal to ventral
V→ D: Ventral to dorsal

CHAPTER 3

Detection of *Cytauxzoon felis* in salivary glands of *Amblyomma americanum*

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ABSTRACT

Cytauxzoon felis is a tick-borne piroplasmid hemoparasite that causes life-threatening disease in cats. Despite the critical role that ticks play in disease transmission and development, our knowledge regarding the *C. felis* life cycle remains limited to the feline hosts and no stage of the parasite has been identified or investigated in ticks. Sporozoites are the infectious stage of piroplasmids that are transmitted by ticks. In other tick-borne piroplasmids, sporozoites have played a key role in disease prevention and management. We believe sporozoites have similar potential for cytauxzoonosis. Therefore, the objective of this study is to evaluate different molecular and microscopic techniques to detect *C. felis* sporozoites in tick salivary glands (SG). A total of 140 *Amblyomma americanum* ticks that were fed on *C. felis*-infected cats as nymphs were included for this study. Specifically, dissected SGs were quartered and subjected to *C. felis* RT-PCR, RNAscope® *in situ* hybridization (ISH), histology, direct azure staining, and transmission electron microscopy (TEM). *Cytauxzoon felis* RT-PCR was also performed on half tick (HT) carcasses after SG dissection. *Cytauxzoon felis* RNA was detected in SGs of 17 ticks. Of these, 7 ticks had microscopic visualization via ISH and/or TEM. The remaining 10 ticks had only molecular detection of *C. felis* in SGs via RT-PCR without visualization. *Cytauxzoon felis* RNA was detected solely in HT carcasses via RT-PCR in 9 additional ticks. In ISH-positive tick SGs, hybridization signals were present in cytoplasms of SG acinar cells. TEM captured rare *C. felis* organisms with characteristic ultrastructural features of piroplasmid parasites. This study describes the first direct visualization of any developing stage of *C. felis* in ticks. Forthcoming studies should employ a combination of molecular and microscopic techniques to investigate the *C. felis* life cycle in *A. americanum.*

Key words *Cytauxzoon felis*, sporozoites, *in situ* hybridization, tick, salivary glands

1. INTRODUCTION

Cytauxzoon felis is a tick-borne hemoprotozoan parasite that causes life-threatening disease in cats. The *Cytauxzoon* genus is classified under the phylum Apicomplexa, order Piroplasmida, and family Theileriidae. Since the first case of fatal cytauxzoonosis was published in 1976 in the United States, reports of *C. felis* and other *Cytauxzoon* spp. continue to emerge throughout the world, including Europe, South America, and Asia [1–5]. While tick vectors have not been identified for most *Cytauxzoon* spp., *Amblyomma americanum* has been recognized as the primary vector for *C. felis* in the United States. Despite the crucial role that ticks play in pathogen transmission and development, our knowledge regarding the *C. felis* life cycle is primarily limited to the feline host. In closely related parasites, identification of life stages in vector hosts have led to important breakthroughs in disease prevention and management. Understanding and utilizing these life stages of *C. felis* in *A. americanum* may allow us to overcome several major hurdles in cytauxzoonosis research, which include establishing an *in vitro* culture system or developing a vaccine.

It is presumed that the life cycle of *C. felis* in ticks mirrors its closest relative, *Theileria*, where sexual reproduction occurs in the ticks' midgut after a blood meal from an infected host. Kinetes then migrate to salivary glands where they undergo sporogony and mature into infective sporozoites [6–8]. Sporozoites represent an important interface between the mammalian and the tick hosts. For this reason, they are frequently viewed as a key target in developing vaccines and therapeutics for other closely related apicomplexan parasites. In *Theileria* spp., tick-derived sporozoites have been widely used to produce vaccines, initiate *in vitro* culture [9,10], discover new therapeutics [11] and investigate disease pathogenesis [12–14]. To date, no stage of *C. felis* has been definitively identified in ticks, including sporozoites, which limits our ability to pursue similar avenues for *Cytauxzoon* research.

Traditional detection methods for *Theileria* sporozoites include direct staining of whole salivary glands, immunohistochemistry (IHC), or transmission electron microscopy (TEM). To our knowledge, there has been very limited work on this front in *C. felis* research. Microscopic features of whole salivary glands from *C. felis*-infected *A. americanum* were described, but sporozoites were not definitively identified [15]. Transmission electron microscopy of *C. felis*-infected salivary glands has also been attempted with inconclusive results (personal communication with Reichard). To convincingly localize the parasite in tissue sections, additional *in situ* molecular techniques like IHC are required. IHC requires monoclonal antibodies or polyclonal antisera that specifically react with the pathogen [16]. Unfortunately, these resources are not readily available for *C. felis*. *In situ* hybridization (ISH) is a viable alternative detection method and target probes are easier to produce and customizable through many commercial manufacturers [17,18]. Additionally, ISH has been successfully used in previous studies to label *C. felis* in both feline and tick tissue sections [19,20].

The objective of this study is to detect *C. felis* in salivary glands of adult *A. americanum* ticks that were fed on *C. felis*-infected cats as nymphs. We evaluated four different microscopic techniques to visualize *C. felis* in this study – direct staining of whole salivary glands, histology, ISH, and TEM in adult ticks.

2. MATERIALS AND METHODS

2.1 Ticks

Amblyomma americanum nymphs were obtained from the Oklahoma State University tick rearing facility. Prior to SG evaluation, ticks were maintained as recommended by the tick rearing facility in a humidity chamber with 90–99% relative humidity at 20–23°C and a 12-hour light/12-hour dark photoperiod.

Acquisition feeding of *A. americanum* nymphs on *C. felis*-infected cats was performed as previously described [21]. Post-feeding, these nymphs were allowed to molt into adults. One hundred and forty of

these ticks were evaluated. Eighty of these ticks were unfed (42 females and 38 males) and 60 of these ticks (30 females and 30 males) were placed into artificial feeding chambers for 12–72 hours prior to SG dissection. Roughly equal numbers of males and females were included in each group to mimic the experimental tick transmission model [21]. Negative controls consisted of 36 unfed *A. americanum* adults (18 females, 18 males) that had not been acquisition-fed on *C. felis*-infected cats as nymphs. Feeding chambers were assembled following a previously described protocol with minor modifications [22,23]. Our modifications included the use of cat hair as an attachment stimulus and the feeding units were floated on a 37°C water bath. Sheep blood (Hemostat laboratories, Dixon, CA) was used for feeding and was changed every 12 hours during the feeding period. Since either heat or feeding had been shown to stimulate sporogony [24,25], all ticks that were placed in the feeding chamber regardless of status of attachment were collected for evaluation. During collection, ticks were categorized as "attached" based on either physical attachment to the membrane at time of collection or if blood was present in the midgut during dissection.

Ticks were surface sterilized with 3% hydrogen peroxide, 10% sodium hypochlorite then 70% ethanol as previously described [26] and dissected for SG removal. Each pair of SG were quartered and placed separately in fixatives or reagents for RT-PCR, direct staining, histology preparation, and TEM (figure 1). After SGs were removed, the corresponding tick carcasses were also halved and stored in RNAlater™ (Invitrogen, Waltham, MA) in -80ºC until RNA extraction.

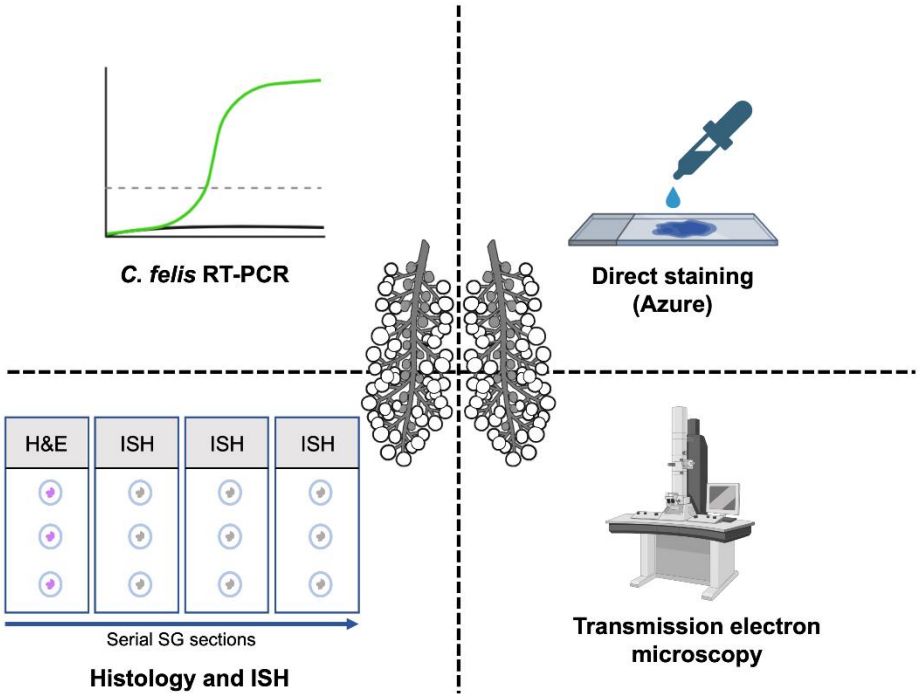

Figure 1. Experimental design. Each pair of *Amblyomma americanum* salivary glands (SG) were quartered and quarters were randomly subjected to one of four methods for the detection of *C. felis:* RT-PCR, direct azure staining, histology and *in situ* hybridization (ISH), and transmission electron microscopy. (RT-PCR: Reverse transcription polymerase chain reaction; H&E: Hemotoxylin and eosin stain.)

2.2 *C. felis* Reverse transcription PCR

Quartered SG and half tick (HT) carcasses from each tick were tested for the presence of *C. felis* 18S rRNA via two-step RT-PCR as previously described [19]. Briefly, total RNA was extracted from tick tissues using an RNeasy Plus Micro kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from 10 µl of extracted RNA using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) and used as template in a SYBR™ green real-time PCR assay as previously described (Bio-Rad, Hercules, CA 94547, USA). CFX96™ Manager Software was used to analyze amplification and melt curves [27].

2.3 RNAscope *in situ* hybridization and histology

2.3.1 Salivary gland processing for histology and ISH

Histologic sections of SG were prepared for standard histologic examination and RNAscope® ISH. Briefly, dissected SGs were fixed either in Bouin's solution (n=80) or 10% neutral-buffered formalin (NBF) (n=60) for 24 hours. Due to some non-specific background that was present in the Bouin's fixed SGs during the ISH assays (figure 3), fixative was changed from Bouin's solution to NBF. After fixation, SGs were washed in 70% ethanol, then embedded in molten Histogel™ in a cryomold until solidification. Histogel™ blocks with SG were then placed in tissue cassettes to undergo standard histologic processing, paraffin embedding, and sectioning. At least 12 serial sections (5 μm thick) were produced per SG for histologic examination or ISH. Specific usage and locations on the slides for each serial section are depicted in figure 2. Slide A was stained with hematoxylin and eosin (H&E) and slides B, C, D were used for ISH. If adequate tissue samples were not present on slide D, additional sections were used which may not be serial to the sections on the previous slide.

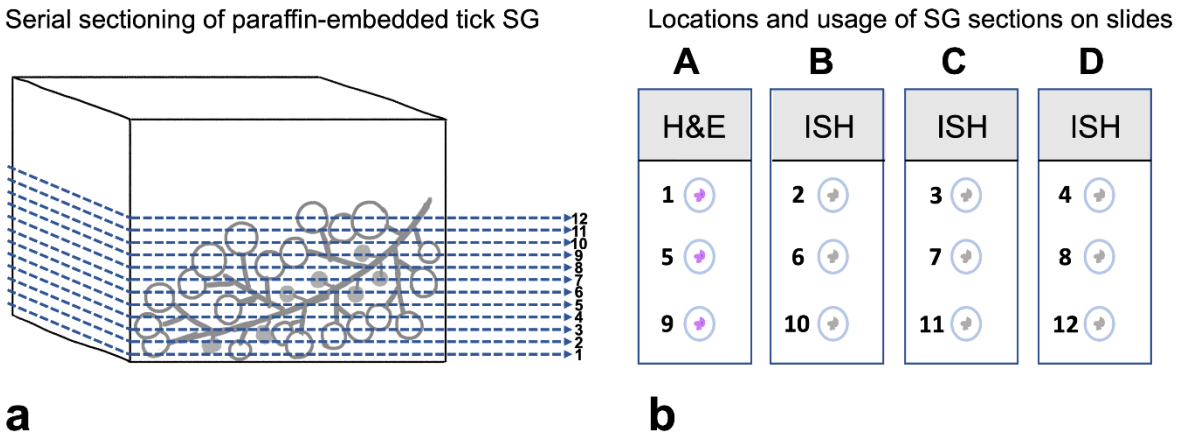

Figure 2. Representative illustration of histologic processing and usage of *Amblyomma americanum* salivary gland (SG). (a) Paraffin-embedded SG were sectioned sequentially, producing at least 12 sections per tissue block. The seriality of sections are illustrated in numerical order. (b) Serial sections were distributed on 4 slides (labeled A–D) and subjected to either routine H&E staining or ISH as shown. (H&E: Hemotoxylin and eosin; ISH: *in situ* hybridization.)

2.3.2 RNAscope *in situ* hybridization assays

RNAscope® 2.5 HD red ISH assay (Advanced Cell Diagnostics; Newark, CA) was performed on slides B and C using a proprietary antisense probe targeting *C. felis* 18S rRNA (Cf-18S-rRNA) as previously described. If *C. felis* was detected on slide B or C, RNAscope® 2.5 HD brown ISH assay was performed on slide D using a proprietary antisense probe targeting *C. felis* GAPDH mRNA (Cf-GAPDH). Positive controls consisted of formalin-fixed paraffin-embedded tissues from cats diagnosed with acute cytauxzoonosis, and negative controls consisted of SGs collected from *A. americanum* adults that had not been acquisition-fed on *C. felis*-infected cats as nymphs (Figure 2). RNAscope® ISH assays were performed according to manufacturer's guidelines with slight modifications after optimizing the pretreatment conditions for salivary glands in different fixatives. For NBF-fixed SG sections target retrieval time of 15 minutes and protease plus treatment of 20 minutes were used. For Bouin's-fixed SG sections, target retrieval time of 10 minutes and protease plus treatment of 30 minutes were used.

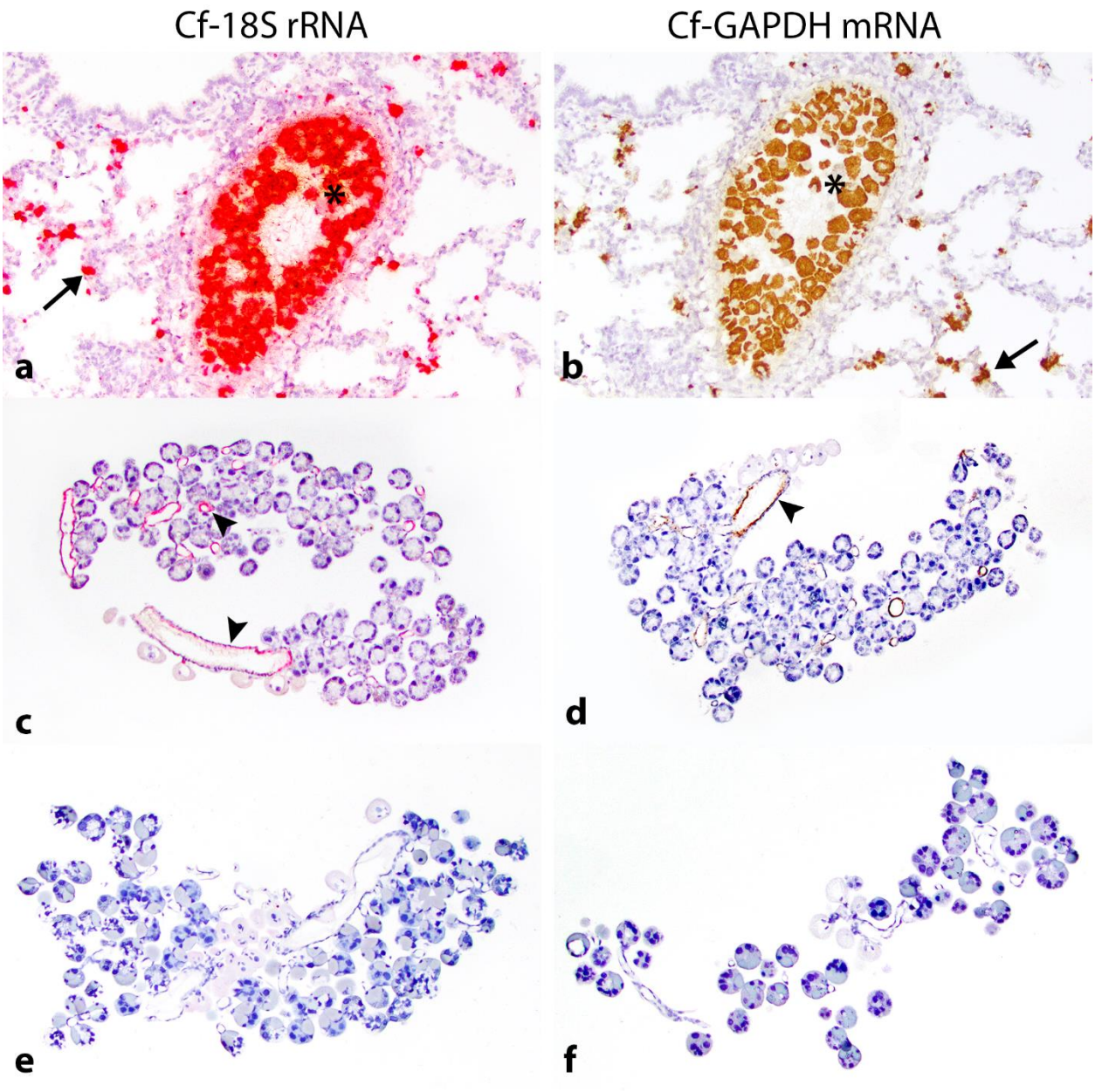

Figure 3. RNAscope ISH assays targeting 18S rRNA (red) and GAPDH mRNA (brown) of *Cytauxzoon felis*. (a, b) Positive controls using formalin-fixed paraffin embedded lung tissue from cats with cytauxzoonosis. Robust hybridization signals for *C. felis* schizonts are abundantly present within the pulmonary vein (asterisk) as well as scattered along the alveolar septa (arrows). (c–f) Salivary glands collected from non-infected *A. americanum* adults with respective ISH probe applied. No hybridization signals are observed within any SG acinar cells in all sections. Bouin's-fixed SGs (c, d) had non-specific chromogen uptake along the salivary ductal linings (arrowheads), and this background staining was substantially reduced in formalin-fixed SGs (e, f).

2.4 Azure staining of salivary glands

To prepare SGs for direct staining, under a dissection microscope, quartered SG was spread out and air-dried on a clean glass slide (Fisherbrand™ Premium Superfrost™). Dried SGs were then fixed with 100% methanol for 5 minutes and stained on the slide by covering the section with 0.1% aqueous azure solution (Sigma Aldrich, Germany) for 30 seconds to 1 minute. Stained SG sections were then examined under a light microscope at 100X–400X magnification.

2.5 Transmission electron microscopy

Immediately after dissection, quartered SGs were stored in Karnovsky's fixative (4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer) at 4ºC for up to 3 months. Salivary glands were processed for TEM following standard procedures with slight modifications [28]. Briefly, SGs were removed from the fixative, washed 3 times (10 min per wash) in 0.1 M sodium cacodylate buffer, then placed in 2% osmium tetroxide for one hour. Following osmication, SGs were washed again with 0.1 M sodium cacodylate buffer and subjected to serial dehydration in 70%, 90%, 95% and twice in 100% ethanol for 10 min each. SGs were sequentially submerged in 100% ethanol-Epon resin mixtures (2:1 ratio overnight, 1:1 ratio for 2 hours, 1:2 ratio for 2 hours, then 100% resin for 2 hours), then flat embedded in resin with accelerator added in a small petri dish and polymerized in 70ºC drying oven overnight. Specimens were then mounted onto blank blocks with Loctite adhesive and allowed to dry. Ultrathin sections (85 nm) were cut with a Leica EM UC7 ultramicrotome using a Diatome diamond knife, collected on 50 mesh formvar/carbon coated grids and post-stained with uranyl acetate and lead citrate. Sections were imaged in a Hitachi HT7800 transmission electron microscope operated at 80 kV.

2.6 Statistical analysis

Total numbers of *C. felis*-positive ticks via any molecular detection method (PCR in SG or HT, or ISH) were compared between the unfed group and the group placed in feeding chambers using Fisher's Exact tests [29]. Significance level was set at 0.05 with a 95% confidence interval.

3. RESULTS

A total of the 140 ticks were evaluated for this study. Twenty-seven ticks with positive test results are described in table 1. *C. felis* RNA was detected in SGs of 17 ticks. Seven of these ticks had microscopic visualization via ISH and/or TEM. The remaining 10 ticks had only molecular detection of *C. felis* via RT-PCR without visualization. Nine additional ticks did not have *C. felis* detected in SGs but had *C. felis* RNA detected by RT-PCR solely in their HT carcasses after SG removal. One tick was positive for azure staining but *C. felis* was not detected via any molecular techniques.

Of the 26 ticks where we detected *C. felis* RNA, the results of the 3 molecular detection techniques did not always agree, and only 3 ticks were positive for *C. felis* RNA via all methods. A summary of the agreement between the 3 molecular assays is outlined in table 2. No significant difference in *C. felis* RNA detection was found between the unfed ticks and the ticks that were placed in the feeding chambers (p = 0.12).

Light microscopy (H&E-stained SG histology and azure-stained whole SGs) did not reveal features that could be definitively associated with *C. felis* infection in tick SGs. However, we were able to partially characterize parasite morphology using TEM. Detailed results of each microscopic evaluation are described below.

Table 1. Summary of ticks with positive *C. felis* test results. (F: Female; M: Male; SG: Salivary glands; HT: Half tick; ISH: *in situ* hybridization; TEM: transmission electron microscopy; A: Attached; NA: Non-attached)

	Tick	F/M	SG PCR	HT PCR	SG ISH	Azure staining	TEM	A/NA
Unfed	1	F	+	+	+	+	+	
	2	F	–	+	–	+		
	3	F	–	+	–	–		
	4	F	+	+	–	–	–	
	5	F	–	+	–	–		
	6	F	+	–	–	–		
	7	F	–	–	+	–		
	8	M	+	–	+	+	–	
	9	M	–	+	–	–		
	10	M	–	+	–	–		
	11	M	+	–	–	–		
Feeding chamber	12	F	+	–	+	–		NA
	13	F	+	–	–	–		A
	14	F	+	–	–	–	+	NA
	15	F	+	+	+	+	+	NA
	16	F	+	+	–	–	–	A
	17	F	+	+	+	–		NA
	18	F	+	–	–	–		NA
	19	F	+	–	–	–		NA
	20	F	+	+	–	–		NA
	21	M	–	–	–	+		NA
	22	M	–	+	–	–		NA
	23	M	+	+	–	–		A
	24	M	–	+	–	–		NA
	25	M	–	+	–	–		NA
	26	M	–	+	–	–		NA
	27	M	+	–	–	–		NA

Table 2. Summary of agreement between molecular detection of *C. felis* (SG RT-PCR, HT RT-PCR, and ISH). (F: Female; M: Male; SG: Salivary glands; HT: Half tick; ISH: *in situ* hybridization)

		Total examined	Total negative	Total positives	SG PCR (+) HT PCR (+) ISH (+)	SG PCR (+) HT PCR (+) ISH (-)	SG PCR (+) HT PCR (-) ISH (-)	SG PCR (+) HT PCR (-) ISH (+)	SG PCR (-) HT PCR (+) ISH (-)	SG PCR (-) HT PCR (-) ISH (+)	SG PCR (-) HT PCR (+) ISH (+)
Unfed	F	42	35	7	1	1	1	0	3	1	0
	M	38	34	4	0	0	1	1	2	0	0
	Total	80	69	11	1	1	2	1	5	1	0
Feeding chamber	F	30	21	9	2	2	4	1	0	0	0
	M	30	24	6	0	1	1	0	4	0	0
	Total	60	45	15	2	3	5	1	4	0	0

3.1 Histology and RNAscope® ISH

Cytauxzoon felis 18S rRNA was detected in SGs via ISH in 6 out of 140 ticks (4.3%).

Hybridization signals were detected in rare SG acini. Within these infected acini, *C. felis* was not detected in every cell. In 4 ticks, signals were limited to single cells of the infected acini; while in the other 2 ticks, signals appeared to be present in 2–3 acinar cells. Positive signals were restricted to cytoplasm of the infected cells. We were able to corroborate the presence of *C. felis* by the detection of an additional ISH target (*C. felis* GAPDH mRNA) in 4 of 6 SGs that were positive for *C. felis* 18S rRNA by ISH. (figures 4, 5, and supplemental figure 1). Failure to detect *C. felis* GAPDH mRNA in the remaining two ticks was correlated with the absence of the infected cells being captured in subsequent serial sections (supplemental figure 1). Positive signals were not detected in any SG acinar cells from negative control ticks. Occasional chromogen uptake was noted in the tracheal and SG ductal linings of the Bouin's-fixed SGs from both positive and negative ticks. This background staining was substantially reduced with NBF-fixed SGs (figure 3).

When corresponding these ISH-positive cells with H&E-stained serial sections, the hybridization signals paralleled with acinar cells that have a hazy pale eosinophilic to amphiphilic cytoplasm and enlarged nuclei with dispersed chromatin (figure 3). No distinct nuclear features of parasites were observable in the cytoplasm of these infected cells. None of the affected acini appeared enlarged when compared to adjacent non-infected acini. These microscopic features were also more apparent on Bouin's-fixed SGs compared to NBF-fixed SGs (figure 4 and 5).

Of the 6 SGs that were *C. felis* ISH-positive, 3 were unfed and 3 were exposed to the feeding chamber. The 3 ticks that were exposed to the feeding chamber were categorized as non-attached at the time of collection.

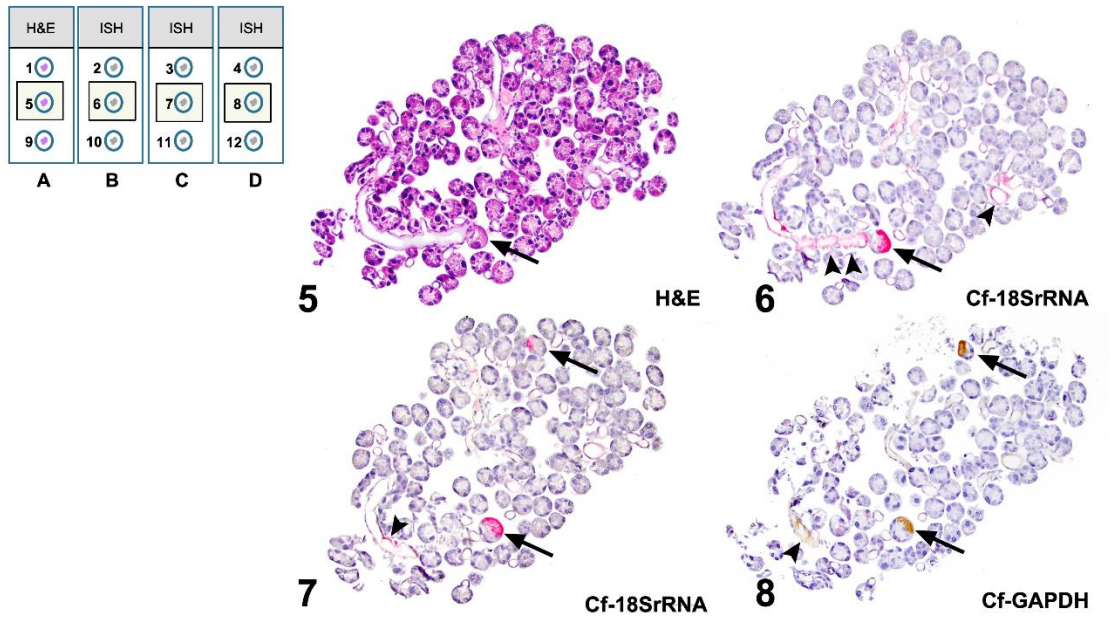

Figure 4. Bouin's-fixed *Amblyomma americanum* salivary gland, capturing *Cytauxzoon felis*-infected acini using H&E corresponding with two RNAscope ISH probes (Cf-18SrRNA and Cf-GAPDH) in serial sections (arrows, 5–8). Locations on the slide of the demonstrated SG sections are indicated in the top left diagram. There is moderate non-specific background staining mainly along the salivary duct linings (arrowheads). 20X
(H&E: Hemotoxylin and eosin; ISH: *in situ* hybridization.)

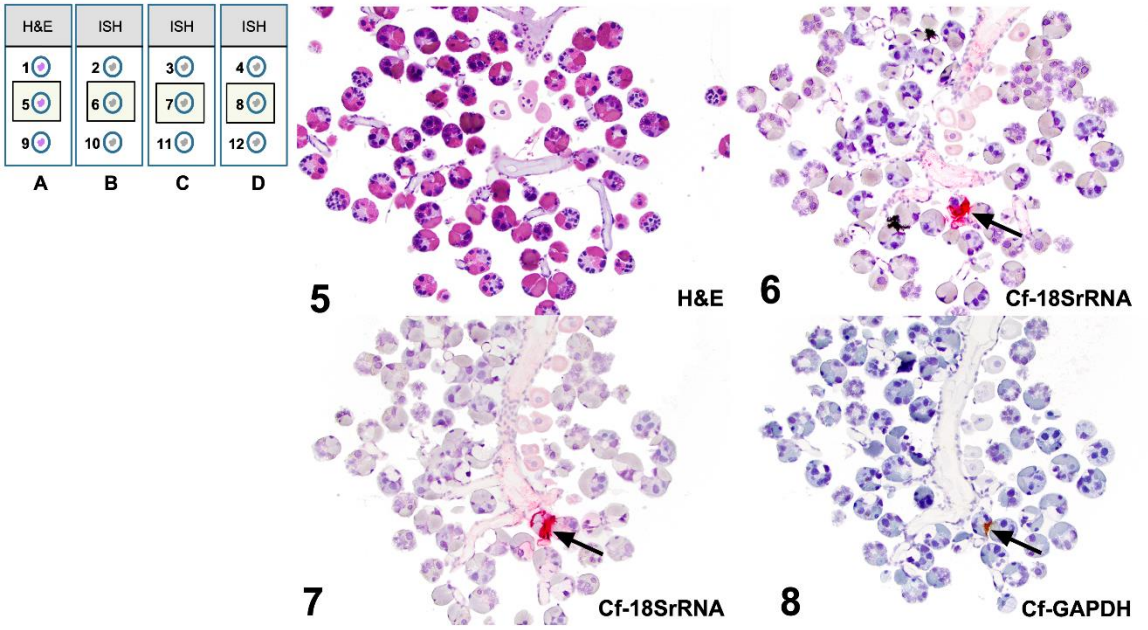

Figure 5. NBF-fixed *Amblyomma americanum* salivary gland, capturing a *Cytauxzoon felis*-infected acinus using two RNAscope ISH probes (Cf-18SrRNA and Cf-GAPDH) in serial sections (arrows, 5-8). There is less background staining in the salivary ducts compared to Bouin's-fixed samples. Locations on the slide of the demonstrated SG sections are indicated in the top left diagram. 20X
(H&E: Hemotoxylin and eosin; ISH: *in situ* hybridization. NBF: neutral-buffered formalin.)

3.2 Azure staining of whole salivary glands

Three of the unfed ticks and 2 of the ticks removed from the feeding chambers had rare enlarged salivary acini detected after azure staining (figure 6). When present, these swollen acini are 2–3 times in size compared to other acini in the same SG section. These enlarged acini appeared to have an increased cytoplasmic volume that is devoid of salivary granules. The cytoplasms of the swollen cells are pale blue with 1–4 dark blue nuclei. Enlarged acini were not noted in SGs from negative control ticks. Of the 5 ticks documented with these features, 3 were unfed and 2 were exposed to the feeding chamber. These 3 unfed ticks were all positive for *C. felis* via RT-PCR (SG or HT) and/or ISH. One SG from two ticks that were exposed to the feeding chamber was positive for *C. felis* via RT-PCR and/or ISH, and one was negative via all molecular detection methods. Of the two SGs that were exposed to the feeding chamber, none of the ticks were classified as attached to the membrane at the time of SG collection (table 1).

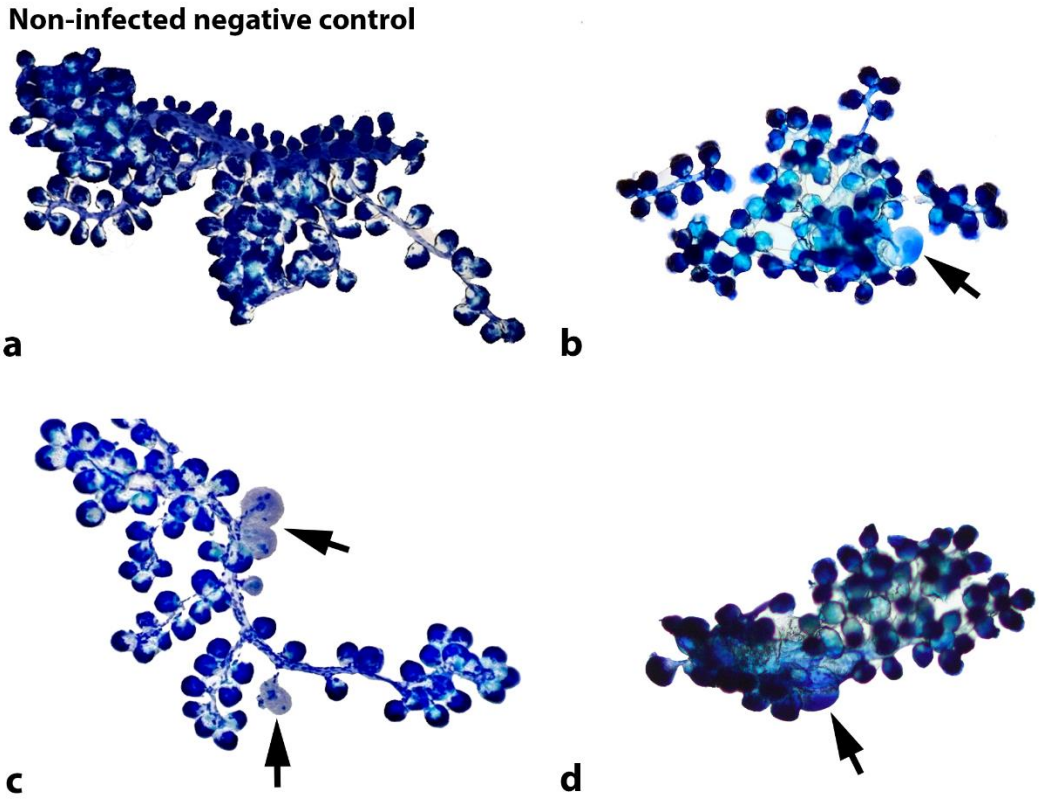

Figure 6. Azure staining of tick salivary gland (SG) wet mounts. (a) SG from a negative control, non-infected *A. americanum* adult. (b-d) SG from ticks that were acquisition-fed on *C. felis*-infected cat as nymphs. Rare SG acini (arrows) were noted to be swollen, pale staining and lack salivary granules. 20X

3.3 Transmission electron microscopy

Six SG quarters that tested positive for *C. felis* via SG RT-PCR were selected for TEM (3 unfed, 3 exposed to the feeding chamber). Three of these 6 SGs contained rare, small ovoid structures that resembled protozoal organisms. One of these 3 ticks was in the unfed group and the other 2 were exposed to the feeding chamber. Neither of the ticks that were exposed to the feeding chamber were categorized as attached at the time of collection. The protozoal organisms were often found in E cells or within intercellular spaces. The host cells appear damaged with winkled nuclei or nuclear remnants (figure 7a). Organisms ranged from 0.5–0.8 μm in diameter, encircled by a single or double-layered membrane, with several variably sized electron-dense secretory organelles within the cytoplasm, and occasionally contained a single nucleus (figure 7b). Three negative control ticks were also evaluated via TEM and structures resembling protozoal organisms were not detected.

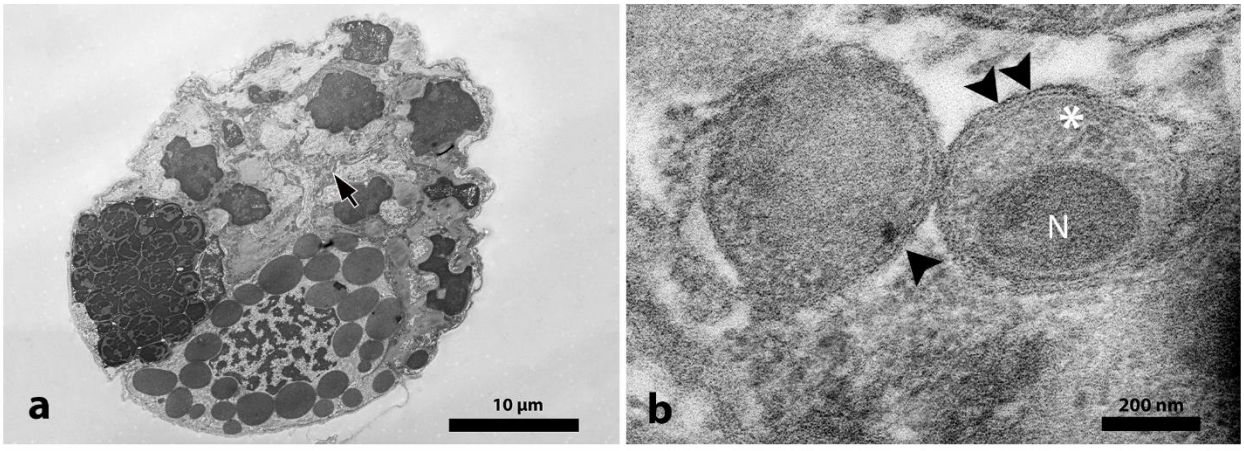

Figure 7. Representative electron micrographs of SG acinus with suspected *Cytauxzoon felis* organisms from an infected *Amblyomma americanum* adult. (a) Lower magnification (2000X) of an infected acinus. Infected cell is swollen and wrinkled, lack distinct salivary granules and host cell nucleus with fragments of nuclear debris. Multiple protozoal organisms are found scattered in cytoplasm and intercellular spaces (arrow), (b) High magnification (70,000X) of arrowed region in (a) capturing two suspect immature *C. felis* sporozoites. Parasites are ~500 nm in diameter, encircled by a double membrane (arrowheads), with intracytoplasmic secretory organelles (asterisk), and a nucleus (N).

4. DISCUSSION

The life cycle of *C. felis* has not been characterized in the vector host and is thought to be similar to other piroplasmids. We demonstrated *C. felis*-infected SGs in *A. americanum* using several microscopic techniques: ISH, light microscopy (direct staining and histology) and TEM. The visualization of two independent parasite RNA transcripts by ISH verifies the presence of *C. felis* in tick SGs. This finding was further supported by RT-PCR, light microscopy and TEM.

Despite being able to localize *C. felis* in tick SGs, it remained challenging to fully characterize the parasite morphology on light microscopy via direct azure staining or H&E. This finding contrasts with what has been described in other related piroplasmids, where infected salivary acinar cells are easily distinguishable under light microscopy. For instance, the *Theileria*-laden salivary acinar cells are often notably enlarged and contain many small parasitic nuclei [30–32]. These features were not observed in the SGs examined in the current study. It is possible that we were simply capturing the parasites at an early stage of sporogony that are difficult to directly visualize because only a few nuclei are present. Our TEM results supported this theory. Even with a low number of infected ticks and acini, we were able to find rare structures in *C. felis*-positive SGs with ultrastructural features that were suggestive of protozoal organisms. These features included double cellular membranes and intracytoplasmic secretory organelles (e.g. rhoptries or micronemes) that resembled those of *Theileria* and *Babesia* sporozoites [33–35]. However, we did not find evidence of syncytium formation, such as multiple nuclei and cytoplasmic partitioning [36] as commonly described in later stages of sporogony.

The ultrastructural features of *C. felis* in ticks have never been reported and only life stages found in the feline host had been documented via TEM [37,38]. Although we were able to identify *C. felis* organisms via TEM, only rare organisms were found, presumably due to the low numbers of infected acini. Unfortunately, screening for rare events, such as this, using TEM can be extremely labor-intensive and

expensive. In our study, the sections used for TEM were 85 nm thick. Therefore, to image the entire quartered SG section from a single tick (~0.5-1 mm^3) would require processing and interpretation of 5,000-10,000 sections. A targeted approach using correlative light and electron microscopy that is supplemented with *in situ* molecular techniques (e.g. ISH, IHC or immunofluorescence) should be explored to identify infected acini for ultrastructural imaging [39,40].

We found discordant results between our three molecular detection techniques: SG RT-PCR, HT RT-PCR and SG ISH. The majority (20/26) of the PCR-positive ticks (either SG or HT) were negative via ISH, and one PCR-negative tick (by both SG or HT) was positive via ISH. There are three primary explanations for these findings. First, the sensitivity for detecting *C. felis* probably varies between our testing modalities. PCR is generally thought to be more sensitive than ISH because it employs exponential amplification of RNA transcripts, whereas the detectability of ISH signals is directly dependent on the amount of targeted RNA present in the examined tissue sections [41,42]. This could explain why most PCR-positive SGs were negative via ISH, as these SGs may have contained too few *C. felis* RNA targets to be detectable. Second, the difference in detection sensitivity is further compounded by the low percentage of infected acini in *C. felis*-positive tick SGs. Positive ISH signals were detected in only one acinus in 3 ticks, and 2 acini in the other 3 ticks. This translates to an acinar infection rate of approximately 1.25–4% as there were ~50-80 acini per examined SG section. Therefore, it is likely that some of the SG quarters from *C. felis*-infected ticks may not have contained any infected acini and yielded a false negative result. Third, the discordant PCR and ISH results in SG and HT carcasses may be due to asynchronous development of *C. felis* in ticks. *C. felis* RNA was detected solely in HT carcasses in 9 ticks via RT-PCR. While it is possible that the SGs were falsely negative as discussed previously, some parasites may have remained in the midgut or other tick organs and have not invaded the SGs. *C. felis* RNA was also simultaneously detected in both HT carcasses and SG in 7 ticks via PCR and/or SG ISH. This could be an indication that the parasites are developing asynchronously and are present in the tick

concurrently in different life stages. This finding of asynchronous development has also been previously described for *Theileria* and *Babesia* in ticks [33,43,44]. Further investigation, including localizing parasites in tick organs using ISH or TEM, should be explored to validate this theory.

Future studies should also evaluate the effects of feeding on *C. felis* sporogony. In *Theileria* spp., infected SGs appear more detectable in ticks that were partially fed when compared to unfed ticks, presumably due to rapid parasite replication with SG acinar cells [45,46]. In the current study, we only examined unfed ticks and ticks that had been exposed to feeding chambers for a short period of time (12–72 hours). While a subset of ticks in our study were placed in artificial feeding units, only 3 infected ticks had clear evidence of feeding (presence of blood in the midgut) visualized at the time of SG dissection. Even though *C. felis* was not detected in any of these ticks via ISH, our sample size was too small to assess whether feeding had any effects on our ability to detect *C. felis* in SGs.

We provided the first conclusive evidence of *C. felis* in tick SGs. Our ability to use ISH to identify and localize the parasites in tick organs and cells suggests that this is a useful platform to characterize the life cycle of *C. felis* in ticks. This would also serve as a basis to facilitating future studies involving sporozoites, including initiating *in vitro* culture or developing sporozoite-based vaccines against cytauxzoonosis.

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Authorship:

T. Yang, A. Birkenheuer, M. Reichard contributed to study conception, execution, and design. L. Miller and A. Bell contributed to optimization of experimental techniques and procedures. All authors contributed to editing and approved the final manuscript.

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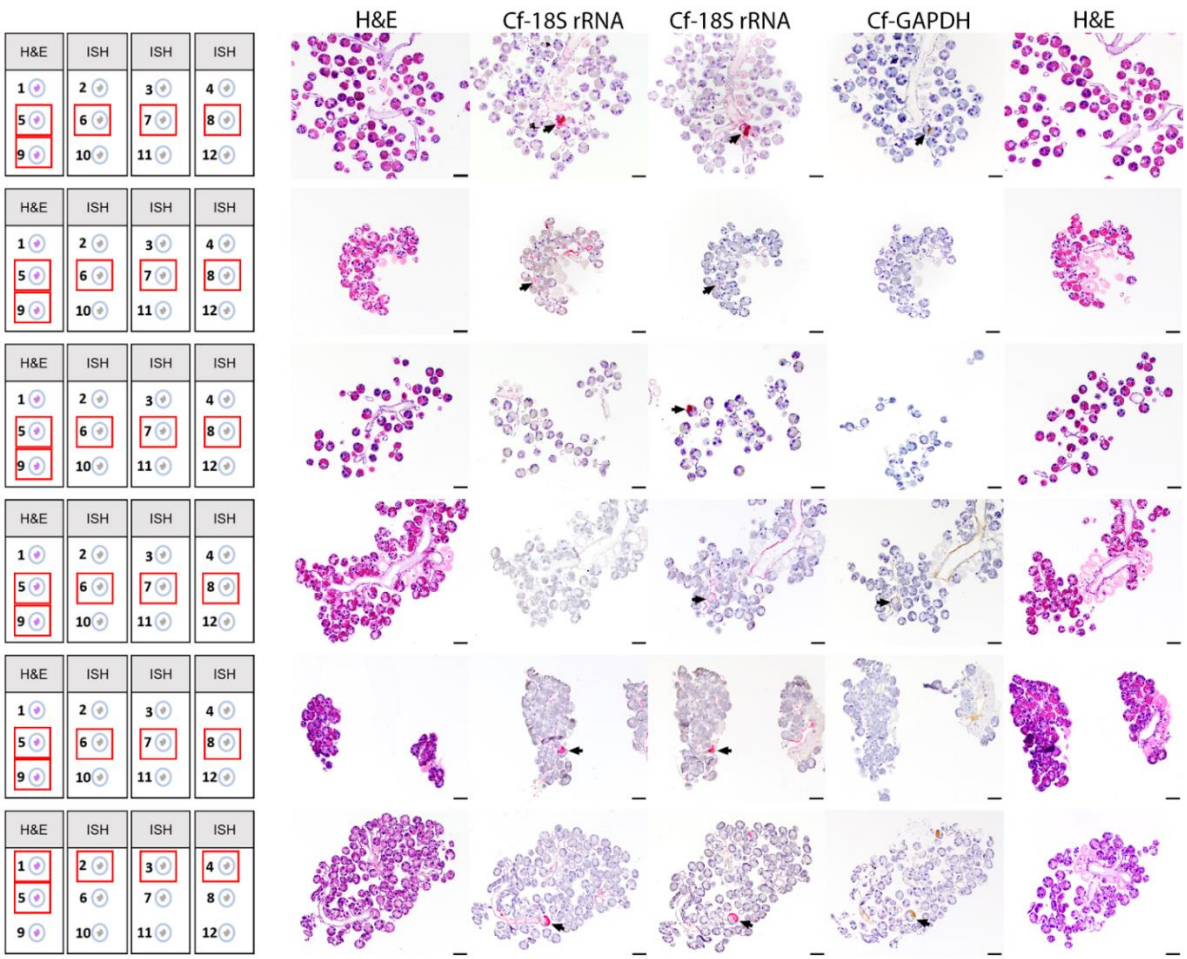
SUPPLEMENTAL MATERIAL



Supplemental figure 1. Histology and ISH results from all six *Cytauxzoon felis*-ISH positive salivary glands. Left panel shows the locations of serial SG sections on slides used for H&E histology and ISH. Red squares reflect the specific SG sections demonstrated to the right. Positive *C. felis* ISH signals are indicated by arrows. (bar = 100 μm)

CHAPTER 4

Direct injection of *Amblyomma americanum* ticks with *Cytauxzoon felis*

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Direct injection of *Amblyomma americanum* ticks with *Cytauxzoon felis*

Tzushan S. Yang, Mason V. Reichard, Henry S. Marr, Leah Cohn, Laura Nafe, Nathan Whitehurst, Adam J. Birkenheuer

ABSTRACT

Cytauxzoon felis is a tick-borne hemoprotozoon that causes life-threatening disease in domestic cats in the United States. Currently, the platforms for *C. felis* research are limited to natural or experimental infection of domestic cats. This study aims to develop an alternative model by infecting *Amblyomma americanum* ticks with *C. felis* via direct injection. *Amblyomma americanum* adults were injected with *C. felis*-infected feline erythrocytes through two routes: directly into the digestive tract through the anal pore, or percutaneously into the tick hemocoel. RNAscope® *in situ* hybridization (ISH) was used to visualize the parasites within the ticks at different time points after injection. Four months after injection, ticks were divided into 3 infestation groups based on injection methods and inoculum type and fed on 3 naïve cats to assess the ticks' ability to transmit *C. felis*. Prior to the transmission challenge, selected ticks from each infestation group were tested for *C. felis* RNA via reverse transcription-PCR (RT-PCR). In both IA- and IH-injected ticks, ISH signals were observed in ticks up to 3 weeks after injection. The number of hybridization signals notably decreased over time, and no signals were detected by 4 months after injection. Prior to the transmission challenge, 37-57% of the sampled ticks were positive for *C. felis* RNA via RT-PCR. While the majority of injected ticks successfully attached and fed to repletion on all 3 cats during the transmission challenge, none of the cats became infected with *C. felis.* These results suggest that injected *C. felis* remained alive in ticks but was unable to progress to infective sporozoites after injection. It is unclear why this

infection technique had been successful for other closely related tick-borne hemoprotozoa and not for *C. felis.* This outcome may be associated with uncharacterized differences in the *C. felis* life cycle, the lack of the feeding or molting in our model or absence of gametocytes in the inoculum. Nonetheless, our study demonstrated the potential of using ticks as an alternative model to study *C. felis*. Future improvement of a tick model for *C. felis* should consider other tick species for the injection model or utilize infection methods that more closely emulate the natural infection process.

1. INTRODUCTION

Cytauxzoon felis is an important tick-borne apicomplexan hemoparasite that can cause fatal disease in domestic cats in the United States [1–4]. Currently, even with the best medical care the mortality rate for acute cytauxzoonosis remains high [5,6]. Additionally, the distribution of *C. felis* appears to be spreading and is no longer limited to the southeast and southcentral U.S. [7,8]. This shift is likely due to the expanding geographic range of the main tick vector, *Amblyomma americanum* [9]. No vaccine is available for *C. felis*, and tick control is currently the only means to prevent infection in domestic cats [10,11]. The greatest barrier facing *Cytauxzoon* research, including the development of a vaccine, has been the inability to culture the parasite. Therefore, most research has relied on experimental or natural infection of domestic cats [12–14]. There is a critical need to establish cost-effective alternative methods to study *C. felis* that minimize the use of live animals.

To date, ticks have mainly been thought of as "just a vector" rather than a host that can be used to study *C. felis.* Ticks typically acquire infective organisms through a blood meal while feeding on live animals. To minimize the usage of live animals for this process, several artificial infection methods have been established and utilized for other tick-borne pathogens [15]. One of these methods is direct injection, which is a technique that has been used to experimentally infect ticks with several tick-borne pathogens [16–19]. Importantly, these include piroplasmids that are closely related to *C. felis*, *Theileria* and *Babesia* [20–22].

The main objective of this study was to develop a model to infect ticks via direct injection with *C. felis* that eliminates the need for acquisition feeding on cats and subsequent molting. To achieve this goal, we injected *A. americanum* adults with *C. felis*, and assessed the viability of

the parasites in the ticks as well as the ability of the injected ticks to transmit the infection to naïve cats.

2. MATERIALS AND METHODS

2.1 Tick maintenance

Pathogen-free adult *A. americanum* ticks were purchased from Oklahoma State University tick rearing facility (Stillwater, OK). Ticks were maintained in conditions recommended by the tick rearing facility with minor modifications. Specifically, ticks were stored in paper cartons in a humidity chamber with 90–99% relative humidity at 20–23°C with a 12 hour light/12 hour dark photoperiod. Ticks were checked every 1–3 days for mortality and fungal contamination. Paper cartons were changed every 3–4 weeks.

2.2 Inoculum

All blood samples were collected in accordance with North Carolina State University Institutional Animal Care and Use Committee (IACUC) approved protocol 20-254 or were diagnostic samples that were scheduled to be discarded. Blood was collected from privately-owned domestic shorthair cats diagnosed with acute cytauxzoonosis. Approximately 1–2 ml of infected blood was collected into EDTA-containing tubes and shipped on ice to the author's laboratory overnight. Upon arrival, thin blood smears were stained with Hema 3 stain (Fisher Scientific, Hampton, NH, USA) and examined via light microscopy for the presence of intraerythrocytic *C. felis*. Upon arrival, thin blood smears were stained with Hema 3 stain (Fisher Scientific, Hampton, NH, USA) and examined via light microscopy for the presence of intraerythrocytic *C. felis*. The percent parasitemia was determined via light microscopy by

counting the number of intraerythrocytic parasites per 1000 erythrocytes. Total DNA was extracted from an aliquot of each sample using a DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions. Eluted DNA was tested for *C. felis* using a species-specific qPCR assay targeting the *cox3* gene of *C. felis* as previously described [23]. Only samples that were positive for both light microscopy (parasitemia 5–8%) and qPCR were used for injection.

Prior to injection, *C. felis*-infected blood was washed twice with equal volumes of either phosphate-buffered saline (PBS) (Gibco, Gaithersburg, MD, USA) or Alsever's solution (Alfa Aesar, Haverhill, MA, USA). Packed cell volume was adjusted to 50–60%. PBS-washed inoculum was used immediately for injection. Alsever's-washed inoculum was either used immediately or stored at 4ºC for up to 10 days until injection.

2.3 Tick injection

One hundred and fifty female and 150 male *A. americanum* adult ticks were used for injection. Males and females were divided into two groups for two different routes of injection: 1) Intra-anal pore (IA) injection into the tick midgut to mimic the natural route of infection; or 2) intra-hemocoel (IH) injection to bypass the midgut. Capillary needles for injection were prepared using a micropipette puller (Narishige, Amityville, NY). The tips were broken under a dissecting microscope and adjusted to match the size of the injection site. Approximately 5–10 µl of inoculum was then loaded into the capillary needles via microloader pipettes (Eppendorf, Hamburg, Germany).

Prior to injection, all ticks were surface sterilized as previously described [24]. The ticks were then placed ventral side up on double-sided tape fixed on a clean glass slide, and the anterior 1/3 of the body secured by cellophane tape. For the IA group, the inoculum was injected directly into the tick's anal pore using a microinjector (pneumatic picopump, WPI) until leakage of the inoculum was observed from the injection site (females ~5 µl, males ~2 µl). For the IH group, the cuticle of the ventral surface of the tick was nicked adjacent to the anal pore with the tip of a tuberculin syringe (27 gauge) and the capillary needle was inserted through the incision for injection until leakage of the inoculum was observed from the injection site. Excess inoculum around the injection site was cleaned with Kim wipes and 70% ethanol. Ticks were then returned to the humidity chamber.

2.4 RNAscope® in situ hybridization

For visualization of *C. felis*, IA- and IH-injected ticks were randomly selected for histopathology and *in situ* hybridization (ISH) at 24 hours (n=2), 1 week (n=8), and 3 weeks (n=8) post injection. At 4 months post-injection, ticks that tested positive for *C. felis* via RT-PCR (see section 2.5.1) were used for ISH (n=18).

A proprietary antisense probe (Cf-18S-rRNA) designed to target a 77 bp fragment of *C. felis* 18S rRNA (position 666-742 of GenBank Accession AF399930.1) was developed. RNAscope® assays are optimized for RNA detection and performed under conditions to minimize nuclear penetrance and therefore do not favor denaturation and hybridization of double-stranded chromosomal DNA (Advanced Cell Diagnostics; Newark, CA) [25]. To validate the specificity of this probe, we used positive controls consisting of formalin-fixed paraffin-embedded tissues

from cats diagnosed with acute cytauxzoonosis, and negative controls consisting of ticks injected with feline blood that tested negative for the presence of *C. felis*.

To prepare ticks for histopathology, ticks were first placed in 70% ethanol for 6–12 hours, and then transferred to Bouin's solution (StatLab, Columbia, MD, USA) and placed in a pressurized vacuum for one hour to facilitate fixative penetration. After fixation, ticks were washed in 70% ethanol for 5 minutes, and embedded ventral side up in molten Histogel™ until solidification. Ticks were then placed in tissue cassettes for paraffin embedding and sectioning (5 μm thick). Three sections per tick were stained with hematoxylin and eosin (H&E) and 3 unstained sections were used for ISH. ISH was performed using RNAscope® 2.5 HD red assay kits according to manufacturer's instructions after optimizing pretreatment times for tick samples (10 minutes for target retrieval and 30 minutes for protease plus treatment).

2.5 Cytauxzoon felis detection of injected ticks

An overview of the transmission challenge is depicted in **Figure 1**. Injected ticks were divided into 3 infestation groups based on injection methods (IA vs. IH) and inoculum type (washed with PBS vs. Alsever's solution).

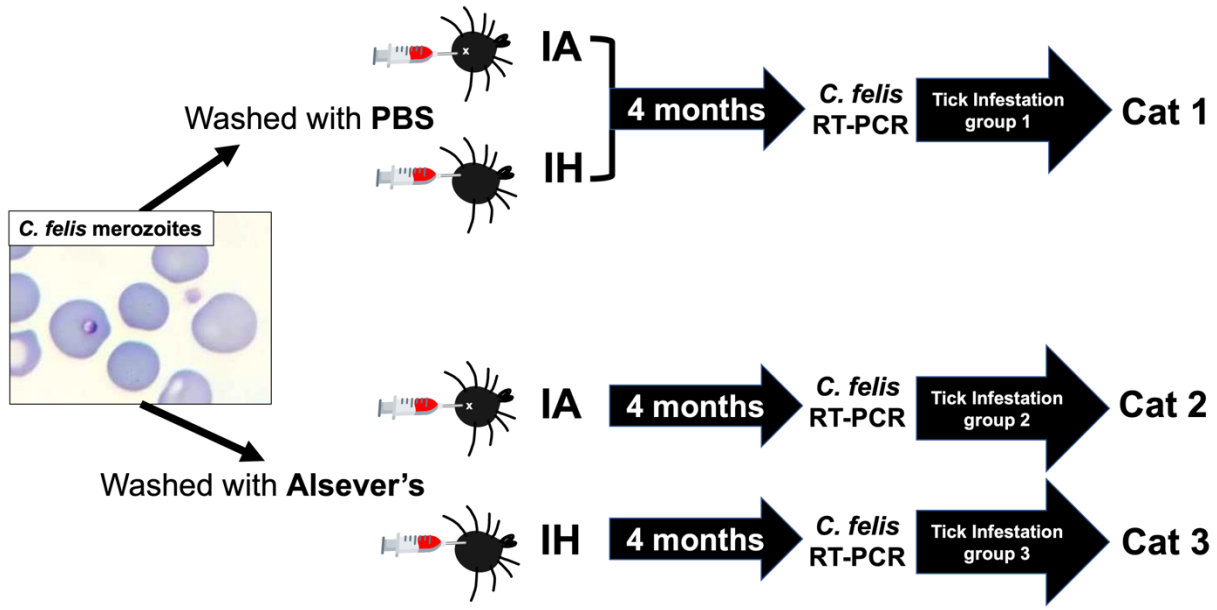

Figure 1. Experimental design of tick *C. felis* transmission challenge. IA: Intra-anal pore injection; IH: Intra-hemocoel injection; PBS: Phosphate-buffered saline; RT-PCR: Reverse-transcriptase polymerase chain reaction.

2.5.1 RT-PCR prior to transmission challenge

Four months after injection, ticks were randomly sampled from each infestation group (group 1, n=8; group 2, n=14; group 3, n=14) for *C. felis* two-step RT-PCR. Briefly, each tick was surface sterilized [24] and cut in half with a new scalpel blade on a clean glass slide. Total RNA was extracted from one half of the tick using an RNeasy Plus Micro kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Ten µl of eluted RNA was used to synthesize complementary DNA (cDNA) with Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) following manufacturer's instructions. One µl of cDNA was then used as template in a *C. felis*-specific real-time PCR assay targeting a 284 bp fragment of the *C. felis* 18S rRNA [23]. This assay was selected as 18S rRNA is likely to be transcribed throughout all *C. felis* life stages. DNA from *C. felis*-positive cats were used as positive control for each set

of reactions. Negative controls included cDNA from sham-injected ticks, RNA samples (no RT controls) and water (no template controls). All reactions were performed in CFX96™ Real-Time PCR Detection System combined with C1000™ or MiniOpticon Thermal Cycler (Bio-Rad, Hercules, CA 94547, USA). Amplification and melt curves were analyzed via CFX96™ Manager software. Amplified products were randomly selected for sequencing (Genewiz, South Plainfield, NJ, USA) and analyzed bi-directionally with Geneious Prime (San Diego, CA, USA).

2.5.2 qPCR after transmission challenge

All ticks that were attached were removed at day 21 post-infestation. Genomic DNA was extracted from these ticks using a QIAamp DNA mini kit (Qiagen) according to manufacturer's tissue extraction protocol with slight modifications. Briefly, male and non-engorged female ticks were cut in half with a clean scalpel blade, frozen in liquid nitrogen for 5-10 minutes and ground into fine powder using a pellet pestle (Fisher Scientific) within a 1.5 ml conical bottom microcentrifuge tube (Eppendorf). For digestion, 250 µl of ATL lysis buffer and 25 µl proteinase K were used at 56ºC overnight (12-18 hours). Engorged females were similarly cut in half. These half ticks were homogenized using Bead Mill 4 (Fisher Scientific) for 90 seconds at 5 m/s after the addition of 500 µl of sterile PBS and five 3.5 mm steel beads (Biospec Inc., OK). This homogenate was digested overnight (12-18 hours) at 56ºC in 500 µl of ATL buffer and 50 µl of proteinase K. For males and non-engorged females, 250 µl of AL buffer was added to each tick lysate. For engorged females, 500 µl of AL buffer was added to each tick lysate. Total DNA was then extracted from these lysates according to manufacturer's instructions. DNA samples were then tested using a *C. felis*-specific *cox3* qPCR assay [23]. Plasmids containing *C. felis cox3* inserts were used as positive controls for each set of reactions. DNA extracted from a non-

injected unfed *A. americanum* female tick and water were used as negative controls. Amplification and melt curves were analyzed via CFX96™ Manager software. Amplicons were visualized in an Agarose gel after electrophoresis to confirm the correct amplicon size. Fourteen representative amplicons were submitted for bidirectional sequencing (Genewiz) and analyzed with Geneious Prime.

2.5.3 Comparison of *C. felis* detection pre- and post-transmission challenge

Prevalence of *C. felis* in injected ticks was estimated according to Bush et al.[26] Ninety-five percent confidence intervals were calculated using QuickCalcs (QuickCalcs, 2017). Proportions of *C. felis* infected ticks were compared using a chi-square tests or Fisher's Exact tests [27]. A Fisher's exact test was used if 20% of the expected values in contingency tables were less than 5. If there was not statistical difference between pre- and post-infestation of *C. felis* in ticks, data were combined for each group to compare injection routes. Significance level was set at 0.05.

2.6 Tick transmission challenge

Approximately 30 male and 30 female ticks were randomly selected from each infestation group and shipped to Oklahoma State University for the transmission challenge. Transmission feeding on naïve cats was performed as previously described [14,28,29] using procedures approved through Oklahoma State University IACUC. Tick attachment was estimated on day 3 and day 7 post-infestation, and unattached ticks were removed. All attached ticks were fed to repletion or removed by day 21 post-infestation and stored in 70% ethanol. Cats were monitored daily for signs of acute cytauxzoonosis, i.e. fever, lethargy, depression, inappetence and hyporexia.

2.7 C. felis PCR of naïve cats during transmission challenge

Whole blood was collected into EDTA tubes on days 0, 3, 7, 13, 17, 21, and 30 post infestation from each cat. Thin blood smears were stained with Hema 3 stain and screened for parasites via light microscopy. Total DNA was extracted as described above in section 2.2. Each sample was then tested in ten replicate reactions using a qPCR assay targeting *C. felis cox3* mitochondrial DNA. This assay was selected as it is more sensitive for the detection of early *C. felis* infection compared to 18S ribosomal DNA [23].

3. RESULTS

3.1 Detection of C. felis ISH signals over time in ticks after injection

The ISH probe (Cf-18S-rRNA) is specific for *C. felis* and does not cross react with ticks injected with *C. felis*-negative feline erythrocytes. (**Figure 2**)

Cytauxzoon felis hybridization signals were detected in ticks 24 hours after injection with *C. felis*-positive inoculum. The number of hybridization signals gradually decreased over time after injection for both IA- and IH-injected ticks (**Figures 3 and 4**) until they were undetectable at 4 months. Subjectively, signal intensity did not differ between inoculum types. Positive hybridization signals frequently correlated with areas where feline erythrocytes were present and *C. felis* signals were never detected within tick cells (**Figure 5**).

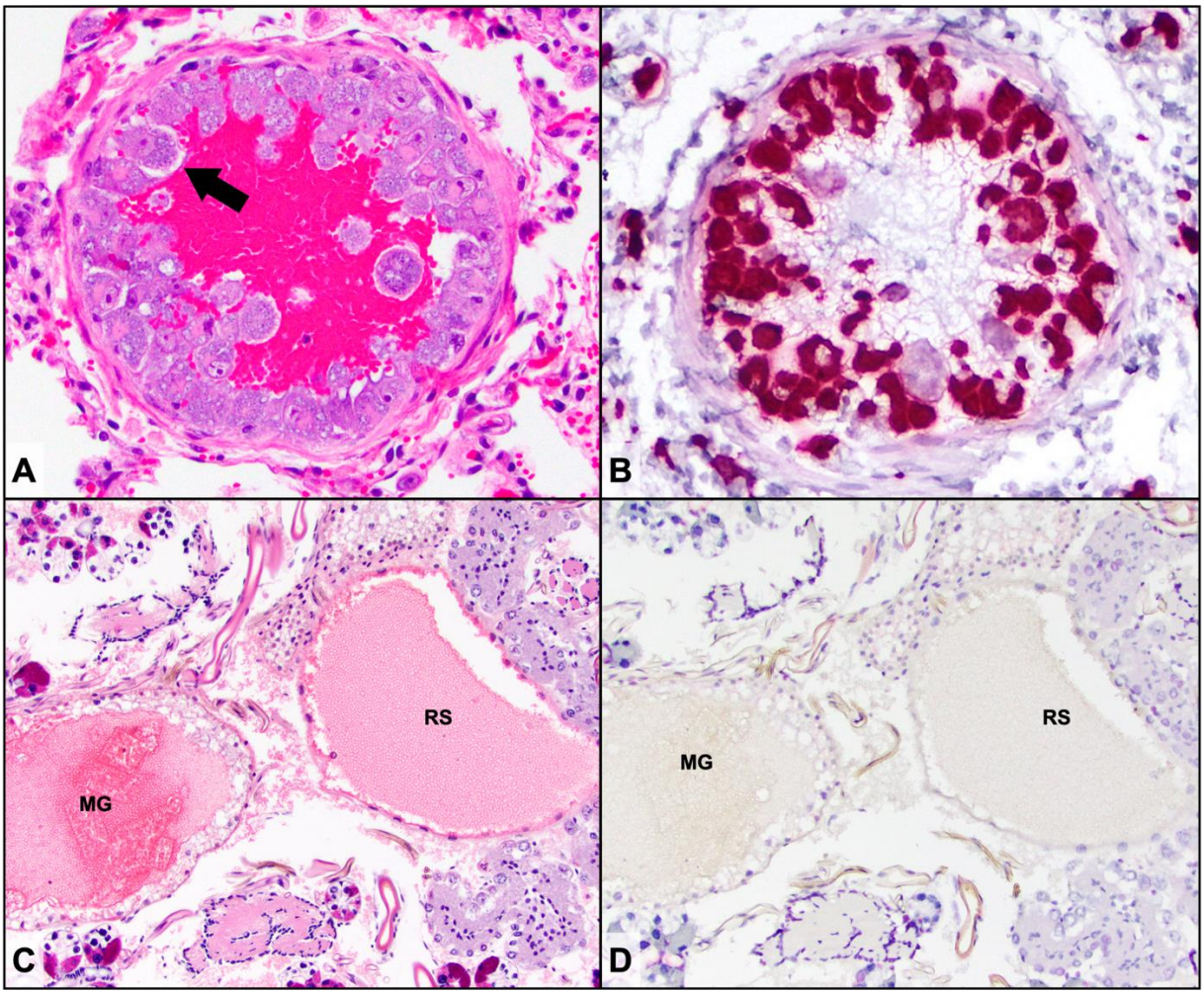

Figure 2. *Cytauxzoon felis* 18S ISH probe is specific to infected feline cells with no cross-reactivity with negative feline erythrocytes or tick cells. **(A)** Positive control: H&E stained feline lung section with intravascular *C. felis* schizont-laden leukocytes (arrow) adhered to vascular endothelium. 40X. **(B)** Serial section of 2A probed with Cf-18S-rRNA showing robust hybridization signals (red) of *C. felis* schizonts. **(C)** Negative control: H&E stained tick injected with *C. felis*-negative feline erythrocytes through the anal pore into the midgut (MG) and rectal sac (RS). MG and RS are filled with feline erythrocytes. 20X. **(D)** Serial section of 2C probed with Cf-18S-rRNA showing no detectable hybridization signals in the injected feline erythrocytes or the surrounding tick cells.

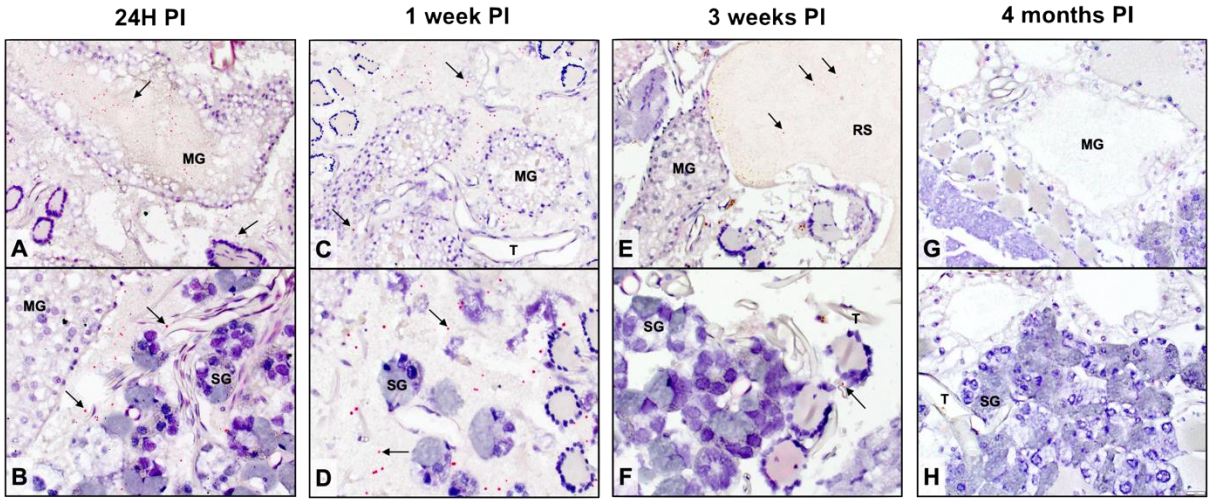

Figure 3. RNAscope® ISH assay in ticks after IA injection with *C. felis*: hybridization signals decreased over time within the ticks after injection. Thin arrows point to representative hybridization signals. **(A,B)** 24 hours post-injection: numerous *C. felis* hybridization signals (red) were seen within the midgut lumen and scattered in the hemocoel **(C,D)** 1 week post-injection: the number of hybridization signals are no longer detected within the midgut lumen and were decreased in numbers in the hemocoel. **(E,F)** 3 weeks post-injection: hybridization signals (arrow) were only seen sporadically in the rectal sac and hemocoel. **(G,H)** 4 months post-injection (immediately prior to transmission challenge): no hybridization signals were detectable. PI: post-injection; MG: midgut; SG: salivary gland; T: tracheae; RS: rectal sac.

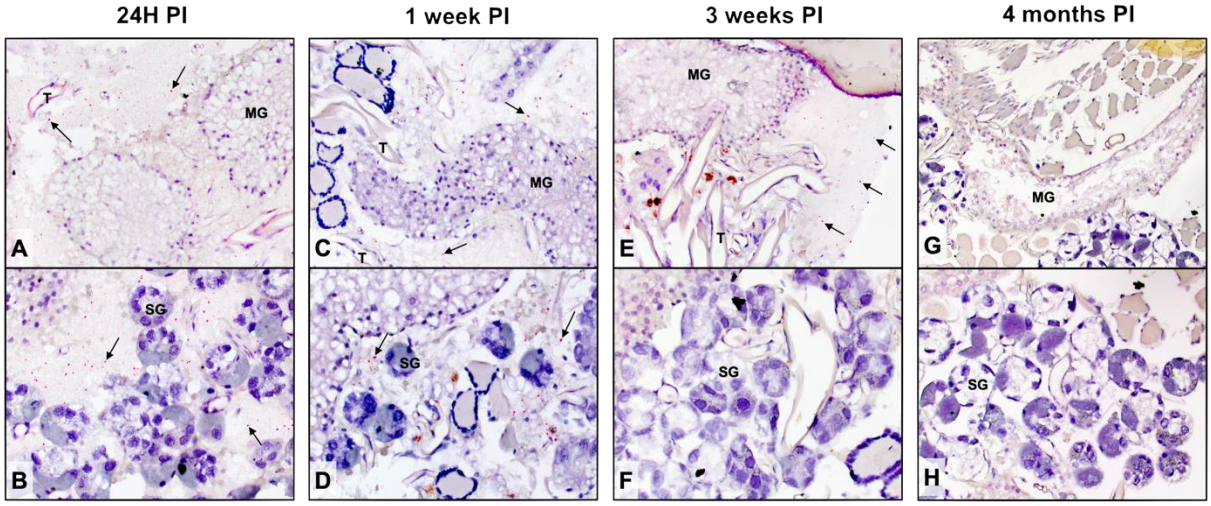

Figure 4. RNAscope® ISH assay in ticks after IH injection with *C. felis*: hybridization signals decreased over time within the ticks after injection. Thin arrows point to representative hybridization signals. **(A,B)** 24 hours post-injection: numerous *C. felis* hybridization signals (red) were seen within the hemocoel. **(C,D)** 1 week post-injection: the number of hybridization signals decreased in numbers in the hemocoel. **(E,F)** 3 weeks post-injection: hybridization signals (arrow) were only seen regionally in the hemocoel. **(G,H)** 4 months post-injection (immediately prior to transmission challenge): no hybridization signals were detectable. PI: post-injection; MG: midgut; SG: salivary gland; T: tracheae.

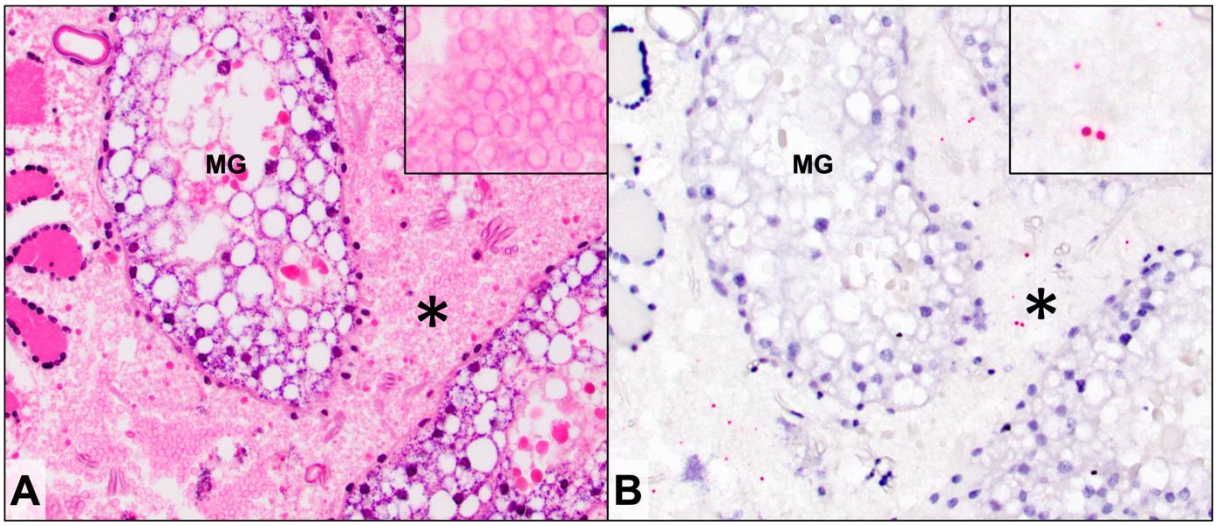

Figure 5. *Cytauxzoon felis* hybridization signals within the ticks correspond with areas where injected feline erythrocytes are present. **(A)** H&E stained tick 1 week after IH injection with *C. felis*-infected feline erythrocytes. Numerous feline erythrocytes maintained its silhouette within the hemocoel (inset: higher magnification of asterisk area) (40X) **(B)** Serial section of 5A when *C. felis* 18S ISH probe is applied. Hybridization signals within the hemocoel correlated with the presence of feline erythrocytes (inset: higher magnification of asterisk area). MG: Midgut; IH: Intra-hemocoel injection; ISH: in situ hybridization.

3.3 Detection of C. felis by RT-PCR and PCR in ticks after injection

Four months after injection and prior to the transmission challenge, 36 ticks were tested for *C. felis* RNA: 3 of 8 ticks (37.5%; 13.5–69.6%) from cat infestation group 1 tested positive; 7 of 14 ticks (50%; 26.8–73.2%) from cat infestation group 2 tested positive; and 8 of 14 ticks (57.1%; 32.6–78.7%) from cat infestation group 3 tested positive. All positive samples matched the melting temperature of the positive control (85±0.5ºC). *C. felis* was not detected in any negative control reactions, including the no-RT controls.

Tick attachment rates were adequate to excellent in all 3 infestation groups, ranging from 60-96% by day 7. At day 21 after tick infestation, 128 attached ticks were removed and tested for *C. felis* DNA by PCR: 10 of 33 ticks (30.3%; 17.3–47.5%) removed from cat 1 tested positive; 23 of 42 ticks (54.8%; 39.9–68.8%) removed from cat 2 tested positive; and 24 of 53 ticks (45.3%;

32.7-58.6%) removed from cat 3 tested positive. All positive samples had a melting temperature and amplicon size that matched the positive control. Representative amplicon sequences (n=14) were confirmed to be *C. felis* (100% identity, GenBank accession KC207821). *Cytauxzoon felis* was not detected in any negative control reactions.

The proportion of the positive ticks detected between pre- and post-infestation did not significantly differ in any group (group 1: p-value = 0.69 [Fisher's Exact test]; group 2: X^2 = 0.0000, df = 1, p = 1.000; group 3: X^2 = 0.239, df = 1, p = 0.625). Similarly, the proportion of positive ticks among groups did not differ statistically (X^2 = 4.496, df = 2, p = 0.106).

3.4 Cytauxzoon felis transmission challenge

Cytauxzooon felis infection was not documented in any of the naïve cats throughout the transmission challenge. All 3 cats tested negative for *C. felis* by PCR prior to the challenge. The cat exposed to infestation group 1 ticks developed no signs of cytauxzoonosis. Fever was the only sign observed in the other 2 cats. For the cat exposed to infestation group 2 ticks, a fever (39.3°C, 102.7°F) was noted on day 13 post-infestation. The cat exposed to infestation group 3 ticks, fever was noted on both days 13 (39.6°C, 103.3°F) and 14 (39.7°C, 103.4°F) post-infestation. The fevers in both cats resolved without medical intervention. *Cytauxzoon felis* was not detected by PCR or microscopy in any blood samples collected throughout the transmission challenge.

4. DISCUSSION

Since its discovery over 40 years ago in the United States, *Cytauxzoon felis* continues to be a significant cause of life-threatening illness in domestic cats living in enzootic areas (Cohn and Birkenheuer, 2012; Wagner, 1976; Wikander et al., 2020). The lack of an *in vitro* culture system for *C. felis* significantly limits our ability to study the parasite. Current experimental models require both acquisition and transmission feeding on domestic cats. Direct injection is an established technique that has resulted in successful infection of ticks for other pathogens and eliminates the need for acquisition feeding (Bonnet and Liu, 2012; Goddard, 2003; Kariu et al., 2010; Taank et al., 2020). Most notably, ticks that were injected with *Theileria* or *Babesia*, the two closest relatives of *C. felis*, were successfully infected and were able to transmit the infections to naïve animals (Battsetseg et al., 2007; Jongejan et al., 1980; Walker et al., 1979). Our goal was to infect *A. americanum* ticks with *C. felis* via direct injection.

In this study, *A. americanum* adults were injected with *C. felis* but failed to transmit this infection to naïve cats. It remains unclear whether or not these ticks actually became infected with *C. felis* after direct injection. Our ability to detect *C. felis* RNA transcripts in these ticks 4 months after injection suggests the presence of living *C. felis*. However, we were unable to determine whether these RNA transcripts indicated infection of these ticks with *C. felis* or just the persistence of erythrocytic life stages from the initial injection. The erythrocytic life stages of *C. felis* are presumed to be long-lived and can be detected in chronically infected cats for years [12,30]. While the exact lifespan of these *C. felis* erythrocytic life stages has not been studied, the erythrocytic life stages of other closely related hemoprotozoons, like *Babesia* and *Plasmodium* sp., are resilient and able to survive up to 60 days *in vitro* [31,32]. In this study, *C. felis* ISH signals were often detected within areas where erythrocytes were present and were

never observed within tick cells. Therefore, it is possible that the ticks never became infected and *C. felis* organisms simply survived for months after injection. Other potential explanations for the failure of parasite development within these ticks include decreased infectivity of the inoculum for ticks or the absence of feeding and molting in the model leading to subsequent lack of stimuli for *C. felis* to infect tick cells and advance to the next life stages.

It is possible that the shipping and storage conditions (4ºC) may have diminished the number of viable parasites within the inoculums or affected the parasites' ability to develop within ticks. While the effects of standard blood storage on *C. felis* viability have not been studied extensively, the authors have used *C. felis*-infected blood samples that were shipped and stored in similar conditions to successfully infect naïve cats via intravenous inoculation. In a *T. parva* infection study, inoculum was stored at 4°C for up to 3 days prior to injection and was still able to successfully infect ticks [21]. Also, *Babesia* spp. and *Plasmodium* spp. remain viable and infectious to vertebrate hosts via intravenous inoculation after 30 days in cold storage at 4ºC [31,33]. Unfortunately, the viability of our inoculums was not assessed prior to tick injection. *C. felis* RNA transcripts were not assessed by RT-PCR as these inoculums were not stored in an RNA-stabilizing solution. We also did not attempt to infect naïve cats with these inoculums directly via intravenous injection. As mentioned above, the detection of RNA transcripts in *C. felis* organisms in ticks after injection is strongly suggestive of live parasites in the inoculums. Based on our experience and previous experiments with related organisms, we believe it is highly likely that our inoculums contained viable parasites, but they either failed to infect the ticks or were unable to complete their life cycle.

Another potential explanation for the lack of parasite development after injection may be the absence of gametocytes in the inoculum. In other closely related piroplasmids, only a small proportion of intraerythrocytic merozoites transform into gametocytes and undergo gametogenesis in the tick midgut lumen once they are ingested [34,35]. For *Babesia* and *Theileria*, intraerythrocytic gametocytes are usually indistinguishable from other asexual erythrocytic stages without electron microscopy [35–37]. Unlike these parasites, gametocytes have not been described in *C. felis*. In a cat that is acutely infected with *C. felis*, merozoites enter erythrocytes after being released from schizont-laden leukocytes. These newly released merozoites may not have had adequate time to become capable of transforming into gametocytes. The duration of time necessary for this process to occur in *C. felis* has never been studied. For *Theileria* spp., experimentally infected calves with acute clinical disease (10–19 days after tick infestation or sporozoite inoculation) and high parasitemia have been used to successfully infect ticks [22,38–40]. *Plasmodium falciparum* can take up to 12 days to fully mature from a merozoite into a gametocyte [41]. All inoculums used in this study were collected from cats with acute cytaxuzoonosis, however we were unable to determine the exact time lapse between tick infestation and onset of clinical signs as these were naturally occurring infections. In experimental tick transmission studies for *C. felis*, it takes 8–14 days after tick infestation for infected cats to develop clinical signs and intraerythrocytic stages were first detected via microscopy 16–18 days after infestation [12,28,29]. Based on these data and our clinical experience, our assumption is that the cats we collected inoculums from were likely to have been infected via tick bite approximately 18–25 days prior to their clinical presentation. Despite our documentation of many intraerythrocytic parasites in our inoculums, it is uncertain whether any of these were gametocytes that were capable of infecting the ticks.

It is also possible that tick feeding, which is absent in our injection model, is required for the development of *C. felis.* Feeding may trigger critical signals for *C. felis* to continue its development within the tick's midgut. Such triggers may include xanthurenic acid (XA), decreased oxygen tension, and a lowered ambient temperature, all of which have been used to induce gametogenesis *in vitro* for other piroplasmids [42–44]. In the current study, we presumed the injected organisms were exposed to decreased oxygen tension in the hemocoele or midgut after injection. Also after injection the ticks were housed at lowered ambient temperature (20–23ºC). While the role of XA in *C. felis* gametogenesis has not been studied, it could be an important element lacking in our model. XA is a molecule that is crucial in facilitating gametogenesis for *Plasmodium* spp. [45,46]. Importantly, increased XA concentrations are directly related to feeding as it is primarily produced by the salivary glands and secreted in the saliva which is ingested into the mosquitoes midgut during a blood meal [47,48]. If XA or other tick-derived molecules play a similar role in the process of gametogenesis for *C. felis*, infection studies that include feeding or the addition of XA to the inoculum prior to injection should be considered in future studies.

Additionally, it is possible that the molting process is essential for *C. felis* to continue its life cycle within the ticks, and the use of adult ticks in our model prohibited parasite development after injection. Successful injection studies for *T. parva* included molting. Jongejan et al. and Walker et al. injected engorged *Rhipicephalus appendiculus* nymphs with infected bovine erythrocytes percutaneouly into the hemocoel or midgut. These *T. parva*-injected nymphs were then allowed to molt into adults that were able to successfully transmit the infection to naïve calves [21,22]. However, the molting process may not be a necessary step for all piroplasmids. In

another injection study for *Babesia gibsoni* and *Theileria equi*, a soft tick (*Ornithodoros moubata*) that is not believed to be a natural vector for either organism was selected for injection. Both nymphs and adult stages of *O. moubata* were directly injected in the hemocoel with *B. gibsoni*- or *T. equi*-infected erythrocytes (collected from experimentally infected dogs and *in vitro* culture respectively). Both parasites were then able to successfully develop after injection without the process of molting and were detected in the ovaries and eggs. Notably, *B. gibsoni* was able to develop into infective sporozoites within the salivary glands and these ticks successfully transmitted the infection to naïve dogs [20]. Future injection studies could include either the molting process or utilize alternative tick species, such as soft ticks or *Dermacentor variabilis*.

An additional limitation in this study is the small number of cats subjected to the transmission challenge. In previous *C. felis* transmission studies using similar numbers of acquisition-fed ticks, 85–100% of naïve cats became infected after transmission challenge [12,14,28]. In these experiments, *C. felis* was detected in 4–20% of the acquisition-fed ticks [10,14] (Reichard, personal communication). Comparatively, we detected *C. felis* in 30–57% of the ticks we injected. One potential explanation for our higher detection level is the difference in sensitivity of molecular assays used. In our study, we used RT-PCR and qPCR assays targeting 18S rRNA and *cox3* DNA of *C. felis*, while the previous studies utilized a conventional nested PCR targeting 18S rDNA [10,14]. Head-to-head comparisons of these assays have not been performed. Despite the higher detection levels in the current study, it is still possible that the transmission rate of injected ticks is lower than that of acquisition-fed ticks, and the number of cats used in this study was not sufficient.

Another potential factor affecting the outcome of our study is the interval between tick infection and transmission. The optimal infection-to-transmission interval has never been evaluated for *C. felis* and *A. americanum.* In previous experiments using acquisition-fed ticks, transmission challenges on naïve cats occurred 6–18 weeks after acquisition feeding (i.e. 2-12 weeks after molting) (Reichard, personal communication). Our transmission challenge occurred in a similar timeframe (16 weeks after injection). The time required for *C. felis* to develop within the ticks after injection is unknown, and it remains unclear whether the 16-week interval influenced the ticks' ability to transmit the infection. To our knowledge, the only other injection study where adult ticks were used, the investigators injected adult *O. moubata* with *B. gibsoni* and the time lapse between injection and transmission challenge was not clearly specified [20].

Even though this experiment was unsuccessful in transmitting *C. felis* to cats, we demonstrated the potential to study *C. felis* using a tick model. Our ability to detect *C. felis* in whole tick sections using ISH illustrates this as a promising platform to investigate the life cycle of *C. felis* in ticks. Additionally, as the injected parasites were able to remain alive for an extended period within the tick, this model could potentially serve as a source to initiate *in vitro* cultivation. To further refine and improve a tick infection model for *C. felis*, forthcoming studies should characterize the *C. felis* life cycle within the vertebrate and tick hosts, including the duration of time needed for merozoites to transform into gametocytes and when those parasites would become infectious to the tick host. These studies could assist with the timing and preparation of blood inoculums. Additionally, models could more closely emulate the process of natural infection (feeding and molting), attempt transmission with a larger number of cats, and determine the optimal interval between tick infection and transmission.

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Authorship:

T. Yang, A. Birkenheuer, M. Reichard contributed to study conception, execution, and design. H. Marr and N. Whitehurst contributed to optimization of experimental techniques. L. Cohn and L. Nafe contributed to recruitment of patient enrollment and sample collection. All authors contributed to editing and approved the final manuscript.

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CHAPTER 5

In vitro membrane feeding of *Amblyomma americanum* nymphs with *C. felis*-infected feline blood

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***In vitro* membrane feeding of *Amblyomma americanum* nymphs with *C. felis*-infected feline blood**

Tzushan S. Yang, Mason V. Reichard, Henry S. Marr, Michael Karounous, Adam J. Birkenheuer

1. INTRODUCTION

Cytauxzoon felis is a tick-borne hemoprotozoa that causes life-threatening disease in cats in the United States. The most significant limitations of *Cytauxzoon* research to date remain the inability to culture the parasites *in vitro* and the lack of experimental models to study *C. felis* that do not infect cats directly or infect ticks via acquisition feeding on live animals. Currently, the only established model to infect ticks with *C. felis* involves tick feeding on an infected cat [1,2]. This process is not only stressful for the cats but animal care before, during and after the experimental period can also be costly. Therefore, there is a need for a model to study *C. felis* that minimizes or eliminates tick feeding on vertebrate hosts as well as the laboratory maintenance of these hosts.

Many innovative techniques to infect ticks with tick-borne pathogens have been established with the aim to circumvent live animal feeding [3]. The techniques that have been most utilized include direct injection and artificial *in vitro* feeding [4–14]. Direct injection is thought of as a "fast track" technique for tick infection, as it eliminates the tick feeding process and requires less time to complete. However, the procedures may result in high mortality rates in ticks, and it is considered to be a biologically unnatural route of pathogen acquisition for ticks. Artificial *in vitro* feeding systems are more widely utilized and described by tick-borne disease researchers, which allow the ticks to undergo the infection process in a more biologically natural state

[15,16]. The close emulation of the natural infection process, including feeding and molting, may be especially critical for tick-borne protozoan parasites. Tick-borne protozoa have complex life cycles within ticks, including multiple life stages that involve sexual and asexual reproduction [17,18]. Artificial feeding systems include physiologic stimuli within the tick which can promote the parasites' ability to progress through their life cycles. The successful use of artificial feeding for other related piroplasmid parasites supports the investigation of this method for *C. felis* [8,19,20].

In a previous study, our laboratory attempted to infect *A. americanum* adult ticks via direct injection of *C. felis*-infected cat blood [21], but these ticks failed to transmit the infection to naïve cats. Therefore, the current study utilized an artificial membrane feeding system to infect *A. americanum* nymphs using blood collected from naturally infected cats. This also aligned with our intention to minimize the chronic laboratory maintenance of vertebrate animals in *C. felis* research. After acquisition feeding on membranes and subsequent molting, the adults were then used in a transmission challenge to assess their ability to transmit *C. felis* to a naïve cat.

2. MATERIALS AND METHODS

1.1 Experimental design

The experimental design of this study is outline in Figure 1. A total of 3 feeding units were used for acquisition membrane feeding, and each unit consisted of a group of approximately 80–90 *A. americanum* nymphs. Nymph groups 1 and 2 received inoculum made from cat 1 and 2, respectively. Nymph group 3 received inoculum made from cats 3 and 4. After molting, adult

ticks were pooled for the transmission challenge to test their ability to transmit *C. felis* to a naïve cat.

1.2 Tick maintenance

Amblyomma americanum nymphs were purchased from Oklahoma State University Tick Rearing facility. The nymphs were kept in a clean syringe body plugged with cotton on both sides and placed in a humidity chamber until feeding. The humidity chamber was kept at 90–99% humidity and 23ºC with a 12-hour light/12-hour dark cycle. After feeding, the repleted nymphs were placed in 8 oz. paper cups in the humidity chambers until molting was completed. Adults were then moved to a new paper cup and kept in the humidity chamber until the transmission challenge.

1.3 Artificial membrane feeding system assembly

The artificial membrane feeding system was assembled as previously described [10] with several modifications (Figure 1a). These modifications include using a thinner silicone membrane (45-50 μm thick), usage of cat hair as an attachment stimulus, and placement of the feeding base (6-well culture plate containing the inoculum) on a 37ºC water bath for heat source (Figure 1b).

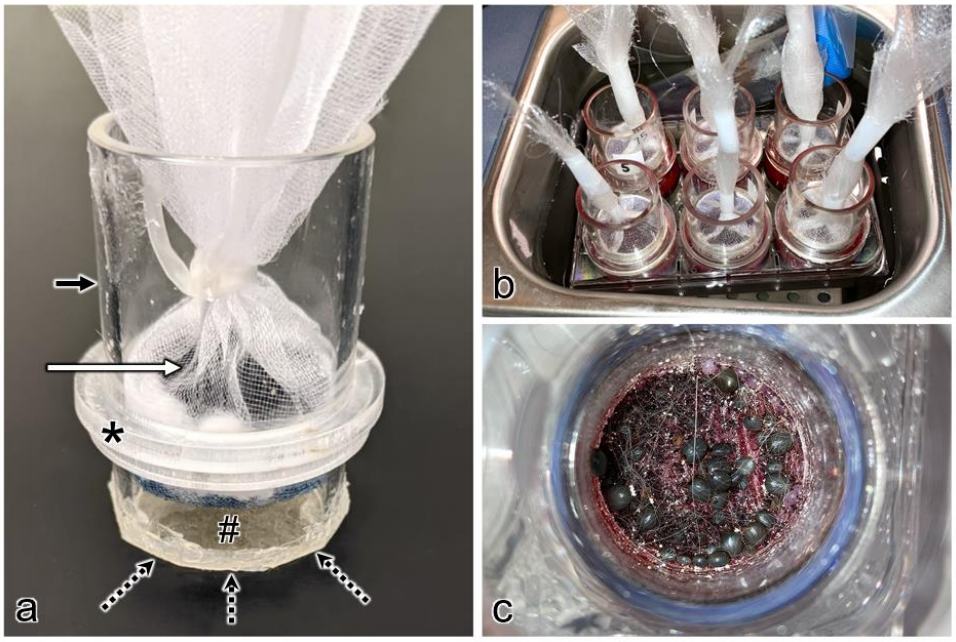

Figure 1. Artificial feeding system for *A. americanum* nymphs
(a) Structure of a typical feeding unit: an acrylic tube (black arrow, ~6-7 cm long, wall thickness 3 mm and 2.5 cm inner diameter) has two wider plastic rings (3 cm inner diameter, 3.7 cm outer diameter) at the waist (asterisk) to secure the unit atop the 6 well culture wells during feeding and the membrane sinks below the blood in the well. The thin silicone membrane is glued to the bottom of the unit (dotted arrows) and the unit is tightly plugged with a tall plastic ring wrapped in organza fabric (white arrow) to prevent ticks from escaping the chamber (#). (b) Typical configuration of a tick feeding experiment. The feeding units are placed within a 6-well culture plate filled with animal blood. The culture place is floated atop a 37ºC water bath. (c) Feeding experiment showing several attached and engored *A. americanum* nymphs.

The membranes were made by mixing 15g silicone glue RTV1 ElastocilE4 (Wacker, Germany), 4.5g silicone oil DC200 (Sigma,Germany), and 2.9g 15% hexane (Sigma, Germany). The mixture was spread evenly on Goldbeater skin that was secured on a layer of plastic wrap on a flat surface. Excess silicone mixture was scraped off with a plastic scraper and left to cure overnight under a UV light. Membranes were measured using a digital micrometer, and areas with the desired thickness were selected for nymph feeding. These selected areas were then gently scrubbed with cat hair (sourced from cats that have not been exposed to ectoparasite

control products). Precut acrylic tubes were secured onto the selected areas on the membrane with silicone glue and left to dry for at least 4 hours.

Prior to tick feeding, excess membrane was trimmed off along the edge of the feeding unit. The outer surface of the unit was sterilized with 70% ethanol, and the units were leaked tested by submerging the membrane side in 70% ethanol for at least 15 minutes. Units were left to air dry and placed in an empty sterile 6 well culture plate until use.

After ticks were loaded into the feeding chamber, the acrylic tube opening was plugged with a circular plastic ring wrapped in organza fabric (Anminy, ASIN: B07ZRCPL1M) to prevent ticks from escaping. The feeding units were place in a 6 well culture plate containing 2.5–3 ml of blood or inoculums. A small gap of at least 3 mm was left between the contact surface of the membrane and the bottom of the culture plate, with the edge of the feeding unit propped along the rim of the well with a plastic ring.

1.4 *C. felis*-infected blood used for acquisition membrane feeding

All feline blood samples used for this study were collected in accordance with protocol approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC). Approximately 1–2 ml of Blood was collected in EDTA tubes from client-owned cats that had been diagnosed with acute cytauxzoonosis and the samples were sent to our laboratory overnight on ice. The presence of *C. felis* organisms was confirmed via PCR testing and the parasitemia of each blood sample was determined by light microscopy as previously described [21]. An equal

volume of Alsever's solution (Alfa Aesar, Haverhill, MA, USA) was added to each sample and they were stored in 4ºC up to 10 days until use.

To keep the parasitemia consistent (1%) and to ensure an adequate feeding volume throughout the experiment, *C. felis*-infected blood with mixed with defibrinated sheep blood (Hemostat, Dixon, CA). Prior to mixing, defibrinated sheep blood was washed and resuspended with an equal amount of Alsever's solution. This step prevented blood clotting when the samples were mixed. Packed cell volume of the blood mixture was adjusted to 35–40%. Blood used for acquisition feeding was mixed fresh before every blood change during the feeding experiment.

1.5 Acquisition feeding

For the first 12 hours of membrane feeding, the ticks were fed uninfected defibrinated sheep blood (Hemostat, Dixon, CA) to ensure the nymphs were able to attach and feed before acquisition feeding commenced. Inoculums were changed every 8–12 hours until infected cat blood is exhausted. For all experiments, ticks were fed the *C. felis*-sheep blood inoculum mixture from hour 12 through day 6, and feeding was terminated at the end of day 7.

Blood used for feeding was changed every 8–12 hours. During each blood change, the side that contacts the membrane was rinsed with warmed 1X Penicillin/Streptomycin solution (Gibco, Gaithersburg, MD, USA) and copious amount (~15 ml) of 0.9% medical grade sodium chloride. At the end of the feeding episode, both fully engorged and partially engorged nymphs were removed from the chamber and allowed to molt into adults.

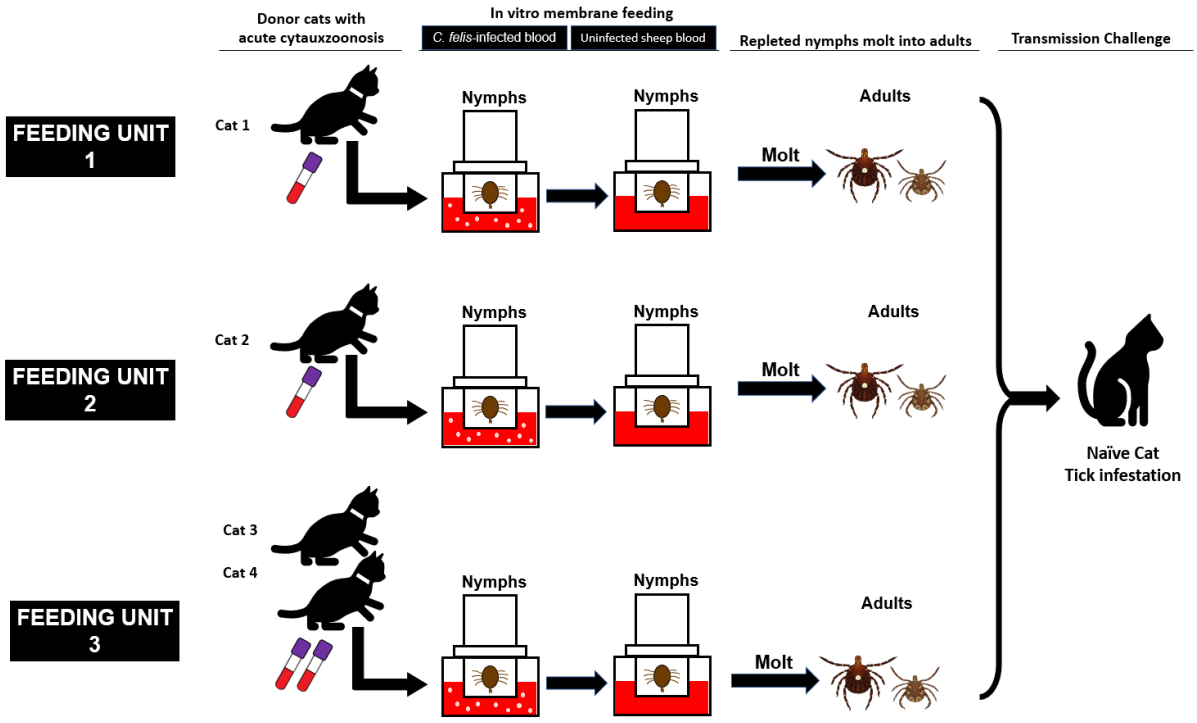

Figure 2. Experimental design

1.6 Transmission challenge

Approximately 3 months after the completion of molting, all ticks were used in a transmission challenge to assess their ability to transmit *C. felis* to a naïve cat. Tick transmission was performed as previously described [1,22,23]. Throughout the transmission challenge, the cat was observed for signs that are typical for clinical cytauxzoonosis, which included fever, lethargy, and anorexia. EDTA blood was collected from the cat at days 0, 3, 7, 13, 17, 21, and 30 post infestation and tested for *C. felis* via a real-time PCR assay targeting the *C. felis cox3* mitochondrial DNA as previously described [24].

1.7 *C. felis* detection in ticks post transmission challenge

All ticks that were used in the transmission challenge were collected and tested for the presence of *C. felis* via PCR. DNA from each tick was extracted following previously described protocol using DNeasy Blood and tissue kit (Qiagen, Valencia, CA) based on their engorgement status at the end of the feeding experiments [21]. For males and partially engorged females, tissues were digested overnight using 250 µl of ATL buffer and 25 µl of proteinase K, and 250 µl of AL buffer was added to each lysate. For fully engorged females, tissues were digested overnight using 500 µl of ATL buffer and 50 µl of proteinase K, and 500 µl of AL buffer was added to each lysate. All extracted DNA was tested via the *C. felis cox3* real-time PCR assay used in section 2.5.

The presence of tick DNA and absence of PCR inhibitors in extracted tick DNA were demonstrated by amplification of a 70 bp fragment of *A. americanum* 18S rDNA (Forward: 5'– CTAATACATGCATTGAGCCTGAAG – 3'; Reverse: 5'– GAACCCGATGGGTCTTGGTC –

3'). Each 25 µl reaction consisted of 12.5 µl of 2X SSO Advanced SYBR Green Supermix (Bio-Rad Laboratories, Inc, Hercules, CA), 10 pmol of each primer, 7.1 µl of molecular grade water, and 5 µl of DNA template. Thermocycling conditions (Bio-Rad CFX96™ Real-Time Detection System) consisted of an initial denaturation step at 95 °C for 3 min, followed by 30 cycles at 95 °C for 10 s, 67 °C for 20 s, and 72 °C for 30 s. Melting temperature (Tm) measurements were made between 65 and 90 °C at 0.5 s intervals. All assays ran included previously extracted DNA from unfed *A. americanum* female adults as position controls, and molecular water as negative controls. Amplification and melt curved were analyzed via CFX96™ Manager Software.

3. RESULTS

3.1 Acquisition feeding of *A. americanum* nymphs

The results of acquisition feeding are summarized in Table 1. Both fully engorged and partially engorged nymphs (total n = 141) successfully molted into adults after 3-4 weeks. During the 3-month period prior to the transmission challenge, 70 molted adults died, leaving only 71 adults (26 females and 45 males) for the transmission challenge.

For all 3 feeding units, by day 4, there was evidence of hemolysis (color change from red to black) and blood change frequencies were increased from every 12 hours to every 8 hours. This is consistent with bacterial contamination based on previous feeding experiments. For Feeding unit 1 and 2, along the tick attachment/puncture site of the membrane, there was small amount of blood leakage into the chamber where the ticks were feeding. Small cotton balls were placed alongside the leakage site and no additional blood pooling was observed within the feeding chamber throughout the rest of the feeding period.

Table 1. Summarized results of *C. felis* acquisition feeding experiments

Feeding Unit	Number of ticks per unit	Fully engorged	Partially engorged	No engorgement	Dead
1	91	45 (49.4%)	14 (15.4%)	20 (22%)	12 (13.2%)
2	85	20 (23.5%)	7 (8%)	36 (42.3%)	22 (25.9%)
3	85	11 (12.9%)	44 (51.8%)	26 (30.6%)	4 (4.7%)

3.2 Transmission challenge

Throughout and after the transmission challenge, the cat remained free of clinical signs of cytauxzoonosis. All blood samples collected during the transmission challenge tested negative for *C. felis* via PCR.

Sixty-six ticks successfully attached to the naïve cat (attachment rate = 93%). Out of 23 females ticks that were attached, 15 of them were fully engorged and 8 of them were partially engorged. Three females and two males were not attached. All attached ticks tested negative for *C. felis* via PCR and *A. americanum* 18s rDNA was amplified in all samples.

4. DISCUSSION

To minimize the use of purpose-bred research cats in *Cytauxzoon* research, we attempted to infect ticks utilizing an artificial membrane feeding system and blood from cats naturally infected with *C. felis*. Our attempt was unsuccessful, as these ticks tested negative for the parasite and failed to transmit the infection to the naïve cat. We suspected this outcome could be

associated with several variables, including the source of the inoculum, the composition of the inoculum, and challenges we encountered with the artificial feeding systems.

All *C. felis*-infected blood used for feeding were sourced from cats that were acutely infected with cytauxzoonosis. This may have contributed to the outcome of our study as it remains unknown whether the parasite stages during this phase of the disease are capable of infecting ticks. Acquisition feeding of *A. americanum* ticks has never been tested on acutely infected cats. Although acutely infected animals with high parasitemia are routinely used to infect ticks for other related piroplasmids and has even been associated with higher infection rates in ticks. It is possible that the blood we used for acquisition feeding was collected before the intraerythrocytic parasites were able to transform into gametocytes, which are the only parasite stage that is capable of further developing within the tick [21,25]. Future artificial feeding studies should consider utilizing blood from chronically infected cats, as these cats have been routinely used to successfully infect ticks in prior studies [1,22,26,27].

Another variable that potentially contributed to our experimental outcome is the composition of the inoculum. We mixed sheep blood in the inoculums to supplement the low volume of *C. felis*-infected blood samples. We adjusted the parasitemia to 1% as this was similar to or higher than most chronically infected carriers used in prior tick transmission studies. However, this may have negatively impacted our experiment in several ways. First, the mixture of blood with different species may have compromised parasite viability or affected parasite development within the ticks via components of the innate immune system in the sheep blood. Although the presence of the parasites was confirmed via light microscopy in all inoculums, parasite viability

was not tested for each feeding. Second, the resulting inoculum may not have contained enough parasites to successfully infect the ticks secondary to the dilution with the sheep blood. Third, due to the small volume of *C. felis*-infected blood samples, we were unable to use the inoculum throughout the entire course of feeding. This along with the low parasite number in the inoculums may have reduced the possibility for ticks to acquire the parasites during acquisition feeding. Additionally, the *C. felis*-infected blood was shipped and stored in 4ºC for up to 10 days (including the feeding period), and it is unknown whether this storage condition impacted gametogenesis.

We also encountered several complications with the artificial membrane feeding system, such as bacterial contamination of the inoculums, fragility of the membrane and the inconsistent feeding behaviors between tick batches. Despite identical setups across all 3 feeding experiments in our study, the percentages for tick attachment, engorgement and mortality widely varied. We attributed this to the biological differences between the batches of ticks used in the feeding system, which made tick behaviors and preferences difficult to predict between each feeding experiment. An additional limitation is the potential for bacterial or fungal contamination of the inoculum, which is mostly associated with the puncture of the tick mouthparts through the membrane. Other similar studies have suggested supplementing the inoculum with antibiotic or antifungals to prevent this problem [16,28]. This was not implemented in our study as we were unsure if these supplements would affect tick attachment rates or alter the tick microbiome. All three experiments in our study experienced inoculum contamination during the course of acquisition feedings. The effects of bacterial contamination on parasite viability are unknown. Lastly, due to the thinness of the membrane that is required for nymph feeding, the membrane

was quite fragile and prone to breakage. The leakages that we encountered in this study were associated with sites of tick attachments, which may have also exacerbated the contamination in the inoculum. Future studies should consider several modifications to further optimize this feeding system for *A. americanum* nymphs. These may include increasing tick numbers in feeding units to compensate for low attachment rates, evaluating if tick attachment and molting rates are impacted by the addition of antibiotics and antifungals in the inoculums, and exploring different materials for membrane assembly to improve the stability of the membranes.

Despite our intentions to minimize the usage of purpose-bred cats for *Cytauxzoon* research, the practicality of using an artificial membrane feeding system for tick infection remains unknown. The large volume of inoculum that is required to complete each feeding experiment may not be feasible for small host species such as cats. For instance, to feed nymphs to repletion, it can take 7–10 days and up to 60 ml of blood inoculum to complete a feeding experiment. Depending on the size of the cat, this could equate to more than 20% of their blood volume, which is the maximum amount of blood recommended for each blood collection [29]. This is in contrast to other related piroplasmids like *Babesia* and *Theileria,* where it is easier to acquire large volumes of blood from their much larger host species (dogs, cattle, sheep, or horse) [8,19]. These successful artificial feeding studies also described collecting infected fresh blood every other day for tick feeding, which is likely another challenging and impractical task when working with cats.

In conclusion, we were able to feed *A. americanum* nymphs to repletion via *in vitro* feeding, and these nymphs were able to molt into adults. However, we were unable to detect *C. felis* within

these ticks and they failed to transmit the infection to a naïve cat. Future attempts should include feeding a larger number of nymphs, optimizing a system that requires lower volume of blood, and utilizing blood with higher parasitemia or blood from chronically infected cats.

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CHAPTER 6

Transmission of *Cytauxzoon felis* by Injection of *Amblyomma americanum* Salivary Glands

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ABSTRACT

Cytauxzoonosis is a life-threatening disease of cats, caused by the tick-borne piroplasmid hemoparasite, *Cytauxzoon felis.* The current experimental models for cytauxzoonosis rely on either tick transmission or direct injection of infected cat tissues. These models require researchers to directly work with ticks or have access to cats with acute cytauxzoonosis. To improve the feasibility and access to study this disease, there is a need to establish more sharable resources. In other related piroplasmid parasites, sporozoites-based inoculums are routinely produced from tick salivary glands, cryopreserved and distributed to other investigators and facilities. For these parasites, sporozoites have also been the basis for vaccine development and *in vitro* cultivation, both of which remain lacking for *Cytauxzoon* research. If infectious sporozoites can be similarly isolated for *C. felis*, it would significantly broaden our capabilities to study this parasite. Therefore, this study aims to determine if *C. felis* sporozoites inoculums collected from the salivary glands of *Amblyomma americanum* ticks are capable of inducing cytauxzoonosis in naïve cats. *A. americanum* nymphs were acquisition-fed on a donor cat that is chronically infected with *C. felis.* Four groups of adult ticks (n = 50/group) were either stimulation-fed for 4 days on naïve cats or were heated at 37°C for 4 days. After these treatments, salivary glands of each group of ticks were collected to create SG inoculums. The infectivity of these inoculums was then tested by subcutaneous injection into naïve cats. The two naïve cats used for stimulation feeding both developed cytauxzoonosis, indicating these groups

of ticks were capable of producing infectious sporozoites. Of the 2 cats that were injected with SGs from these stimulation-fed ticks, one cat developed cytauxzoonosis and had definitive confirmation of infection by both light microscopy and PCR. The other cat did not develop cytauxzoonosis and only had equivocal evidence of infection. Neither cat that was injected with SGs from the heated ticks developed cytauxzoonosis. One of these cats had equivocal evidence of infection and one had no evidence of infection. This study validates the feasibility of collecting infectious sporozoites from *C. felis*-infected ticks that can be used to infect naïve cats. While this model requires further optimization, it has the potential to expand resources to study *C. felis* and further advance research in this field.

1. INTRODUCTION

Cytauxzoonosis is a life-threatening tick-transmitted disease in domestic and wild felids (1–5). In the United States, the disease is caused by the piroplasmid hemoparasite, *Cytauxzoon felis,* and is primarily transmitted by *Amblyomma americanum* (6,7). Since the disease was first reported nearly 50 years ago (8), *C. felis* continues to be a significant cause of morbidity and mortality in cats living in enzootic regions. The more recent discovery of *Cytauxzoon* species in Europe, Asia and South America makes cytauxzoonosis a disease of worldwide concern.

Unfortunately, *Cytauxzoon* research remains largely hindered by the inability to culture the parasite *in vitro.* For this reason, *Cytauxzoon* researchers currently rely on either natural or experimental infections of domestic cats to study this disease, which leads to an inherent set of limitations. For instance, to perform studies using naturally infected cases and samples, investigators are limited as naturally occurring cases of acute cytauxzoonosis in the United States occur almost exclusively between late spring to early fall (4). Additionally, as cytauxzoonosis is geographically restricted, it may reduce the access to case materials for investigators living outside of enzootic regions. While cytauxzoonosis can be induced experimentally via either transmission through tick feeding on cats or direct inoculation of cats with *C. felis*-infected feline tissues, these techniques are also associated with drawbacks that may prevent the widespread study of this infection (7,9,10). The tick transmission model requires researchers to work directly with ticks. This calls for specialized biocontainment facilities for arthropods, which may not be available at all institutions. Alternatively, clinical cytauxzoonosis can also be induced through injection with tissue or blood harvested from cats with acute cytauxzoonosis. However, this method is considered less natural as it bypasses tick feeding and cats are simultaneously injected with multiple parasite stages. These inoculums include schizont-infected monocular cells and

intra-erythrocytic life stages (10,11). It is unknown whether or not these inoculums also contain sporozoites. As a result, infecting cats with these inoculums may impact parasite development and host immune responses as it deviates from natural infections that are initiated with the injection of sporozoites only.

For all piroplasmid parasites, sporozoites play a central role in tick transmission and parasite development in the mammalian hosts. For *Theileria,* a close relative to *C. felis,* sporozoites isolated from ticks are a vital resource for research and vaccine production (12–15). The isolation and cryopreservation of sporozoite inoculums created from tick salivary glands are a common process in *Theileria* research. These studies include titrating appropriate vaccine dosages in cattle, investigating host-parasite interactions *in vitro,* and identifying potential vaccine and therapeutic targets through proteomics and transcriptomics (16–18). The ability to similarly isolate *C. felis* sporozoites would broaden the capabilities and resources for *Cytauxzoon* research. Importantly, this could also improve the accessibility to *Cytauxzoon* research for more investigators and institutions.

The objective of this study is to determine if *C. felis* sporozoites inoculums produced from the salivary glands of adult *A. americanum* ticks are capable of infecting naïve cats.

2. MATERIALS AND METHODS

2.1 Animals

The experimental design for is outlined in Figure 1. All procedures were approved by Oklahoma State University Institutional Animal Care and Use Committee. Seven cats were used for this study, including 6 specific pathogen-free cats that were naïve to *C. felis* infection and one cat that was chronically infected with *C. felis*. This *C. felis*-infected donor cat was used for acquisition feeding of *A. americanum* nymphs. Two of the naïve cats were used for stimulation feeding of the *C. felis*-infected *A. americanum* adults prior to inoculum preparation. Four of the naïve cats were used to test the infectivity of the SG inoculums. Two of these naïve cats received inoculums from heated ticks (inoculum 1 and 2) and the other two naïve cats received inoculums from the stimulation-fed ticks (inoculum 3 and 4). Prior to the injection or blood collection, cats 1 and 4 were sedated with dexmedetomidine (0.03 mg/kg) intramuscularly; cats 2 and 3 were sedated with dexmedetomidine (0.03 mg/kg) and butorphanol (0.02 mg/kg) intramuscularly.

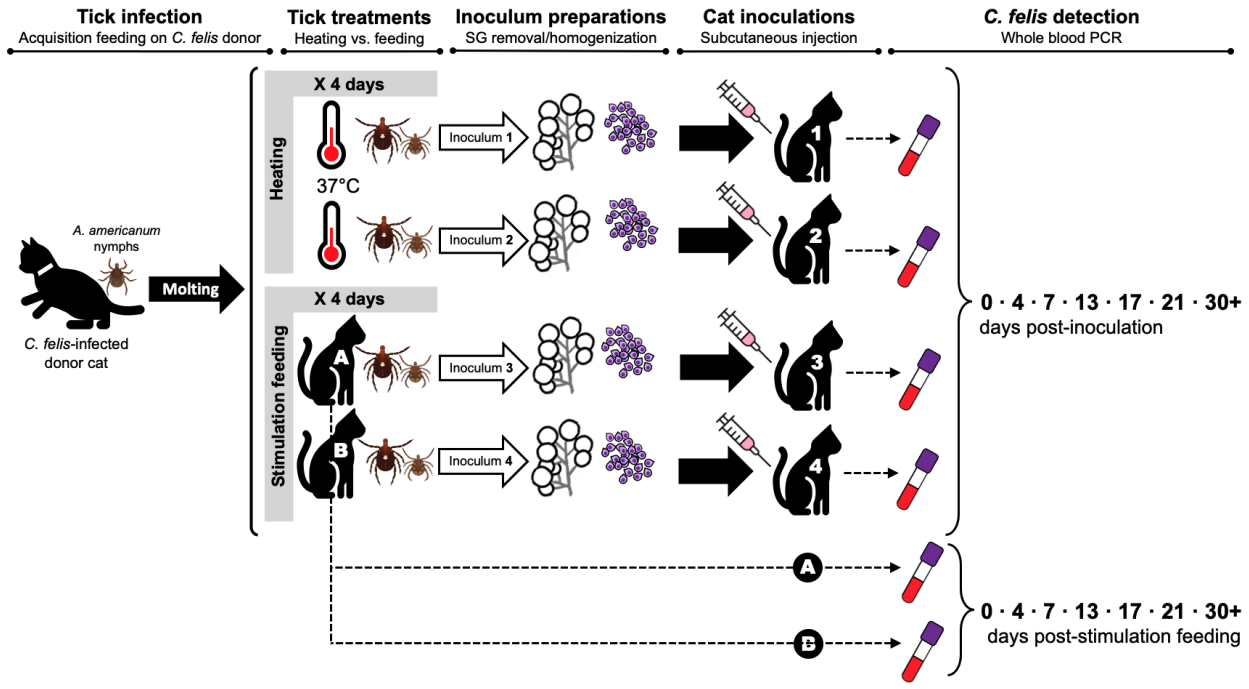

Figure 1. Experimental design

2.2 Tick maintenance, infection and treatments prior to inoculum preparation

Specific pathogen-free *A. americanum* nymphs were purchased from Oklahoma State University tick rearing facility (Stillwater, OK). All ticks were maintained in humidity chambers with conditions that were recommended by the tick rearing facility.

Approximately 500 *A. americanum* nymphs were acquisition-fed on the chronically infected *C. felis* donor cat (parasitemia ~1%) described in section 2.1 (7,19). Nymphs first fed to repletion at 3 days post infestation, and ~98% of nymphs were fed to repletion at day 6 post infestation. After repletion, all engorged nymphs were placed back into the humidity chamber and allowed to molt into adults.

Seven to eight weeks after molting, adult ticks were randomly selected for inoculum preparation. Similar to prior successful transmission experiments, each inoculum group consisted of 25 males and 25 females (6,7,19–21) . Two inoculum groups were placed in a 37°C incubator with 80-90% humidity for 4 days and two inoculum groups were placed on two naïve cats and allowed to feed for 4 days prior to inoculum preparation.

2.3 Inoculums preparation and injections

Four individual inoculums were prepared as follows. Salivary glands were dissected and placed on ice in RPMI culture medium (Sigma Aldrich, Germany) until homogenization. RPMI culture medium was supplemented with 10% feline serum (GeminiBio, Sacramento, CA), 20 mM HEPES buffer, 50 µM β-mercaptoethanol, 2 mM L-glutamine and 50 µg/ml gentamicin (all supplements except for the serum were purchased from Sigma Aldrich). Dissected SGs were

homogenized using a glass homogenizer (DWK Life Sciences, Millville, NJ) in 1.25 ml of the aforementioned RPMI culture medium. 100 μl of each inoculum was removed for DNA extraction and *C. felis* PCR as detailed below. The remaining inoculum was collected using a 16 gauge needle into a 3 ml syringe and immediately used for injection. The inoculum (~1 ml) was injected subcutaneously into each cat along the dorsum through a new 16 gauge needle.

2.4 Monitoring and blood collection

After stimulation feeding (cats A and B) and SG injection (cats 1–4), cats were monitored daily for clinical signs of cytauxzoonosis (fever, inappetence, and hyporexia). Cats were scheduled to have whole blood collected into EDTA tubes on days 0, 4, 7, 13, 17, 21 and 36 (±3) post stimulation feeding or SG injection. If a cat tested positive for *C. felis* by PCR and exhibited clinical illness, in order to minimize stress, blood samples were not obtained until after the animals were clinically recovered. A last blood sample was collected from each cat at the end of the study period after day 30 to document infection status at the end of the study period.

2.5 Treatments for clinical cytauxzoonosis

Once a cat tested positive for *C. felis* by PCR and exhibited clinical signs for cytauxzoonosis, they were treated with Atovoquone (15 mg/kg orally every 8 hours) or Ponazuril (30 mg/kg orally every 24 hours) and azithromycin (10 mg/kg orally every 24 hours). Subcutaneous fluids (0.9% medical grade sodium chloride), appetite stimulant (Mirtazapine), and analgesic medication (buprenorphine) were given to infected cats based on the clinical veterinarians' assessment during the experimental period.

2.6 Detection *of C. felis*

Blood samples were screened for *C. felis* infection via real-time PCR and light microscopy of thin blood smears. For PCR, total DNA was extracted from 200 µl of whole feline blood using a QIAsymphony® SP robot (QIAGEN, Valencia, CA) according to manufacturer's instructions. Extracted DNA was then tested in ten replicate reactions using a *C. felis*-specific real-time PCR assay targeting *cox3* as previously described (22). Plasmids that contain *C. felis cox3* inserts were used as positive controls for each assay. Two different negative controls were used: no template controls (molecular grade water) and DNA extracted from cats that were not infected with *C. felis*. Amplification and melt curves were analyzed using CFX96™ manager software and results were confirmed via gel electrophoresis and amplicon sequencing. For light microscopy, thin blood smears were prepared and stained with Hema 3 stain to screen for parasites. The feathered edge was scanned at low power (200X) for presence of schizonts, and entire monolayer was examined at high power (1000X) for intraerythrocytic life stages. If possible organisms were identified on the blood smear from a sample that tested negative for *C. felis* via PCR, that sample was subjected to a broad range PCR assay targeting the apicomplexan 18S rDNA as previously described (5).

Total DNA was extracted from 100 µl of each SG inoculum using a Qiagen blood and tissue kit following manufacturer's instructions. The concentration of the extracted DNA was estimated using a spectrophotometer (Nanodrop™, ThermoFisher Scientific) and the sample was tested for *C. felis* in triplicate reactions with the aforementioned *C. felis cox3* real-time PCR assay. The relative *C. felis cox3* copy number was compared between the inoculums using the $2^{-\Delta Ct}$ method

(23). The relative quantification (RQ) ratio was calculated by using the sample with the highest Ct value as the baseline.

3. RESULTS

A summary of the *C. felis* PCR results from the cats and their clinical evidence of cytauxzoonosis is outlined in Table 1. All SG inoculums tested positive for *C. felis* via PCR and their relative quantification results are shown in Table 2. The inoculum that induced cytauxzoonosis in its recipient cat had the highest copy number of *C. felis* mtDNA and this was not directly related to the total amount of DNA present in the sample. Despite using identical numbers of ticks to make each inoculum and extracting DNA from an identical volume of each inoculum, the total amount of DNA extracted varied between samples. This could reflect the different amounts of tick SGs that were extracted from each batch of ticks or a result of an unevenly distributed tick SGs in the inoculums despite consistent homogenization and mixing of the samples.

Table 1. PCR results of cat blood samples and disease development in cats post-stimulation feeding and salivary gland injection

Cats			Days post stimulation feeding or salivary gland inoculation						
			0	4	7	13	17	21	36 (±3)
Stimulation feeding		A	–	–	–	+	/[a]	/[a]	+[b] (D33)
		B	–	–	–	+	/[a]	/[a]	+[b] (D39)
SG inoculation	Heated	1	–	–	–	–	–	–	– (D37)
		2	–	–	–	–	–	–	– (D38)
	Stimulation-fed	3	–	–	+	/[a]	/[a]	/[a]	+[b] (D35)
		4	–	–	–	–	–	–	– (D36)

/ : Blood not obtained due to clinical cytauxzoonosis
D: Days post infestation or inoculation
[a] Cats exhibiting clinical signs of cytauxzoonosis
[b] Cats recovered and exhibiting no clinical signs of cytauxzoonosis

Table 2. Relative copy numbers of *C. felis cox3* mtDNA in each SG inoculum and their total DNA concentrations

Inoculums	Cat inoculated	Developed cytauxzoonosis	Mean Ct*	RQ ($2^{-\Delta Ct}$)	Total DNA (ng/µl)
1	Cat 1	No	27.4	1.0	15.7
2	Cat 2	No	23.4	16.1	49.0
3	Cat 3	Yes	22.1	39.7	40.8
4	Cat 4	No	25.6	3.5	24.2

*Mean Ct across 3 replicate reactions
RQ – relative quantification

All cats tested negative for *C. felis* by PCR prior to stimulation feeding or SG injection (day 0). Both cats used for stimulation feeding (cats A and B) developed cytauxzoonosis and tested positive for *C. felis* by PCR. Neither cat (cat 1 and 2) that was injected with SG from heated ticks developed cytauxzoonosis nor tested positive for *C. felis* by PCR throughout the study. One of the cats (cat 3) that was injected with SG from stimulation-fed ticks developed cytauxzoonosis and tested positive for *C. felis* by PCR. The other cat (cat 4) that was injected with SGs from stimulation-fed ticks did not develop cytauxzoonosis and tested negative for *C. felis* by PCR throughout the study. All infected cats recovered from acute infection and remained PCR positive for *C. felis* at the end of the study (after day 30).

Infected cats tested positive for *C. felis* by PCR 2–4 days prior to developing clinical signs. Cat A showed clinical signs (lethargy and hyporexia) from day 15 through day 22 post-stimulation feeding and was febrile from day 15 through day 18 (39.2–39.9ºC, 102.6–103.9ºF). Rare intraerythrocytic stages of *C. felis* (5 per 200 high powered fields) were detected on the blood smear from day 13 post-stimulation feeding and none were found in the last blood sample collected at day 33. Cat B developed the same clinical signs from day 14 through day 19 post-stimulation feeding and was febrile from day 15 through day 17 (39.2–40.4ºC, 102.6–104.8ºF). Rare intraerythrocytic stages of *C. felis* were detected on the blood smear from day 13 (7 per 200 high powered fields) and day 39 (17 per 200 high powered fields) post-stimulation feeding. Cat 3 experienced the longest course of clinical signs starting on day 11 and lasting through day 21 post-SG injection and was febrile from day 11 through day 17 (39.1–40ºC, 102.5–104ºF). Intraerythrocytic stages of *C. felis* were detected on the blood smears from day 7 (3 per 200 high powered fields) and day 35 (10 per 200 high powered fields) post-stimulation feeding. No

schizonts were found on blood smears from any infected cat at any timepoint. Cats 2 (heated SG inoculum) and 3 (stimulation-fed SG inoculum) did not develop clinical signs throughout the study but two intraerythrocytic structures that resembled *C. felis* were found in each blood smear from blood samples collected on day 37 (cat 2) and 38 (cat 3). On these days, both cats tested negative for *C. felis* by PCR in all ten replicate reactions and also tested negative for other piroplasmid infections using a broad range PCR assay. Therefore, the infection statuses for these two cats were considered equivocal. After the study concluded, one of these cats (cat 2) was available for additional sampling at day 65 post injection. On this day, no organisms were identified on blood smear, and the sample tested negative using both the *C. felis* PCR and the broad range PCR targeting apicomplexan 18S rDNA.

4. DISCUSSION

In this study, we successfully demonstrated that SGs isolated from *C. felis*-infected ticks were capable of inducing cytauxzoonosis. We evaluated two methods (stimulation feeding and heating) to induce sporogony in *C. felis*-infected ticks and the infectivity of the inoculums was tested in naïve cats. Of the 2 cats that were injected with SGs from stimulation-fed ticks, only one cat developed cytauxzoonosis and had definitive confirmation of infection by both light microscopy and PCR. Neither cat that was injected with SGs from the heated ticks developed cytauxzoonosis. As all inoculums were confirmed to contain *C. felis* by PCR, the cause for the differences in infectivity between the inoculums is unknown and will require further investigation.

Two cats that never developed cytauxzoonosis (one cat that received heated inoculum and one cat that received stimulation-fed inoculum) had rare intraerythrocytic structures that resembled *C. felis* organisms observed on a single blood smear each. However, even with replicate testing of serial samples and two independent assays, *C. felis* DNA was never detected via PCR in either of these cats. We deemed the infection status of these cats to be equivocal at best. These intraerythrocytic structures may represent something other than hemoprotozoal infections, such as Howell-Jolly bodies with an atypical appearance (24). We have previously observed similar structures in healthy cats when blood smears are intensively screened at high magnification. An infection model needs to be consistently reproducible with verifiable evidence of infection or exposure to the pathogen. Therefore, even if these cats were truly infected with *C. felis*, the utility of their "silent" infections for *Cytauxzoon* research remains unknown. Currently, there are no widely available serologic assays to detect *C. felis* antibodies. Therefore, it remains unknown whether these cats have developed an immune response against *C. felis* suggesting that they were

infected. In future experiments, cats with similar outcomes after SG injection could be challenged by tick transmission to determine whether they had protective immune responses.

Arguably one of the most interesting findings of our study is that only one of the two cats that were injected with stimulation-fed SGs developed cytauxzoonosis. This is despite the fact that both cats used for stimulation feeding (cat A and B) developed cytauxzoonosis making it clear that these ticks produced infective sporozoites. As mentioned above, one of the cats could have had a silent infection, but we believe this cat did not actually become infected. Based on our real-time PCR results, the SG inoculum that was able to induce cytauxzoonosis contained almost tenfold more copies of *C. felis* mtDNA than the other stimulation-fed inoculum. Therefore, it is possible that the outcome of these cat infections was simply associated with the dose of infective *C. felis* sporozoites. There are several possibilities to explain these differences in the outcome between the two inoculums. First, it is possible that the viability of the collected parasites was compromised during the inoculum preparation process. This may have reduced the numbers of infectious sporozoites within the inoculum and subsequently lowered the infectivity in one of the inoculums below the minimum dose required to cause clinical disease. If this is the case, reevaluation of the inoculum preparation procedures (e.g. testing different reagents and storage conditions) may provide insights on ways to maximize parasite survival during this process. Alternatively, we could also counteract the loss of parasites by using an increased number of ticks in each batch of inoculum. Another possible explanation is that there may be a difference in the infectivity between the sporozoites that were secreted in the saliva compared to those that remained in the SGs. In this scenario, the majority of the sporozoites may have rapidly egressed from the SGs as they matured, leaving fewer numbers of infectious organisms or immature forms

of *C. felis* in the SGs. Alternative methods that focused on collection of saliva for inclusion in the inoculums such as *in vitro* stimulation feeding or induction of salivation could be explored. Lastly, there may be a difference in immune responses between these two naïve cats, and perhaps one cat was able to overcome the initial sporozoite challenge without developing cytauxzoonosis. While this infection method has not been attempted previously, prior *C. felis* infection studies have not described any domestic cats that were innately immune to *C. felis*. In naturally occurring infections, there does appear to be a population of cats that get infected with *C. felis* yet never experience acute cytauxzoonosis. While some investigators have suggested that there are virulent and avirulent strains (25,26), it is possible that these differences in outcomes are related to the initial dose of *C. felis.*

Neither of the cats that were injected with SGs from heated ticks developed cytauxzoonosis. It is therefore possible that heating was less effective than stimulation feeding for promoting *C. felis* sporogony. This was the case with other related piroplasmids when these two methods were compared head-to-head. For *T. parva* and *T. annulata*, while SG inoculums from heated ticks were able to transmit infections, compared to stimulation-fed ticks the transmission rates were lower and the heated ticks contained fewer infected SGs on light microscopy (27–29). When heat-induced sporogony was evaluated ultrastructurally for *Babesia microti*, the maturation of sporozoites in heated ticks was notably delayed compared to the ticks that were stimulation-fed (30). The reasons for the differences between these two methods remains unclear. It is possible that when ticks are heated, they lack the endogenous stimuli from the tick and exogenous stimuli from the blood meal that might normally promote sporogony (31). Despite the apparent advantages of stimulation feeding, heat treatment should still be considered for *C. felis*, as this

would allow us to minimize the use of vertebrate animals for the collection of sporozoites. Some techniques might increase the likelihood of developing infectious sporozoites with heating alone. For example, it has been shown that incubating ticks under a higher ambient temperature (28ºC vs. 23ºC) while they are molting prior to being heated (37ºC) as adults resulted in higher transmission rates for *Theileria* (Young et al., 1984). The effects of this additional step and/or extending of the heating time for adults should be assessed in future studies when heat is utilized to induce sporogony.

The amounts of *C. felis* DNA present within the 4 inoculums varied widely. Whether this variation is due to the biological differences associated with the individual batches of ticks or is a result of differences in sporogony secondary to the heating and stimulation feeding remains unclear. To our knowledge, all previous molecular detection of *C. felis* in ticks has only been qualitative not quantitative (32,33). Therefore, to further assess the effects of heating or stimulation feeding on *C. felis* sporogony, parasite loads in ticks prior to and after these treatments should be performed using larger sample sizes. This variation of *C. felis* dosages in the inoculums could also be attributed in part to the different prevalence rates between batches of ticks. While we did not specifically test the percentage of infected ticks in our study, prior successful tick transmission studies of *C. felis* that utilized the same acquisition feeding technique had documented tick infection rates of 12–25% (Reichard personal communication and unpublished data). Based on these data, the estimated 95% confidence interval for the number of infected ticks that could be present within a batch of 50 ticks can range greatly from 2 ticks (4% positivity rate) to 19 ticks (38% positivity rate) (34). This variation in tick positivity rate may increase the possibility of having batches of ticks that contain parasites that are either

just at or below the minimum infectious dose. Based on published tick transmission studies, this appears to be corroborated as ~90% of the challenged cats became infected. Future studies could explore methods to maximize the number of infected ticks per acquisition feeding by either using the natural reservoir host (bobcat) or raising the parasitemia level in *C. felis*-infected domestic cats through splenectomy or immunosuppressive drugs (9,35,36).

In conclusion, this pilot study is the first to validate the feasibility of collecting viable and infectious sporozoites from *C. felis*-infected ticks. However, this process should be optimized to determine the conditions necessary for the consistent production and collection of infectious *C. felis* sporozoites. The success of this model could greatly expand the resources we have to study *C. felis* and significantly advance research in this field.

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CHAPTER 7

CONCLUSIONS

Cytauxzoon research is hindered by the inability to culture the parasite *in vitro.* An additional challenge is the limited number of models to study *C. felis*. This dissertation investigates tick-based experimental models for *C. felis*.

Our processing technique for whole tick histology for Ixodid ticks is easy to follow uses tools, materials, and equipment that are standard in the field of histotechnology. This technique will not only continue to advance *C. felis* research but will also help us further understand tick internal anatomy and host-pathogen interactions for other tick-borne diseases. This technique will also benefit other investigators who are interested in tick histopathology to achieve their research goals.

We were also able to definitively detect and visualize *C. felis* for the first time in tick tissues by combining multiple microscopic and molecular detection methods. The validation of these various techniques provided us with a foundation to further investigate other parts of the uncharacterized development of *C. felis* within the tick host.

While the two *in vitro* detection techniques were unsuccessful in infecting *A. americanum,* both models have great potential to further our mission in minimizing the use of purpose-bred laboratory cats to infect ticks. For this reason, future work should continue to make modifications and aim to establish systems that are suitable for *C. felis*. These techniques may also be gateways to establishing *in vitro* cultivation for *C. felis* or serve as a basis to investigate the complex parasite-vector interactions.

Lastly, we were able to demonstrate the feasibility of producing sporozoites-based inoculums from *C. felis*-infected ticks. While this model requires further optimization, the

availability of sporozoites-based inoculums can greatly expand our capabilities in *Cytauxzoon* research. These may include utilizing sporozoites to initiate culture in feline cells, investigate disease pathogenesis and parasite development, or discover potential vaccine or therapeutic targets.

In conclusion, the utilization of these tick-based experimental models could significantly advance *Cytauxzoon* research and allow us to develop new strategies to treat, prevent and diagnose cytauxzoonosis.