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Infection Potential of *Rickettsia felis* via Ingestion

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INFECTION POTENTIAL OF *RICKETTSIA FELIS* VIA INGESTION

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Pathobiological Science

by

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May greater glory, love unending
be forever thine.

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ABSTRACT

Rickettsia felis is the etiologic agent of flea-borne spotted fever (FBSF) in humans and a poorly described cause of fever in animals. It is transmitted by its primary arthropod vector and reservoir host, the cat flea *Ctenocephalides felis*. Known routes of *Rickettsia felis* transmission between *Rickettsia felis*-infected cat fleas and vertebrate hosts include cutaneous bites and contamination of cutaneous wounds with infective flea feces. The bulk of FBSF infections occur in young children in Africa, though infections of people at all ages all over the world have been confirmed. As mammals and young children frequently come into contact with flea feces through routine grooming and indiscriminate oral hygiene, respectively, we speculate that ingestion of infective flea feces may potentially account for a portion of natural *Rickettsia felis* infections. To investigate the potential role ingestion of *Rickettsia felis* plays in transmission of the bacterium to vertebrates, we designed sister experiments using a BALB/c mouse model to determine 1) if *Rickettsia felis* could establish an infection via an oral route and 2) if *Rickettsia felis* could establish an infection via an oral route in the form of infective cat flea feces. For our first objective, *Rickettsia felis* was cultured in ISE6 cells, purified, and administered orally in SPG buffer. Our second objective was executed by feeding a viable *Rickettsia felis*-spiked blood meal to *Ctenocephalides felis*, collecting infective flea feces, and administering the feces orally in SPG buffer. In both experiments necropsy was performed at 1, 7, and 14 days post-exposure and heart, liver, spleen, stomach, and intestine were collected for DNA extraction, RNA extraction, and qPCR analysis. Tissues with positive results were subsequently submitted for histopathology using hematoxylin and eosin staining as well as anti-*Rickettsia* immunohistochemistry. Select tissues were tested via RT-qPCR and a single serum sample via an indirect immunofluorescence assay. Our results indicate that *R. felis* from culture is transmissible via ingestion in mice and

may be found in organs distant from the gastrointestinal tract at all time-points tested without evoking inflammation. Further studies are needed to characterize all aspects of *R. felis* transmission via ingestion in vertebrates.

CHAPTER 1. THE CASE FOR INGESTION AS A POTENTIAL ROUTE OF *RICKETTSIA FELIS* INFECTION

1.1. A Review of *Rickettsia*

The genus *Rickettsia* belongs to the order Rickettsiales (phylum Proteobacteria, class Alphaproteobacteria) which derives from a distant ancestor that diverged from mitochondria after colonizing single-celled organisms 2,000-1,500 million years ago [1]. As a consequence of the Cambrian Explosion ushering in the majority of characteristically multicellular metazoan phyla, members of Rickettsiales started infecting eukaryotes, notably early arthropods, around 525-425 million years ago [2, 3]. *Rickettsia* began to diverge from the closely related genus *Orientia* around 225-150 million years ago and subsequently evolved to occupy ecological niches as the hydra, torix, and arthropod-associated clades [2]. The former two clades are known parasites of many protists and leeches, however, there is debate whether the arthropod-associated *Rickettsia* initially infected protists and later adapted to infecting arthropods [2]. This theory is logically supported by the idea that early arthropods were exposed to Rickettsiales via ingestion of their protist hosts. Protists emerged far sooner than arthropods, the first multicellular protists as early as 1,200 million years ago [4-6], indicating a significantly longer period of co-habitation with Rickettsiales. Moreover, genomic sequencing of *Rickettsia bellii* has revealed genes related to amoebal symbionts, suggesting an ancient horizontal gene transfer event occurred [1, 7, 8].

Rickettsia first came under scientific scrutiny through the eyes of Howard Taylor Ricketts, an American pathologist whose 1907-1909 investigation into Rocky Mountain Spotted Fever led him to correctly describe the etiologic agent *Rickettsia rickettsii* as a pleomorphic bacillus isolated from the salivary glands, alimentary sac, and ovaries of ticks [9, 10]. In the following decades small intracellular bacteria that were unable to be isolated or otherwise

grouped were described as rickettsiae [1], particularly if they were gram negative, difficult to culture, and borne by hematophagous arthropods. Classically, *Rickettsia* have been placed into two key groups based on arthropod vector, optimal growth temperatures, antigenic characteristics, and clinical signs associated with disease [10-12]. These main groups include the spotted fever group (SFG) composed of *Rickettsia* species typically transmitted by ticks and the typhus group (TG) made up of *Rickettsia* species transmitted predominantly via fleas or lice [1, 11]. With the dawn of molecular diagnostics, whole genome sequencing, gene banking, and computational phylogenetics, over 30 *Rickettsia* species and many strains have been identified [2, 10] and a few species erroneously classified as *Rickettsia* – such as *Coxiella burnetti*, the causative agent of Q fever – have been removed [10]. A landmark phylogenetic study published by Gillespie *et al* in 2007 established the transitional group (TRG) and rooted *Rickettsia* phylogeny to the ancestral group (AG), defining four distinct modern lineages of *Rickettsia* [8, 13, 14]. TRG rickettsiae, consisting chiefly of *Rickettsia felis*, *Rickettsia akari*, and *Rickettsia australis*, share features of both the SFG and TG rickettsiae [1, 8, 13]. SFG rickettsiae demonstrate increased sequence homogeneity relative to other clades and are capable of entering the host cell nucleus through the polymerization of actin [1]. TG rickettsiae are partially characterized by highly reductive genomes [13], a common theme amongst intracellular symbionts as they become increasingly reliant on host metabolic pathways [3] – and increasingly pathogenic [1]. In contrast with the other clades, members of the basal AG are largely considered non-pathogenic to vertebrates [2].

1.2. The Current Understanding of *Rickettsia felis* as an Emerging Pathogen

1.2.1. Discovery, Characterization, and Organization

A minor debate persists regarding the original point of discovery of *Rickettsia felis*. It has been suggested that the organism was first described upon isolation from a European cat flea (*Ctenocephalides felis*) in 1918 by H. Sikora who named it ‘*Rickettsia ctenocephali*’ [15, 16] before it was subsequently forgotten. As this occurred prior to the molecular age, it is impossible to determine if the organism Sikora described was truly the modern-day *R. felis* or simply the other known flea-borne rickettsia, *Rickettsia typhi* [10]. Rather, the discovery is commonly credited to Adams *et al* for their 1990 electron microscopy-based description of a rickettsia-like organism in the midgut epithelial cells, myocytes, fat body, ovaries, and epithelial sheath of the testes of a laboratory cat flea colony [15, 17-22]. Ironically, the flea colony was being used to identify a vector for *Ehrlichia risticii* (renamed *Neorickettsia risticii*), the causative agent of Potomac Horse Fever [22]. This incidentally identified organism – temporarily termed ‘ELB agent’ for the flea supplier Elward Laboratory [17, 20-22] – was characterized as part of *Rickettsia* in 1992 by Azad *et al* after successful amplification of a conserved genera-specific 17-kDa protein antigen and a citrate synthase gene (*gltA*) [17, 20]. Through a series of specific polymerase chain reaction (PCR) amplifications and restriction enzyme assays, Azad *et al* determined that ELB agent more closely resembled TG *Rickettsia* than SFG *Rickettsia* [20], a classification overturned in 2001 by Bouyer *et al* [22] who successfully demonstrated amplification of the SFG-associated 190-kDa antigen gene (*rompA*). Meanwhile, Higgins *et al* investigated eight laboratory colonies of cat fleas, identified a 43-93% prevalence range of ELB agent, and subsequently proposed ELB agent be deemed *Rickettsia felis* [17, 22-24], which was soon placed in the newly organized TRG [8].

1.2.2. Distribution and Arthropod Association

R. felis exhibits a worldwide distribution, with positive identification on every continent except for Antarctica [10, 11, 15, 17, 25, 26]. This sprawl is largely due to the nearly ubiquitous presence of the cat flea (*Ctenocephalides felis*) [17, 26, 27], which has been considered the primary vector of *R. felis* as well as its reservoir host owing to its ability to maintain the bacterium in flea populations [24, 25, 28]. While *R. felis* bears a strong relationship with the cat flea, it is only considered to be a facultative parasite of this species [18]. Furthermore, *R. felis* is unique among rickettsiae in that it can infect both insect and acarine arthropod hosts [17, 29-32]. These arthropods have come to include over 10 other flea species, several species of ticks, mosquitoes, mites, and chiggers, as well as a single report in a bed bug [15, 17, 25, 33, 34] and a single non-hematophagous host, the book louse *Liposcelis bostrychophila* [35-37].

Transmission of *R. felis* within cat fleas has been shown to occur both vertically and horizontally [19, 26, 28, 38-40]. Vertical transmission of *R. felis* between generations of cat fleas was deemed plausible when the bacterium was noted in ovaries and the epithelial sheath of testes [21]. In 2002 Wedincamp and Foil [28] demonstrated vertical transmission for up to 12 generations of cat fleas, indicating that both transovarial and transstadial maintenance of *R. felis* occurs. They additionally noted that there were no negative effects of infection on host fitness, such as reduced egg counts [28]. They did, however, report waning levels of *R. felis* past the F8 generation [28], suggesting that vertical transmission alone was unlikely to perpetually maintain the infection in a single flea colony [26, 41]. In a separate study, Wedincamp and Foil reported PCR detection of *R. felis* in feline blood and that laboratory cats became seropositive within four months of exposure to *R. felis*-infected cat fleas, providing additional support for horizontal transmission [42]. In 2008 Macaluso *et al* reported the presence of *R. felis* in the salivary glands

of cat fleas observed with transmission electron microscopy and confirmed via PCR [19]. Finally, in 2011 Hirunkanokpun *et al* showed that horizontal transmission between cat fleas occurs via both cofeeding – the sharing of a blood-meal between infected and naïve hosts – and mating [38], while Reif *et al* demonstrated that cat fleas could become infected with *R. felis* via an infective blood meal [43]. Brown *et al* went further in 2015, demonstrating cofeeding transmission of *R. felis* between two different arthropod species (*C. felis* and *Xenopsylla cheopis*) [26]. This hints at a greater complexity of *R. felis* transmission that is undoubtedly occurring in sylvatic, suburban, and urban cycles [17]. Moreover, horizontal transmission for maintenance in an arthropod host has been associated with increased pathogenicity in rickettsiae [14, 44].

1.2.3. Intraspecific Variation and Virulence

Several strains of *Rickettsia felis* have been identified to date. *R. felis* was first isolated in pure culture from *Ctenocephalides felis* by Raoult *et al* in 2001 using a *Xenopus laevis*-derived XTC-2 cell line [45]. This cultured line, designated as *R. felis* str. URRWXCal2 (alternatively Marseille-URRWXCal2 or California 2), grew best at 28°C and failed to grow at temperatures >32 °C – a characteristic that supported the early placement of *R. felis* in the SFG rickettsiae [17, 45, 46]. In 2006 a novel isolate of *R. felis* str. URRWXCal2 (Pedreira) was successfully grown in a C6/36 *Aedes albopictus* cell line [47]. In the same year a new strain of *R. felis*, *R. felis* str. LSU, was isolated from *C. felis* and cultured in an ISE6 *Ixodes scapularis* cell line [48]. In 2010 Behar *et al* identified *R. felis* in a non-hematophagous booklouse, *Liposcelis bostrychophila*; this strain is not officially named and is referred to as “Aus-Lb” [37]. Similarly, *R. felis* str. LSU-Lb was isolated from the same species of booklouse and was successfully grown in ISE6 cells by Thepparit *et al* in 2011 at 32 °C [35]. Finally, a *Rickettsia felis*-like organism (RFLO) from Asembo, Kenya, known as *Candidatus Rickettsia asemboensis*, was described by Jiang *et al* in

2013 [49]. Currently, over 30 RFLOs have been identified in wild-caught arthropods worldwide based on 0.2% divergence of the 16S rRNA (*rrs*) gene [25], however, caution has been advised for future speciation based on low thresholds of genomic divergence [50], as phenotypic and ecological relevance may be insignificant [25].

The genome of *R. felis* str. URRWXCal2 was first sequenced in 2005 by Ogata *et al* [46, 51]. Subsequently, Gillespie *et al* sequenced the genomes of *R. felis* str. LSU and *R. felis* str. LSU-Lb in 2014 [14]. Comparison of the genomes revealed that the cat flea-associated strains were minimally divergent and that the differences observed in LSU-Lb likely resulted from adaptation to an endosymbiotic relationship with the booklouse [14]. Interestingly, it has been shown that the booklouse requires *R. felis* for development of the oocyte, indicating a different type of vector association as compared to the facultative parasitic relationships of flea-borne rickettsiae [25, 36, 52].

The initial team sequencing URRWXCal2 estimated the presence of ~1,500 open reading frames and two plasmids they named pRF and pRF δ , the first plasmids to be discovered in a *Rickettsia* species [46]. Pornwiroon *et al* noted that strain LSU contained pRF but lacked pRF δ [48], a finding later confirmed by Fournier *et al* who additionally discovered that strain URRWXCal2 may lose pRF δ at high passage [53]. Similarly, other strains of *R. felis* have been identified that occasionally lack plasmids altogether (e.g. strain RF2125) [53, 54]. Interestingly, strain LSU-Lb contains pRF and a unique plasmid pLbaR [14]. In order to determine the effect of strain (i.e. presence of pRF and pLbaR) on transmission, infection, and host fitness, Healy *et al* infected *C. felis* with *R. felis* str. LSU-Lb for comparison with *R. felis* str. LSU [18]. Surprisingly, they found that strain LSU-Lb infected *C. felis* more efficiently than strain LSU

[18], suggesting either that vector adaptation occurs despite variance in plasmids or that plasmids play a minor role in host relationships of *R. felis* strains.

While the functions of these plasmids are still being explored, their presence and putative as well as confirmed genes suggests several possibilities, including potential virulence genes as well as release of effector molecules and horizontal gene transfer via components of a Type IV Secretion System [8, 14, 46, 55-57]. Although *R. felis* and other vertebrate-associated rickettsiae lack pathogenicity islands [1, 8, 58], prospective virulence genes of *R. felis* notably include several *sca* genes, surface antigens variably involved in adhesion and actin-based motility (*sca2*), including *ompB* and *sca4* which have been universal among *Rickettsia* [46, 59-64]; a hyaluronidase homolog pRF56 which bears sequence homology to *Clostridium perfringens* hyaluronidase NagI [8, 46]; *pat1* and paralog *pat2* potential phospholipases [8, 46, 65, 66]; and several genes for patatin-like proteins associated with hemolytic activity – a finding functionally confirmed by dithiothreitol inhibition [46]. Of these, the most concerning for higher eukaryotes is hyaluronidase, which is considered a “spreading factor” owing to its ability to digest the extracellular matrix of animal tissues [8]. Similarly, *R. felis* also contains a chitinase, which explains its ability to colonize arthropods [46]. Additionally, *R. felis* has several genes devoted to antibiotic resistance, including resistance to streptomycin, class C and D B-lactamases, and penicillin [46]. Finally, pathogenicity is further implied by the reductive nature of the *R. felis* genome, which has been associated with pathogenic phenotypes [1]. It has been proposed that these deletions occurred either through relaxation of purifying selection [1, 67-69] or through failed horizontal gene transfer [1, 70], each resulting in degraded genes or pseudogenes.

1.2.4. Disease Association in Animals and Humans

While *Rickettsia felis* has been isolated from many arthropods, its primary vector and reservoir host is the cat flea [15, 25], which has been found worldwide in close association with vertebrates of sylvatic, suburban, and urban ecosystems. It is important to note that a definitive mammalian host of *R. felis* has yet to be identified [17] and that the bacterium has not been isolated from a vertebrate host [11, 25], leaving Koch's postulates of disease causality unfulfilled. Furthermore, Brown *et al* demonstrated that horizontal transmission of *R. felis* occurs between cofeeding arthropods in the absence of bacteremia, suggesting that vertebrate transmission and a subsequent systemic infection are not required for maintenance of *R. felis* in arthropod hosts [26]. Consequently, the clinical significance of *R. felis* has been questioned [26, 71]. Though this may be, there remains a significant body of evidence that supports the role of *R. felis* as a pathogen of both animals and humans.

While many companion animals are protected from hematophagous arthropods due to diligent owners and preventative medications, neglected, stray, and wild animals are completely exposed to such vectors. These animals play an important role in the health of humans as sentinels of infectious disease, particularly in suburban and urban settings [72]. Since being definitively identified in 1996, *R. felis* has been reported in association with mammals worldwide. Stray cats and dogs, opossums, raccoons, and rats are considered reservoir hosts [22, 25]. Surveillance studies conducted in several countries have investigated the prevalence of *R. felis* in the fleas of these species with rates that are mostly in agreement with the <25% for wild-caught fleas reported by Reif *et al* [38, 73-84]. Importantly, animals have been shown to mount an antibody response to *R. felis*, as first documented by Wedincamp and Foil when their research cats seroconverted within four months [17, 42, 85, 86], suggesting infection occurs in

vertebrates. Furthermore, Mongkol *et al* correlated *R. felis* with fever, anemia, and petechia in dogs by specifically collecting fleas and blood from dogs diagnosed with fever of unknown origin (FUO) [76]. While their results provide evidence of *R. felis*-association in these clinically ill dogs, it is uncertain which diagnostic tests these dogs received in order to rule-out other etiologies, thus further studies are needed to refine this association. Currently, no articles related to *R. felis*-associated disease in animals can be found in the *Journal of the American Veterinary Medical Association* or the *American Journal of Veterinary Research*, suggesting that it has largely been overlooked as a significant cause of concern in veterinary medicine. Likewise, *R. felis* is not mentioned in the latest edition of *The Textbook of Veterinary Internal Medicine* [87].

The diagnosis of arthropod-borne illness in veterinary medicine is largely dependent on microimmunofluorescence, direct immunofluorescence, and to a lesser extent serology and PCR identification [87], methods with potential for interspecies cross-reactivity that have not been fully delineated [10]. Moreover, empirical treatment is common practice in veterinary medicine, and, considering the treatment for most arthropod-borne illnesses is a tetracycline antibiotic – chiefly doxycycline – there is little pressure on practitioners to reach an absolute diagnosis.

Furthermore, one survey of veterinarians found that only 55.8% polled knew of *R. felis* and only 51.2% thought fleas posed a health risk to humans [88]. Therefore, the sparsity of hard evidence supporting the affiliation of *R. felis* with animal disease is likely due to a combination of effective preventative medicine against companion animal ectoparasites, a lack of need for an absolute diagnosis in the treatment of arthropod-borne animal disease, a paucity of correlative studies, and potentially a lack of primary and continuing education for veterinarians in regards to arthropod-borne disease.

The incidence of *R. felis*-associated disease is better documented in humans as compared to animals and has been reported worldwide, notably in several endemic areas including large swathes of sub-Saharan Africa, parts of Texas, and Los Angeles, California [11, 15, 89-91]. In humans, *R. felis*-related disease is termed flea-borne spotted fever (FBSF) and has been associated with clinical signs such as headaches, fever, chills, cough with or without pneumonia, malaise, and myalgia, dermatologic signs such as a maculopapular rash or eschar, and rarely more severe gastrointestinal or neurological signs such as photophobia, hearing loss, or meningitis [11, 15, 89, 92-94]; many signs that occur with murine typhus (endemic typhus) infections [25]. Murine typhus is caused by *Rickettsia typhi* and is similarly transmitted by fleas in regions worldwide that commonly overlap with *R. felis* [11]. Consequently, FBSF has likely been misdiagnosed as murine typhus for years [45]. Indeed, in 1994 the first confirmed human case of FBSF was initially misdiagnosed as murine typhus in 1991 due to poor specificity of serological testing for rickettsial diseases [95]. Since that time serology has improved and many reports have identified seropositivity in humans, providing further evidence of *R. felis* exposure and possible infection [76, 96-103]. Interestingly, a 2017 report by Teoh *et al* investigated the incidence of seropositivity in veterinarians – a unique human niche consistently exposed to fleas over the span of many years [88]. They found 16% of veterinarians were unequivocally positive for exposure to *R. felis* and an additional 35.1% tested were seropositive either for *R. felis* or *R. typhi* [88]. In a comical turn of biological retribution, veterinarians that failed to advocate for flea prevention were significantly more likely to be seropositive [88].

Perhaps the most compelling case for zoonotic horizontal transmission of *R. felis* via fleas was reported by Richter *et al* in 2002, detailing a couple and their dogs [104]. The couple each developed a fever and maculopapular rash. A series of negative diagnostic results led physicians

to eventually test for flea and tick-borne diseases as the couple reported that their dogs had been infested with ectoparasites. Both the woman and one of her dogs had high titers for *R. felis*. The presence of *R. felis* was later confirmed with Western blot and PCR analyses. Although no ectoparasites were available from the dogs for testing, the physicians came to the logical conclusion that the couple had been bitten by fleas or ticks from their dogs. While no conclusion can be drawn regarding zoonotic horizontal transmission, the case provides solid evidence that domestic exposure to ectoparasites can result in human disease [104].

In Africa fever rates have remained stagnant even though cases of malaria have declined [105]. In the effort to discern causes for FUO, investigators have identified *R. felis* as a significant entity in febrile children less than five years of age, especially those younger than three [101, 105]. Mourembou *et al* found that *R. felis* is significantly more associated with febrile children than afebrile children and that several febrile children presented with detectable levels of *R. felis* in their blood, suggestive of bacteremia in a situation of active infection [105]. Conversely, Mediannikov *et al* revealed that some patients bacteremic with *R. felis* lack an antibody response and that others are asymptomatic [106, 107], leading them to propose the possibilities of relapse or recurrent infections or that people may be reservoir hosts. Still another possibility to consider is cutaneous contamination during sampling, as skin swabs of African patients have been PCR positive for *R. felis* [108]. In spite of the inconsistent data surrounding FBSF, a group of researchers that correctly predicted the chikungunya and Zika virus outbreaks of the past ten years believe *Aedes* spp. mosquitoes transmitting *R. felis* out of Africa will account for the next global epidemic [109].

1.2.5. Routes of Transmission to Vertebrates

The most widely accepted method for transmission of *R. felis* from an arthropod to a vertebrate is via an infectious bite. This route was presumed with initial identification of FBSF, further supported by seropositivity in vertebrates post flea-feeding [42], gained traction when Macaluso *et al* identified *R. felis* in the salivary glands of cat fleas [19], and was finally proven by Brown *et al* when *R. felis* transcripts were recovered from flea-feeding sites on mice skin [26]. Additionally, it has been suggested that contamination of cutaneous wounds (i.e. flea-feeding sites) with infectious flea feces could constitute an alternate route of transmission, as is the case with *Rickettsia prowazekii* and *Rickettsia typhi*, the causative agents of epidemic typhus and murine typhus, respectively [110-112]. This hypothesis was supported by Reif *et al* who established that cat flea feces contains *R. felis* DNA as far out as 28 days post exposure (DPE) and additionally that the *R. felis* was viable, as RNA transcripts were recovered in cat flea feces at 21 DPE [43]. Further evidence for deposition of *R. felis* in cat flea feces was provided by Thepparit *et al* in 2013 when they used immunofluorescence assays and transmission electron microscopy to demonstrate the dissemination of *R. felis* into cat flea excretory organs including the hindgut, Malpighian tubules, and rectal ampulla [40]. Finally, this route was tested by Legendre *et al* [113] who conducted an experiment wherein mice were intradermally inoculated with infectious doses of *R. felis* from culture and flea feces. They found sites of inoculation were significantly positive for *R. felis* DNA at 24 and 48-hours post exposure, both from culture and infective flea feces [113]. To date, a timeline of significant milestones in research related to *R. felis* is presented (Figure 1).

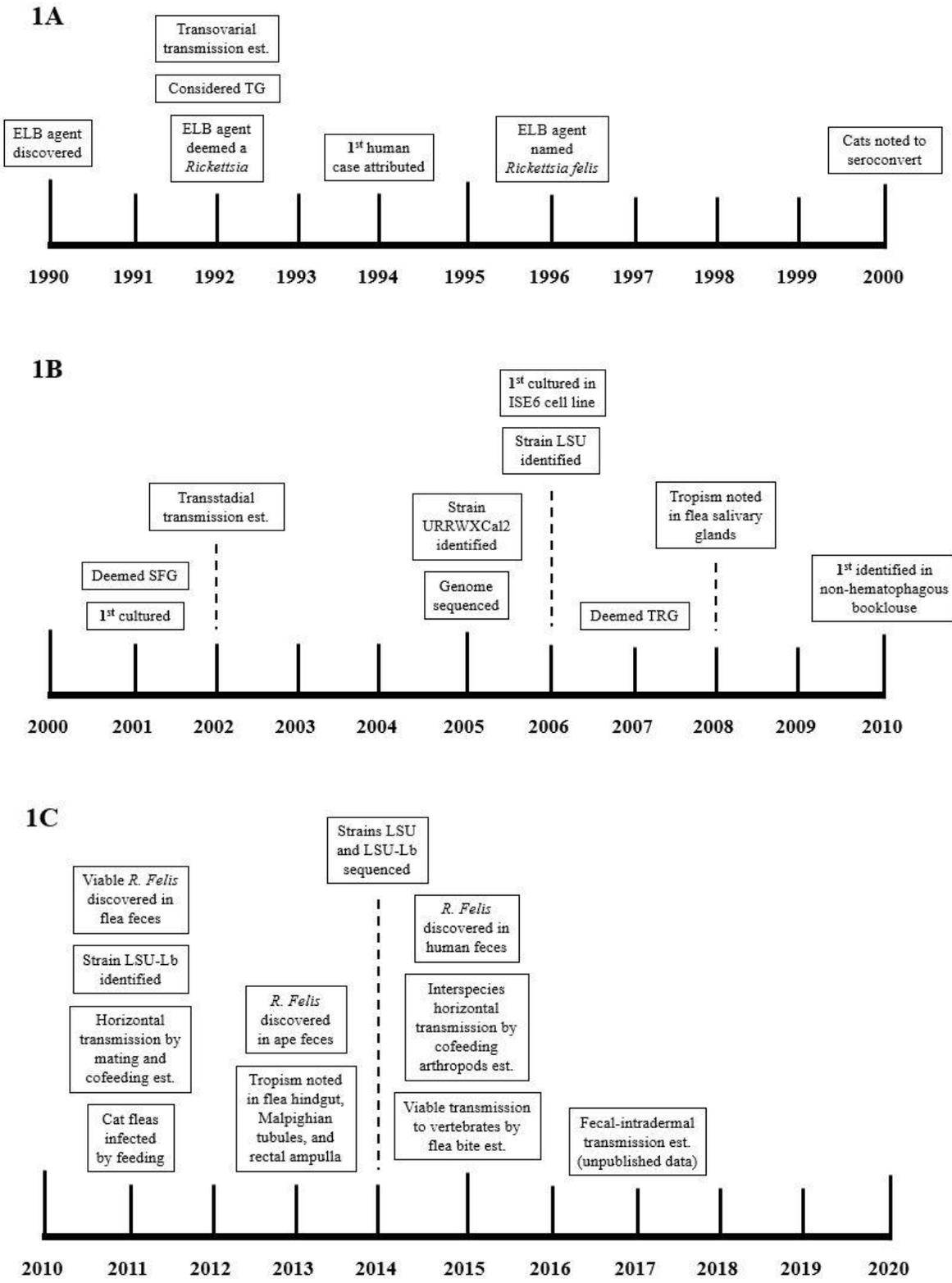


Figure 1. A timeline of *Rickettsia felis* milestones. 1A 1990s. 1B 2000s. 1C 2010s.

The current study seeks to further investigate the infectious nature of flea feces, hypothesizing infectivity of *R. felis* to vertebrates via ingestion, as is the case for similar organisms covered in the following section. Preliminary evidence of alimentary exposure to *R. felis* is provided by Keita *et al* who established the presence of *R. felis* in the feces of both apes and humans across Africa [114, 115], minimally suggesting exposure via ingestion and, more ominously, alimentary infection and the possibly of shedding of *R. felis* in feces – the implications of which would be considerable for animal and human health globally. From a veterinary perspective, mammalian grooming behaviors provide ample exposure to fleas and flea feces; indeed, Wade and Georgi demonstrated that as many as 49.5% of the fleas on a cat end up in its feces [116]. This route also supports the age bias in humans identified by Mourembou *et al*, as younger children – a niche of humans with poor hygiene and indiscriminate oral exposure – were statistically more likely to contract FBSF [105].

1.3. Ingestion as a Route of Infection of Similar Pathogens

Transmission via ingestion has been observed in several other obligate and facultative intracellular Alphaproteobacteria. Both *Neorickettsia helminthoeca* and *Neorickettsia risticii* are ultimately ingested by their vertebrate hosts in which they are the causative agents of salmon poisoning disease and Potomac Horse Fever, respectively [117-119]. *Bartonella henselae*, the causative agent of cat-scratch disease, has been reported as transmissible via ingestion in cats [120], though this finding was not supported by a subsequent investigation [121]. While these species provide examples of orally transmitted bacteria, other members of Rickettsiales, chiefly the TG members *R. prowazekii* and *R. typhi*, provide the most compelling comparisons.

1.3.1. *Rickettsia prowazekii*

Known as the “scourge of armies” *Rickettsia prowazekii* is of great historical importance as the louse-borne causative agent of epidemic typhus. It is estimated to have been the cause of death of up to 20% of Napoleon Bonaparte’s Grand Armée [122], three million people during the Russian Revolution of 1917 [123], and many innocents in concentration camps during World War II [124]. Although in 1995 it was thought to be functionally extinct, an outbreak occurred in 1997 in the African country of Burundi after a civil war displaced a large percentage of the population into close-quartered refugee camps [125-127].

Transmission of *R. prowazekii* to humans by the body louse *Pedunculus humanus humanus* (*Pedunculus humanus corporis*) occurs due to contact of infective lice feces or crushed lice with wounds (i.e. lice-feeding sites), conjunctiva, or mucous membranes, including contact with the airways via inhalation – a common route of infection for health care workers, as fecal matter can become particulate [128]. Frighteningly, the infective feces remain viable for up to 100 days [128]. Humans are considered the reservoir host of *R. prowazekii*, as infection is fatal to lice; the bacteria rapidly replicate by binary fission, leading to rupture of the midgut epithelial cells and leakage of the bloodmeal, creating a “red louse” appearance [128]. The initial infection is treatable with doxycycline, however, a relapse known as Brill-Zinsser disease is known to occur, even years after the original course of disease [129]. In its recrudescence epidemic typhus consists of bacteremia and may continue the transmission cycle, making the possibility of eradication extremely difficult [128]. This type of recrudescence has been proposed as an explanation for the inconsistent bacteremia associated with FBSF [33, 106].

1.3.2. *Rickettsia typhi*

Similar to *R. felis*, *Rickettsia typhi* is found worldwide, causes a near identical menagerie of clinical signs and disease severity, and is transmitted by fleas. While its chief vector is the oriental rat flea *Xenopsylla cheopis* [130], *R. typhi* is also found in the cat flea [131] and may coinfect fleas along with *R. felis* [132]. Moreover, *R. typhi* and *R. felis* are transmitted vertically and horizontally among fleas in a similar manner [17] and neither is detrimental to the flea [130]. Transmission of *R. typhi* to humans results in murine typhus (endemic typhus) and occurs through several routes: an infective arthropod bite from fleas infected for more than 21 days [133], contamination of infective flea feces into wounds (i.e. flea-feeding sites) [134], exposure of oronasal mucosa including via inhalation [89], and contact with conjunctiva [133]. Infected fleas will begin shedding infective feces after 10 days [130] and their feces will remain viably infective for 10 days [112]. Considering the existing similarities and their ability to coinfect, the possibility of horizontal gene transfer between *R. typhi* and *R. felis* that could result in increased pathogenicity is not beyond the imagination.

1.4. Summary

Rickettsia felis has been identified as an emerging disease marked with the potential to become the next global epidemic. Predominantly transmitted by the cat flea, *Ctenocephalides felis*, it is the causative agent of flea-borne spotted fever in people and is an underrecognized etiologic agent of fever in animals. Once infected, fleas shed *R. felis* in their feces. Transmission from infected arthropods to vertebrates is known to occur via infectious flea bites and strong evidence suggests transmission occurs via inoculation of wound sites with infective flea feces, as is the case with typhus group rickettsiae which also have the potential for oronasal transmission via infective feces. The possibility that *R. felis* might also be transmitted via ingestion is further

supported by evidence of *R. felis* in the feces of both humans and animals. Therefore, our current study hypothesized that *R. felis* from culture and from infective cat flea feces would be infective to mice via ingestion.

CHAPTER 2. INFECTION POTENTIAL OF *RICKETTSIA FELIS* VIA INGESTION

2.1. Introduction

Rickettsia felis is a gram negative, obligate intracellular bacteria (class Alphaproteobacteria, order Rickettsiales) of the transitional group (TRG) of rickettsiae that is present mostly within its primary vector and reservoir host, the cat flea (*Ctenocephalides felis*) [8, 25]. Transmission within arthropods occurs both vertically and horizontally, suggesting that vertebrates play a role in the life cycle of *R. felis* [28, 38]. Further evidence for its association with vertebrates includes seropositivity in both animals and humans [25, 42]. Case series of disease in vertebrates that detected only *R. felis* by means of polymerase chain reaction (PCR) and/or serology have led to its characterization as the etiologic agent of flea-borne spotted fever (FBSF) in humans as well as a poorly recognized cause of fever in animals [25, 76, 105]. One known transmission route to vertebrates is via the bite of infected fleas during feeding [26], however, closely related members of the typhus group (TG) of rickettsiae, namely *Rickettsia prowazekii* and *Rickettsia typhi*, are shed in feces and can subsequently be transmitted in wounds, mucus membranes, conjunctiva, and oronasally via inhalation [89, 128, 133]. Our lab previously investigated the transmission of *R. felis* via intradermal inoculation to simulate fecal transmission in cutaneous wounds such as flea-feeding sites. The unpublished results indicate that the route is viable. Furthermore, there is evidence to support vertebrates are exposed to *R. felis* via ingestion, as *R. felis* has been detected in both human and ape feces [114, 115]. This is possibly due to the grooming habits of mammals and the poor hygiene of young children – the human group most infected with FBSF in Africa [105]. In the current study it is hypothesized

that ingestion of *R. felis* from culture and from infected cat flea feces will result in the transmission of *R. felis* to mice.

2.2. Materials & Methods

2.2.1. Source and Strain of Bacteria, Fleas, and Mice

The experiments performed utilized *Rickettsia felis*, *Ctenocephalides felis*, and BALB/c mice. *Rickettsia felis* strain LSU (Louisiana State University, passage 3) was cultured in *Ixodes scapularis* embryonic cells (ISE6) with L15B complete medium (L15B, 10% ΔFBS, and 10% tryptase phosphate broth at a pH of 6.8-7.0) in an incubator set at 32°C and 5% CO₂ as originally described by Pornwiroon *et al* [48]. Each week a concentrated smear (Figure 2) was made and stained with Diff-Quik to assess *R. felis* growth [48]. Fleas used were newly emerged *Rickettsia*-uninfected cat fleas from Elward II (El-Labs, Soquel, CA). BALB/c mice were chosen, as previous work has described their ability to become infected with *R. felis* [135]. All mice were obtained from the Louisiana State University Division of Laboratory Animal Medicine for use as a murine model of mammalian disease; their gender was randomized, and they were juveniles with ages ranging from six to seven-week-old mice at the time of procurement.

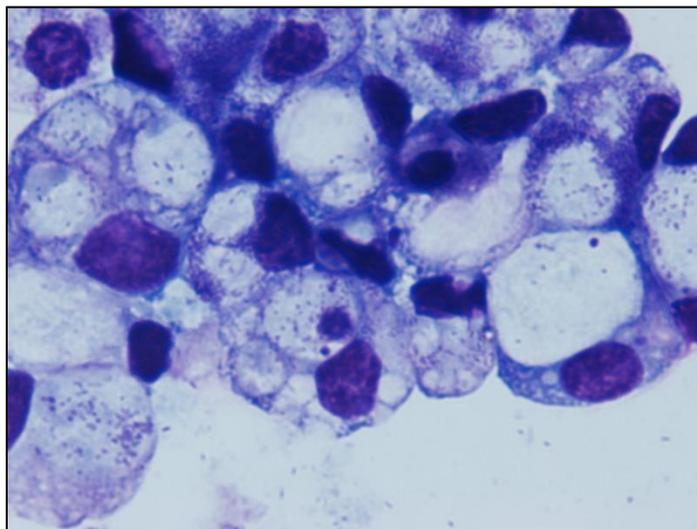


Figure 2. *Rickettsia felis* inside ISE6 cells. 1000x magnification oil-immersion, Diff-Quik stain.

2.2.2. Ethics Statement

This study was conducted in compliance with the Animal Welfare Act (9 CFR Ch. 1 Subpart C 2.31 (c) 1-8), the Guide for the care and use of Agriculture Animals in Agricultural Research and Training (Ch. 1), and the Public Health Service Policy on Human Care and Use of Laboratory Animals (Section IV. B. (1-8)). Additionally, all animal-related research was conducted with approval of the LSU Institutional Animal Care and Use Committee (IACUC; Protocol Number: 15-115).

2.2.3. Per Oral Administration of *Rickettsia felis* from Culture

For this experiment 15 test mice and three control mice were organized into three groups, each consisting of five test mice and one control mouse (Figure 3). Mice were caged according to gender and treatment status, keeping infected and control mice separated for the duration of the experiment. *R. felis* was cultured in ISE6 cells, enumerated with the backlight *BacLight* viability staining kit, and sucrose purified. Each test mouse received a solution containing 3.0×10^6 *R. felis* organisms suspended in sucrose phosphate glutamate (SPG) buffer while each control mouse received an equivalent volume of SPG buffer only. This number was based on a study that indicated 3.0×10^6 was the upper range of *R. felis* per milligram of flea feces using high passage *R. felis* (passage 8) [113]. Therefore, this study was designed to simulate ingestion of 1 mg of high passage *R. felis*-infected flea feces, aiming to recreate a natural exposure event wherein the dose of *R. felis* administered mimics the amount that may be ingested via grooming or poor hygiene in animals or humans, respectively. This experiment was performed in duplicate, independently, and with *R. felis* from different culture flasks during the second trial.

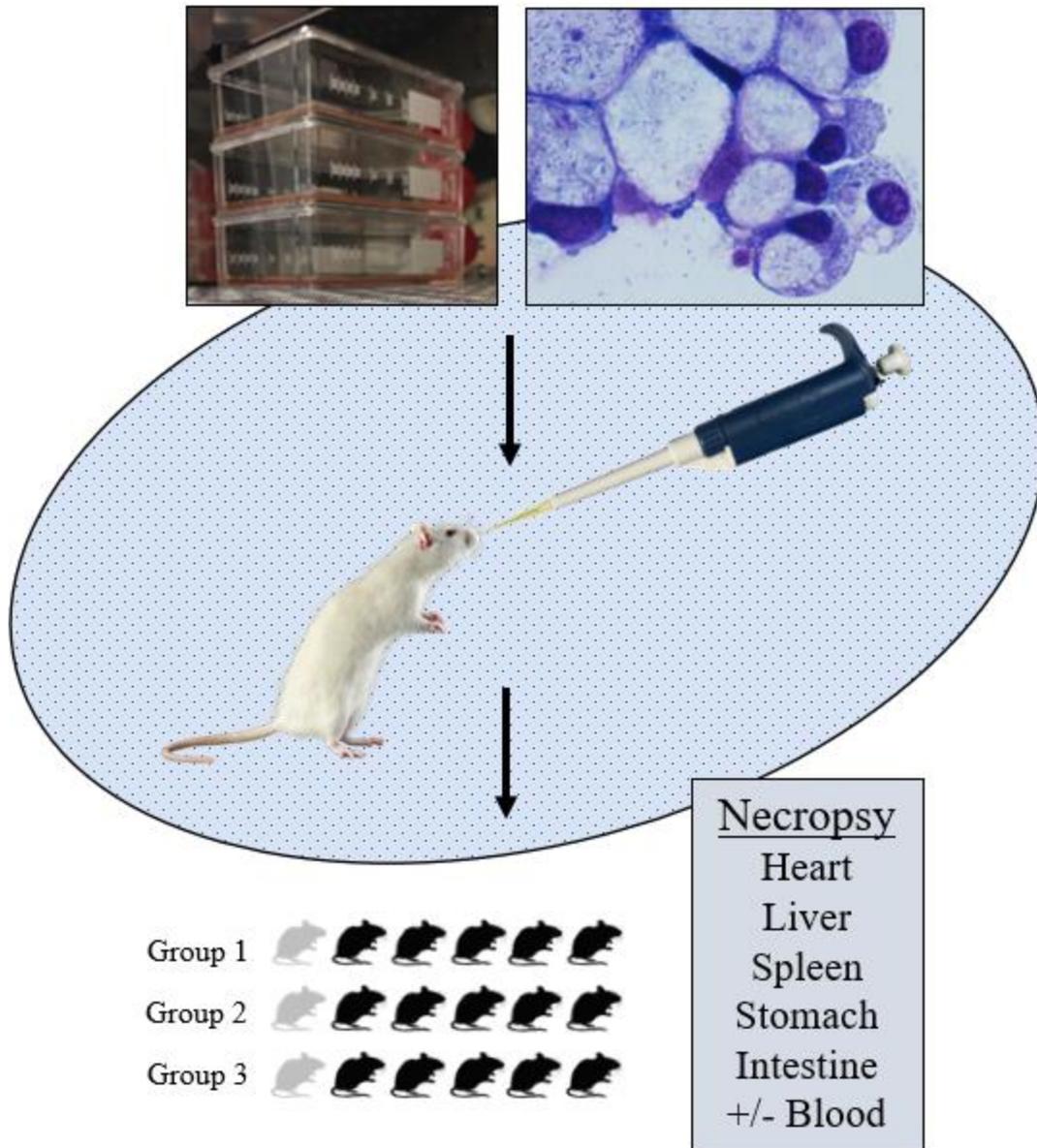


Figure 3. Per oral administration of *Rickettsia felis* from culture. *R. felis* was grown in ISE6 cells (culture flasks pictured top left, *R. felis* within ISE6 cells picture top right), quantified, sucrose purified, and then suspended in SPG buffer. Test mice were pipette-fed 3.0×10^6 *R. felis* organisms while control mice received an equivalent volume of SPG buffer only (central illustration). Mice were arranged into 3 test groups (bottom left), each with 1 control mouse (colored gray) and five test mice (colored black). Group 1 was sacrificed 1-day post ingestion, Group 2 at 7 DPI, and Group 3 at 14 DPI. A full necropsy was performed and heart, liver, spleen, stomach, and a jejunal segment were collected for gDNA extraction, RNA extraction, and histopathology. Blood was retrieved via intra-cardiac aspiration in 14 DPI mice.

2.2.4. Per Oral Administration of *Rickettsia felis* from Flea Feces

This experiment mirrored the first, consisting of 15 test mice and three control mice once again arranged into three groups, each with five test mice and one control mouse (Figure 4). Mice were caged by gender and treatment status, keeping infected and control mice separated for the duration of the experiment. *R. felis* was cultured in ISE6 cells, enumerated via the BacLight viability staining kit, purified, and then resuspended in 600 μ L of heat-inactivated bovine blood. This infectious bloodmeal was provided to a colony of approximately 200 *R. felis*-uninfected fleas that had been previously withheld a bloodmeal for 6 hours. The fleas were allowed to feed on the infectious bloodmeal for 48 hours before it was changed for an uninfected bloodmeal, which the fleas received for the rest of the experiment. A control colony of fleas was also maintained in order to procure uninfected flea feces.

Flea feces collection coincided with cage changing and was performed once weekly for the duration of the experiment. Using a dissecting microscope, cage debris was examined and the flea feces were carefully separated from flea eggs, larvae, dead fleas, and dried blood. A small portion of flea feces was weighed and its gDNA was extracted using a Qiagen DNeasy Tissue Kit (Qiagen, Germantown, MD) per the instructions for tissue extractions then eluted with 25 μ L of PCR-grade H₂O. The extracted gDNA was tested via quantitative polymerase chain reaction (qPCR) using primers for rickettsial *ompB* [136] and the *C. felis* 18S rRNA gene [79]. Subsequently, the density of the *R. felis* load was identified and then used to calculate the weight of feces required to achieve 3.0×10^6 *R. felis* organisms. The flea feces were then suspended in SPG buffer and the correct volume pipette-fed to each test mouse. Control mice received a solution prepared using the same weight of *R. felis*-free feces collected from the control colony of fleas. These feces were verified *R. felis*-negative via qPCR. As with the first experiment, this

experiment was performed in duplicate, independently, with *R. felis* from different culture flasks, and different flea colonies during the second trial.

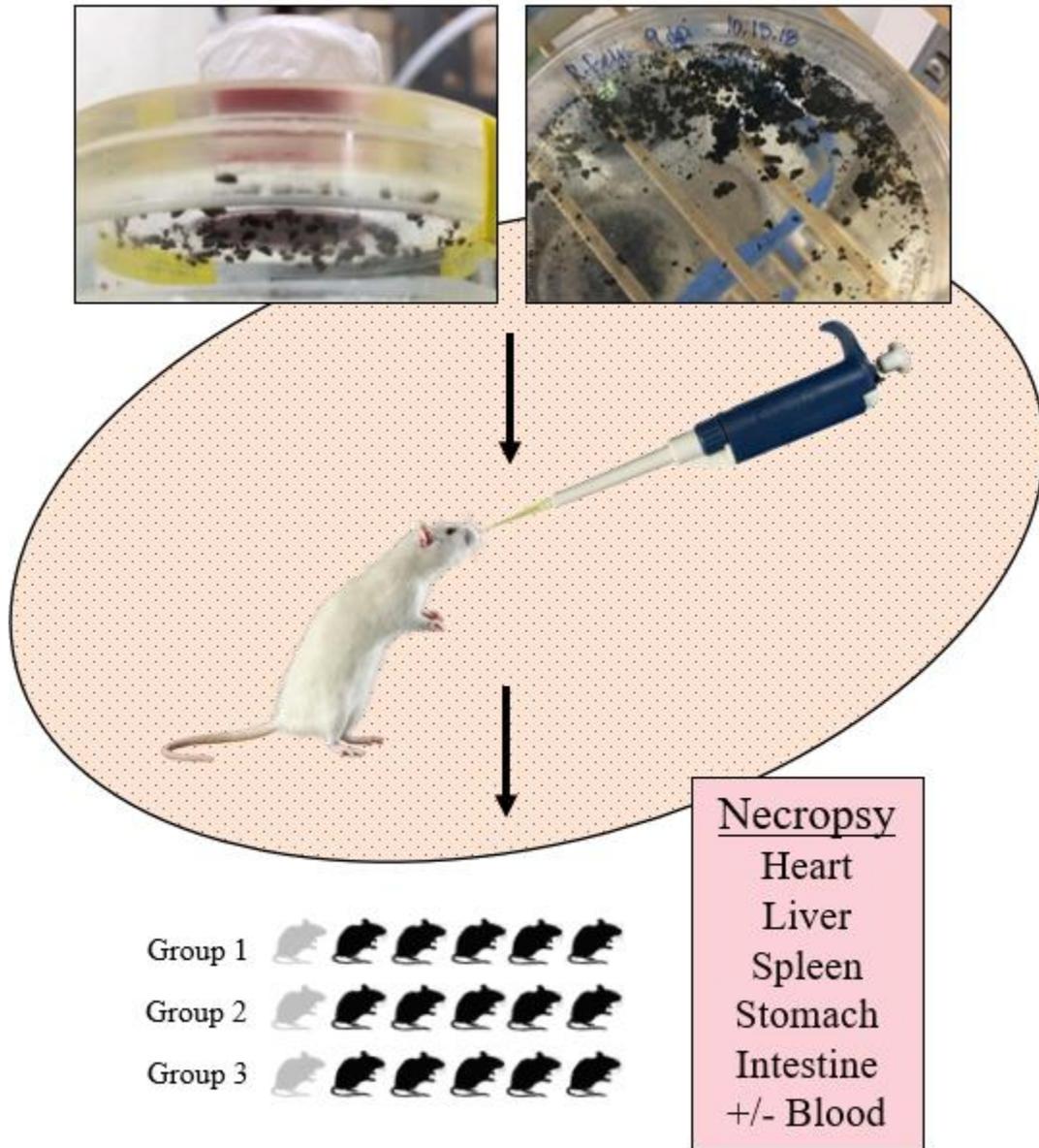


Figure 4. Per oral administration of *Rickettsia felis* from flea feces. Colonies of cat fleas were fed an infectious bovine bloodmeal (top left). Their feces were isolated (top right) and proven to be *R. felis* positive via qPCR. A flea feces dose containing 3.0×10^6 *R. felis* organisms was calculated and suspended in SPG buffer. Test mice were pipette-fed infective flea feces and control mice were fed the same weight of qPCR-proven *R. felis*-free flea feces in an equivalent volume of SPG buffer (central illustration). Mice were arranged in 3 groups and sacrificed as in the previous experiment. Tissues and blood were also collected in the same way.

While the amount of *R. felis* each test mouse received remained constant across each trial, due to an inability to standardize the density of *R. felis* fleas excrete in their feces, the total weight of flea feces varied between trials. The variation is largely based on the amount of an infectious bloodmeal the colony ingests.

2.2.5. Necropsy and Sample Collection

Mice were examined daily up until their sacrifice date for the duration of each experiment. Group 1 mice were sacrificed at 1-day post ingestion (DPI), Group 2 mice at 7 DPI, and Group 3 mice at 14 DPI. Mice were humanely euthanized individually, primarily via carbon dioxide chamber and secondarily via cervical spinal dislocation. Blood was collected from Group 3 mice via intra-cardiac aspiration, spun, and the serum removed. A full necropsy was performed on each mouse. Select organs were harvested, including heart, liver, spleen, stomach, and a jejunal segment, in that order to avoid gastrointestinal contamination. The organs were sectioned; one piece placed in a cassette and stored in 10% neutral buffered formalin, a second piece stored in a 1.7 mL Safe-Lock microcentrifuge tube for genomic DNA (gDNA) extraction, and a third piece stored in 1 mL of TRIzol for RNA extraction. The samples for gDNA extraction and serum samples were stored at -20 °C and the samples for RNA extraction were stored at -80 °C.

2.2.6. Detection of *Rickettsia felis* in Tissues

Initial detection of *R. felis* in heart, liver, spleen, stomach, and intestine samples was performed via qPCR. First, the mouse tissues were homogenized. This process involved the addition of two stainless steel beads, proteinase K, and buffer ATL to each of the 1.7 mL Safe-Lock microcentrifuge tubes. The samples were then run twice through the Tissue-Lyser (Qiagen, Valencia, CA) at 30 Hz for three minutes each cycle. Genomic DNA was then extracted per the

manufacturer's instructions using the Qiagen DNeasy Tissue Kit, eluting with 25 μ L PCR-grade H₂O. Tissue samples were processed in batches of 30, which included all samples from a single timepoint of one experiment (i.e. 1 DPI *R. felis* from culture, replicate 1) and an environmental control containing only extraction reagents was included in every batch of extractions. Likewise, all samples from a timepoint were run on a single qPCR plate, along with the environmental control from the extraction process, a negative control consisting only of PCR-grade H₂O and master mix, as well as two positive controls, one for *R. felis* and one for mouse. The qPCR was performed using a LightCycler 480 Real-Time PCR system (Roche), standard sets for both mice and *R. felis*, as well as primers for rickettsial *ompB* [136] and the mouse *cfp* [137]. Results were viewed as rickettsial copy numbers per sample and were considered positive if two of three replicates showed amplification before cycle 35, if negative and positive controls were correctly amplified, and if the standard curve was appropriate.

Select test samples, as well as their control sample counterparts, were chosen for histopathology and immunohistochemistry (IHC) based on qPCR results. Once formalin fixation was complete, these tissues were submitted for paraffin-embedding and were subsequently sectioned for hematoxylin and eosin (H&E) staining and IHC using polyclonal anti-*Rickettsia* antibodies diluted to 1/1000 as described previously by Grasperge *et al* [137]. All H&E and IHC slides were read and interpreted by an anatomic pathologist certified by the American College of Veterinary Pathologists.

2.2.7. Detection of *Rickettsia felis* in Serum

Based on initial tissue sample qPCR findings, the serum of one mouse was selected for qPCR detection of *R. felis* as well as an indirect immunofluorescence assay for detection of an antibody response to *R. felis*. Genomic DNA was extracted from the serum and an environmental

control consisting only of extraction reagents using a Qiagen DNeasy Tissue Kit (Qiagen, Germantown, MD) and the protocol for gDNA extraction from non-nucleated blood. The sample was eluted with 25 μ L of PCR-grade H₂O and qPCR was performed using primers for rickettsial *ompB* and mouse *cfp* on a LightCycler 480 Real-Time PCR system (Roche). Samples were considered positive if two of three replicates showed amplification before cycle 35.

The serum was also tested using an indirect immunofluorescence assay as described in previous studies [35]. *R. felis* from culture was fixed to a glass slide with 4% PFA and allowed to sit for 15 minutes. The slide was then washed three times five minutes apart with PBS. A blocking solution consisting of 3% BSA in PBS was applied and allowed to incubate for 1-hour at room temperature. Serum was diluted 1:100, applied to the slide, and allowed to sit at room temperature for one hour. Once again, the slide was washed three times five minutes apart with PBS. Finally, a secondary antibody (goat anti-mouse IgG conjugated to horseradish peroxidase diluted 1:1000 in blocking buffer) was applied and the slide was incubated at room temperature in the dark for 30 minutes. A mounting solution with added DAPI was then applied prior to cover-slipping. Samples were read using fluorescence microscopy (Leica).

2.2.8. Assessment of *Rickettsia felis* Viability

PCR-positive tissue samples stored in TRIzol were further processed using the Bio-Rad iScript™ cDNA Synthesis Kit for reverse transcription quantitative PCR (RT-qPCR) to test the viability of *R. felis* organisms detected. First, the tissue samples were homogenized as previously described for gDNA extraction from tissue samples. Next, 200 μ L of chloroform was added before vortexing and incubating the samples at room temperature for three minutes. The samples were then subjected to 15 minutes of centrifugation at 12,000 x *g* and 4°C. The aqueous phase of the samples was pipetted-off, transferred to a new microcentrifuge tube, and 500 μ L of

isopropanol was added. The samples were incubated overnight at -20°C for increased RNA yield. Once precipitated, the samples were centrifuged at 12,000 x g and 4°C for 10 minutes. The supernatant was pipetted-off, the pellet was washed with 1 mL of 75% ethanol, and the samples were vortexed and then centrifuged at 7,500 x g and 4°C for 5 minutes. The supernatant was discarded and the RNA was allowed to air-dry for 10 minutes before resuspending with 25 µL of pre-warmed PCR-grade H₂O. The samples were then incubated at 60°C for 15 minutes. The samples were then subjected to two cycles of DNase treatment, by combining with 5 µL 10x DNase buffer, 1 µL Turbo DNase, and 24 µL PCR-grade H₂O before incubating at 37°C for 30 minutes. An additional 5.77 µL 10x DNase buffer and 2 µL Turbo DNase were added before being incubated at 37°C for 30 minutes once again. The samples were then subjected to an RNA clean-up step as previously described [26]. A preliminary RT-qPCR was performed on all samples without the addition of iScript™ reverse transcriptase in order to establish the samples were free of DNA. Finally, the complementary DNA (cDNA) was generated from the samples with the addition of iScript™ reverse transcriptase as previously described [26], and RT-qPCR was performed on the samples, an environmental control from the RNA extraction process, a negative control consisting only of reagents and PCR-grade H₂O, a negative control lacking iScript reverse transcriptase, and *R. felis* and mouse positive controls using rickettsial *ompB* and mouse *cfp* on a LightCycler 480 Real-Time PCR system (Roche). Samples were considered positive if two of three replicates showed amplification before cycle 35.

2.3. Results

2.3.1. Per Oral Administration of *Rickettsia felis* from Culture

Two replicates of the experiment were performed. All mice made it to their intended sacrifice dates without developing clinical signs of disease. Moreover, gross lesions were not

appreciated during the necropsy of any mouse. Genomic DNA was successfully extracted from the heart, liver, spleen, stomach, and intestines of each mouse and qPCR was successfully performed on all samples (Table 1). Of the five mice that had detectable levels of *R. felis*, three of them had two organs with detectable levels. Furthermore, all mice with detectable *R. felis* in more than one organ had detectable *R. felis* in their spleen. No mice had detectable levels of *R. felis* in either their stomach or intestine. The serum of an individual mouse that had detectable levels of *R. felis* in its heart and spleen was tested and found to be negative.

Table 1. Detection of *R. felis* from culture via qPCR

Sample Type	Number of Test Mice Samples Positive for <i>R. felis</i>			
	1 DPI	7 DPI	14 DPI	Total
Heart	1/10 (10%)	0/10 (0%)	1/10 (10%)*	2/30 (7%)
Liver	0/10 (0%)	2/10 (20%)*	0/10 (0%)	2/30 (7%)
Spleen	1/10 (10%)	2/10 (20%)*	1/10 (10%)*	4/30 (13%)
Stomach	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/30 (0%)
Intestine	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/30 (0%)
Serum	—	—	0/1 (0%)	0/1 (0%)

Quantitative PCR results of test mice from both replicates of the *R. felis* from culture model of ingestion. Detectable levels of *R. felis* occurring before PCR cycle 35 were considered positive. Two mice had detectable *R. felis* in their hearts, two in their livers, and four in their spleens. No mice had detectable levels of *R. felis* in their stomach or intestine. Samples marked with (*) indicate they came from a mouse with more than one positive sample. Percentages shown parenthetically. All samples from the control mice of both replicates were negative for *R. felis*.

All tissue samples of the two test mice with the highest *R. felis* densities (data not shown) and presence of *R. felis* in more than one organ were submitted for histopathology (Figure 5). These mice included “Rf10.1” from the 7 DPI time-point with detectable *R. felis* in its

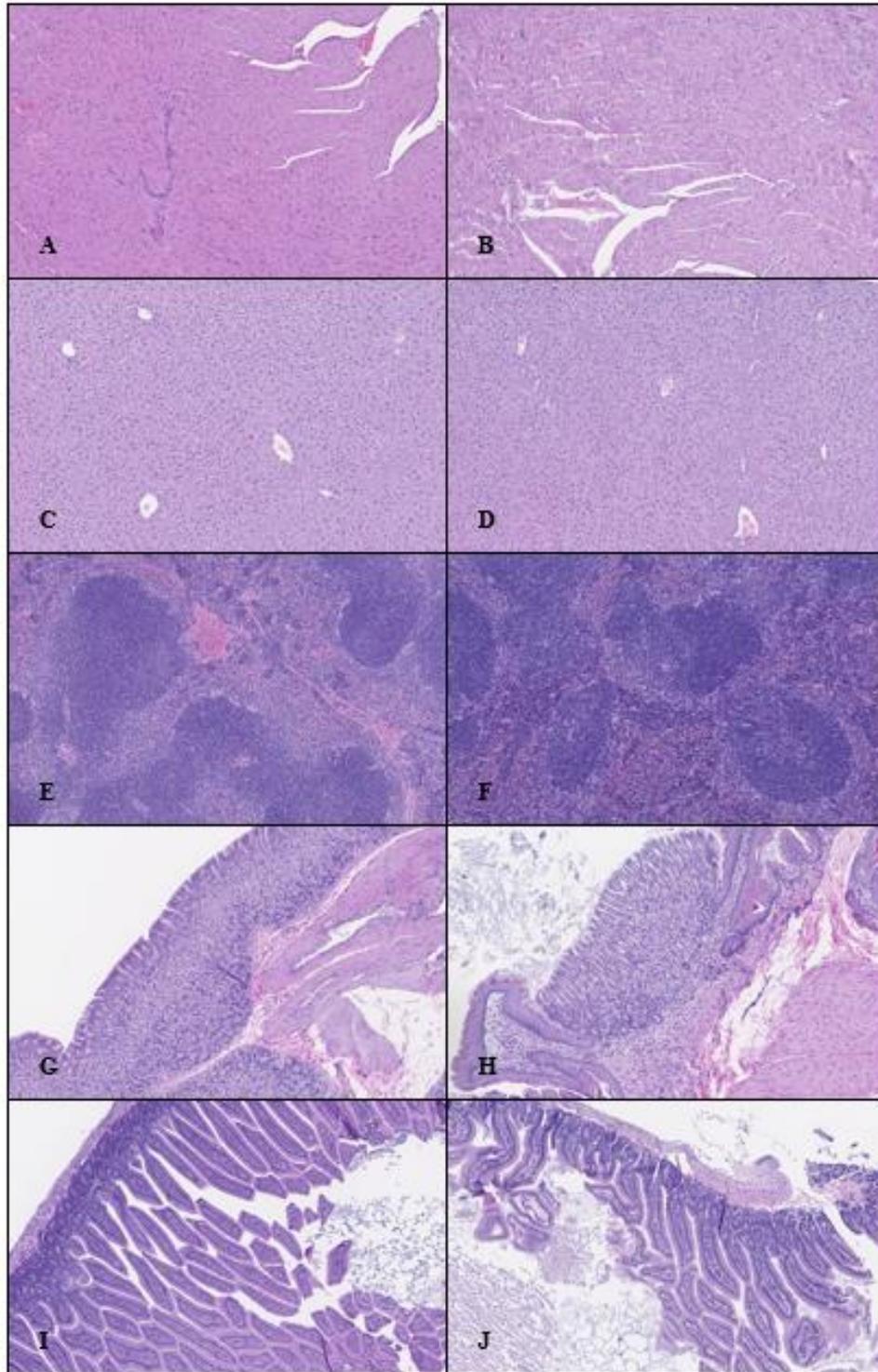


Figure 5. Histopathology of mouse samples post-ingestion of *R. felis* from culture. All images to the right are of test mouse Rf13.1 (sacrificed 14 DPI) and images to the left are from the associated control mouse (hematoxylin and eosin stain, 100x). **A, B.** Heart. **C, D.** Liver. **E, F.** Spleen. **G, H.** Stomach. **I, J** Intestine. No evidence of disease is appreciated in any organ. Rf10.1 had similar findings (histopathology not shown).

liver and spleen and “Rf13.1” from the 14 DPI time-point with detectable *R. felis* in its heart and spleen. All tissue samples from the control mice associated with these test mice were submitted for comparison. Histopathology performed by a board-certified veterinary anatomic pathologist concluded that no evidence of disease was present in any samples.

Additionally, the qPCR positive organs of Rf10.1 and Rf13.1 were sectioned for immunohistochemistry (IHC) with a polyclonal anti-*Rickettsia* antibody diluted 1:1000 as previously described [137]. Of the four test organs analyzed with IHC, the spleen of Rf10.1 had few positively staining bacteria interpreted as *R. felis* (Figure 6). No other samples were considered positive.

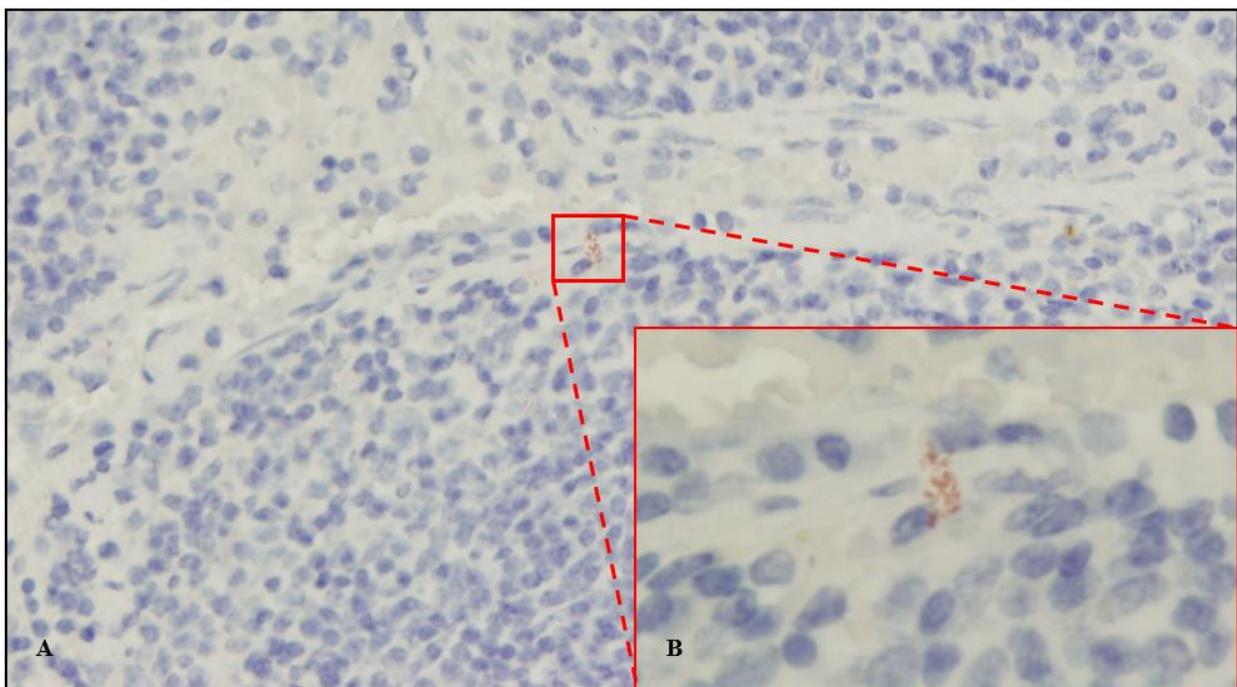


Figure 6. Anti-*Rickettsia* immunohistochemistry of mouse spleen post-ingestion of *R. felis* from culture. **A.** 400x photomicrograph of the spleen of the *R. felis* from culture 7DPI test mouse Rf10.1. *R. felis* organisms are observed both intracellularly and possibly in the extracellular space (red box). **B.** Inset with an expanded view of *R. felis* organisms in the red box. *R. felis* organisms are observed intracellularly and possibly also in the extracellular space.

An indirect immunofluorescence assay was performed on serum from Rf13.1 in order to determine if antibodies against *R. felis* were present. The serum was positive, as indicated by the fluorescent secondary antibody binding the mouse antibodies against *R. felis* which in turn bound a primary fixed layer of *R. felis* from culture (Figure 7).

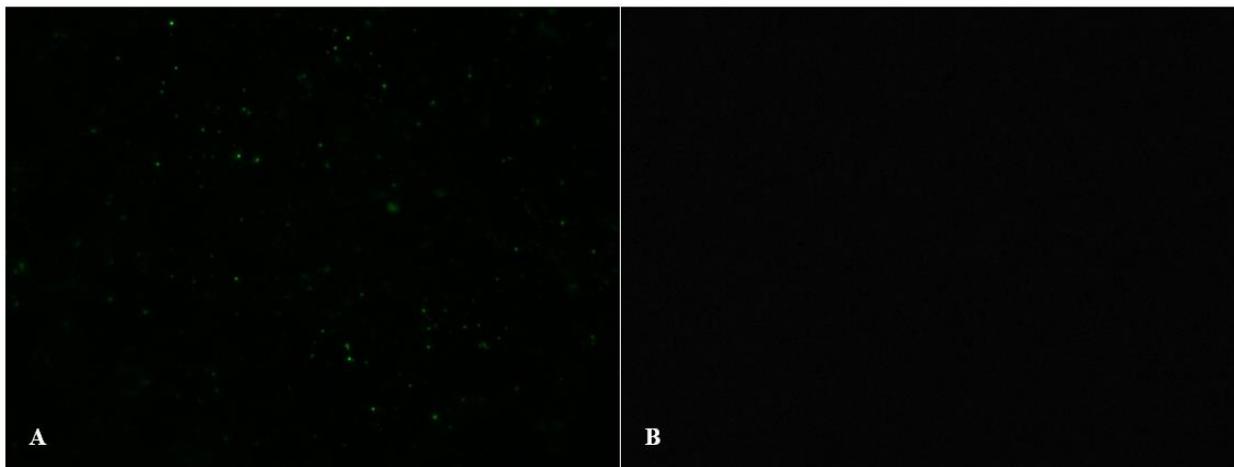


Figure 7. Indirect immunofluorescence assay of mouse serum post-ingestion of *R. felis* from culture. **A.** 1000x oil immersion photomicrograph of the test sample (serum from 14DPI *R. felis* from culture test mouse Rf13.1) demonstrates green fluorescence associated with binding of goat anti-mouse secondary antibody (dilution 1:1000) to mouse antibodies directed at *R. felis*. **B.** 1000x oil immersion photomicrograph of the negative control lacks immunofluorescence.

All tissue samples of Rf10.1 and Rf13.1, as well as the tissue samples of their associated control mice, were analyzed using RT-qPCR. The samples were initially processed without iscript[®] reverse transcriptase to ensure no background amplification of rickettsial *ompB* was present. Once processed with iscript[®] reverse transcriptase, no cDNA samples exhibited detectable levels of *R. felis*.

2.3.2. Per Oral Administration of *Rickettsia felis* from Flea Feces

As with the first experiment, two replicates of per oral administration of *R. felis* from flea feces were performed. Once again, all mice made it to their intended sacrifice dates without developing clinical signs of disease. Moreover, gross lesions were not appreciated during the necropsy of any mouse. Genomic DNA was successfully extracted from the heart, liver, spleen, stomach, and intestines of each mouse and qPCR was successfully performed on all samples (Table 2). As all samples were negative, no additional tests were performed.

Table 2. Detection of *R. felis* from flea feces via qPCR

Sample Type	Number of Test Mice Samples Positive for <i>R. felis</i>			
	1 DPI	7 DPI	14 DPI	Total
Heart	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/30 (0%)
Liver	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/30 (0%)
Spleen	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/30 (0%)
Stomach	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/30 (0%)
Intestine	0/10 (0%)	0/10 (0%)	0/10 (0%)	0/30 (0%)

Quantitative PCR results of test mice from both replicates of the *R. felis* from flea feces model of ingestion. No samples exhibited detectable levels of *R. felis* occurring before PCR cycle 35. The control mice associated with both replicates of this experiment were also negative for *R. felis*.

CHAPTER 3. DISCUSSION & FUTURE DIRECTIONS

Owing to its similarities with typhus group *Rickettsia*, *Rickettsia felis* has been identified as a pathogen that is potentially transmitted in flea feces by contact with mucus membranes. The supporting evidence for this route of infection includes the tropism of *R. felis* for flea midgut epithelial cells, Malpighian tubules, and rectal ampulla [40], proof that fleas shed viable *R. felis* organisms in their feces, and the discovery of *R. felis* in mammalian feces, suggesting alimentary passage [114, 115]. Theoretically, this route of transmission may also account for the predilection of young children in sub-Saharan Africa to contract *R. felis*-induced flea-borne spotted fever (FBSF) [105], due to frequent oral exposure. Along those lines, mammals such as cats and non-human primates with meticulous grooming habits would stand to have exceptionally increased risk for oral exposure to infective flea feces, potentially contributing to disease as a poorly characterized cause of fever.

Interestingly, several putative genes have been identified that could ameliorate the role of *R. felis* as a pathogen transmissible via ingestion. Chiefly, *R. felis* contains a hyaluronidase that has been shown to be highly similar to that of *Clostridium perfringens*, one of the most common causes of food poisoning largely due to the actions of its hyaluronidase on the gastrointestinal epithelial cells. Also known as a “spreading factor,” hyaluronidase is proposed as a means for degrading polysaccharides such as those holding mammalian tissues together. Additionally, *R. felis* contains an ortholog (Rf0371) to *RickA* of *Rickettsia conorii* which is tied to mobility via actin polymerization [46]. Best demonstrated by *Listeria monocytogenes*, actin polymerization is an effective means for entering cells by force subsequent to the creation of a posterior actin trail. Ogata *et al* were [46] able to visualize *R. felis* actin filaments and differentiate them from host

cell stress fibers with immunofluorescence microscopy, providing further support for functional actin mobility in *R. felis*.

In light of this mounting support, we hypothesized that *R. felis* would be infective to vertebrates via ingestion. Specifically, we designed sister experiments to test this theory with *R. felis* from culture as well as *R. felis* in cat flea feces. We utilized BALB/c mice as a model of vertebrate disease in order to compare results with a previous transmission study investigating the infectivity of *R. felis*-laden cat flea feces via dermal inoculation [113]. Likewise, our infectious dose of 3.0×10^6 *R. felis* organisms was chosen to represent the upper range of *R. felis*-burden of 1 milligram of cat flea feces [113], approximating the amount that a small child or other small mammal could be expected to ingest through indiscriminate oral exposure or grooming behaviors.

Our results indicate that *R. felis* is capable of infecting mice via ingestion, as determined by positive qPCR findings in the *R. felis* from culture experiment. Specifically, 5 out of 30 test mice orally administered *R. felis* from culture had rickettsial DNA at sites distant from the gastrointestinal tract, indicating an infection rate of 17%. Of the infected mice, we recovered rickettsial *ompB* in 80% of spleens (4/5 mice) and 40% of hearts and livers (2/5 mice each). Moreover, the majority of mice that were infected exhibited more than one organ with detectable levels of *R. felis* (3/5 mice). Furthermore, *R. felis* detected via qPCR was additionally corroborated by the positive staining of low numbers of *R. felis* organisms with anti-*Rickettsia* immunohistochemistry in the spleen of an individual mouse. While detection of *R. felis* in mammalian viscera has been previously demonstrated [26, 95, 138], the ability of ingested *R. felis* to disseminate to distant organs is a novel finding with broad implications for how *R. felis* is potentially transmitted to, among, and within vertebrate hosts.

Transmission of *R. felis* to vertebrate hosts has been established through infectious cat flea bites [26] and intradermal inoculation of infective flea feces [113]. Here we have been able to demonstrate alimentary transmission of *R. felis* from culture, though our attempts to transmit *R. felis* to mice via ingestion of infective cat flea feces failed. While no conclusions may be drawn regarding the infectivity of *R. felis* in cat flea feces via ingestion in mice, we can speculate on the ways in which mammals might come to ingest *R. felis* outside of cat flea feces. One possible route would be via ingestion of whole cat fleas – or other arthropods – infected with *R. felis*. This model of transmission was explored with negative results by Foil *et al* [121] as a means for transmission of *Bartonella henselae* in cats, though to the author’s knowledge this route has yet to be explored for *R. felis*. Transmission of *R. felis* may also occur via ingestion of cutaneous *R. felis*, as Mediannikov *et al* [108] demonstrated that *R. felis* may be ubiquitous in environments of high prevalence, such as parts of Senegal. Specifically, cotton swabs of non-diseased skin of Senegalese people revealed detectable levels of *R. felis* via qPCR [108]. Thus, through routine grooming or indiscriminate oral habits, animals and young children might come in contact with free *R. felis* organisms; whether or not these organisms are viable remains to be seen.

The possibility of *R. felis* transmission directly between vertebrates has been poorly explored and not yet proven, however, if transmission of ingested material can result in infection, additional avenues of exposure arise. One route might be the ingestion of infectious feces of other vertebrates. Some species, such as dogs, regularly exhibit coprophagia behavior, and subsequently might become infected via a fecal-oral route. Though *R. felis* has been shown to be viable in flea feces, viability of *R. felis* in vertebrate feces has yet to be established. Another potential route of transmission between vertebrates is via ingestion of other vertebrate organisms

infected with *R. felis*, as suggested by De Nys *et al* [139] regarding the presence of *R. felis* in the feces of non-human primates. This route would be especially applicable to cats due to their predatory nature and is further supported by evidence that many species of small mammals can be infected with *R. felis* [73, 74, 77, 138].

The current study also raises questions regarding the dissemination of *R. felis* within vertebrate hosts. Our results suggest that *R. felis* was not only able to survive the extreme acidity of the stomach, it was able to successfully contend with several innate barriers of the intestines, including mucus, anti-microbial peptides, and commensal bacteria well-adapted to monopolizing available space and resources [140]. The mucus present within the gastrointestinal tract variably consists of two specialized layers: a thin, easily-removed top layer present throughout the gastrointestinal tract and a dense bottom layer firmly adhered to the epithelial cells that is absent only in the small intestines [141]. One means of breaking through the mucus is via glycosidases, enzymes necessary for the degradation of mucin oligosaccharides [140], which have not been attributed to *R. felis* at this time. Therefore, unless the single mucin layer of the small intestine is removed through pre-existing disease, it is likely that *R. felis* requires an alternative route to infection. One option would be the microfold cells (M-cells) located throughout the gastrointestinal tract in mucosal-associated lymphoid tissues (MALT) and in the Peyer's patches of the small intestine. As these cells are uniquely uncovered by mucus at all times, they may provide a consistent surface by which *R. felis* might attach through the actions of its *sca* genes, including *ompB*. Furthermore, the M-cells might provide critical access to cells of the immune system, chiefly macrophages, for which *R. felis* has known tropism [142]. Subsequently, as the infected macrophages circulate, *R. felis* might be able to disseminate to distant sites, such as the spleen, liver, or heart. Alternatively, *R. felis* has also demonstrated tropism for endothelial cells

[142]. Therefore, if the gastrointestinal tract has pre-existing ulcerative disease, *R. felis* might gain easy access to these cells and move cell-to-cell via actin polymerization in a manner similar to *Listeria monocytogenes*, or gain entry to the bloodstream for metastatic spread, again leading to *R. felis* presence in distant organs. Lastly, *R. felis* might employ hyaluronidase to burrow its way to endothelial cells or macrophages, a process that may even occur in the oral cavity or esophagus.

While our study demonstrates the ability of *R. felis* to infect mice via ingestion, it notably does so without inducing an inflammatory response. All sections of test mice tissue positive for *R. felis* via qPCR lack overt histopathologic changes as compared to control mice sections. Furthermore, the splenic tissue positive via immunohistochemistry (IHC) for *R. felis* demonstrates a complete lack of inflammation at the site of the bacteria. This finding suggests that *R. felis* is capable of effectively evading the immune system, a trait shared with other obligate intracellular bacteria. Considering its low numbers despite a lack of immune pressure, it is plausible that *R. felis* bides its time and replicates slowly in infected mammalian cells, potentially further avoiding notice by the host immune system. This would explain the relatively low number of organisms detected by all methods in infected mice throughout all time-points. Additionally, this would explain the negative RT-qPCR results from tissues that were positive for *R. felis* DNA via qPCR, as the low total number of organisms might preclude detection. Furthermore, even though RT-qPCR was negative, the *R. felis* observed are thought to be viable considering their transit from the gastrointestinal tract to a distant site of infection. This is supported by the finding that *R. felis* from IHC were observed in groups, suggesting some replication did occur.

Although the infected test mice failed to mount a cell-mediated immune response, we demonstrated the presence of a humoral response in the individual infected mouse we tested via an indirect immunofluorescence assay. This finding is in line with many earlier studies that have documented antibodies to *R. felis* in vertebrates [42, 85, 96, 97]. Humoral responses are ineffective at eliminating intracellular bacteria, in contrast to cell-mediated responses that deploy cytotoxic T-lymphocytes. Interestingly, some bacteria are capable of modulating the host immune system in order to direct the host towards an ineffective immune response, thus increasing odds of survival [143]. While it is unknown at this time whether *R. felis* is capable of this, it would help to explain the observed lack of a cell-mediated immune response as well as the inconsistent presence of humoral responses observed in the literature [33, 106].

Further studies are needed to better characterize all aspects of transmission of *R. felis* to vertebrates via ingestion. Here we tested a single dose and strain of *R. felis* in a single strain of mice, leaving much to be elucidated. While we were unsuccessful at transmitting *R. felis* to mice via ingestion of infective flea feces, the dose used is still relatively low depending on how much infective flea feces is consumed. Our model is based on consumption of 1 mg of flea feces with high passage *R. felis*, though it is conceivable that both animals and young children could consume more flea feces by weight or that the *R. felis* may be of a lower passage. Furthermore, mammalian feces – particularly from animals and people with documented *R. felis* infection – should continue to be inspected for *R. felis*, not only via qPCR but also with RT-qPCR to determine *R. felis* viability. If such feces are found to contain viable *R. felis*, increased measures protecting both animals and people from exposure could then be employed. Additionally, the pathogenesis of *R. felis* via ingestion requires further investigation. While we have provided

several possibilities based on the known genes of *R. felis*, the function of these genes remains putative in *in vivo* mammalian cells and must be further characterized.

It is important to note the many variables at play with disease transmission, including rickettsial strain, type of arthropod vector, type of mammalian target, and route of infection. Each of these factors may affect the outcome of transmission, whether or not disease ensues, and the severity of such disease. Considering this complexity, it is not surprising that Koch's postulates have gone unfulfilled. Our study identified that organs infected with *R. felis* exhibit a low burden up to 14 DPI, therefore highly efficient methods of recovering these bacteria, potentially incorporating a host cell lysis step and separation via flow cytometry, might improve chances for culturing *R. felis* from infected animals.

In conclusion, the present study has demonstrated that ingestion of *R. felis* serves as a viable route of transmission and subsequent infection in mice. Additionally, this study has provided evidence that *R. felis* is capable of disseminating to distant organs from the starting point of the gastrointestinal tract and that it can do so without inducing inflammation. We have also provided further evidence of a humoral response post-infection with *R. felis*. Our findings have increased the plausibility of several additional avenues of transmission, including transmission between vertebrates via ingestion of infective vertebrate tissues or infective vertebrate feces. Moreover, we have posited theories detailing how *R. felis* is able to escape the gastrointestinal tract and metastasize. All considered, this study has expanded the current understanding of *R. felis* to include transmission via ingestion, opening many avenues for further investigation.

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VITA

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