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Graduate School

2009

Arthropod and vertebrate determinants for horizontal transmission of Rickettsia felis

Kathryn Elizabeth Reif
Louisiana State University and Agricultural and Mechanical College, katie1826@yahoo.com

ARTHROPOD AND VERTEBRATE DETERMINANTS FOR HORIZONTAL
TRANSMISSION OF *RICKETTSIA FELIS*

A Dissertation

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
Doctor of Philosophy

In
The Interdepartmental Program in
Veterinary Medical Sciences
Through the Department of Pathobiological Sciences

by
Kathryn Elizabeth Reif
B.A., Ohio Wesleyan University, 2004
MSPH, Tulane University, 2005
May 2009

ACKNOWLEDGEMENTS

I would like to dedicate this dissertation to my parents, Luke and Susan Reif, who have always believed that outside of love, an education is one of the greatest gifts they could give their children. I would also like to thank my other family members, especially my brother and sister, Nick and Sarah, my grandparents, and my Aunt Carol, who were always ready with an open ear to hear what I was working on, even if they thought it was strange that I spent a significant portion of the last four years 'playing' with fleas.

I don't know if I would have ever pictured this career for myself if it wasn't for my first mentor, Stephanie Levi.   After giving seminar in one of my high school biology classes, I asked Stephanie, a research associate in the Medical School at Case Western Reserve University, if I could work on a science fair project with her. She immediately agreed and it was her excitement that first sparked my interest towards a career field I hadn't considered. Since then, I have been fortunate to work with several people that also were as passionate about research as Stephanie, including my undergraduate mentors Dr. Ramon Carreno and Dr. Tuhela-Reuning, who took me to my first scientific meetings and with whom I published my first papers. In my Master's program at Tulane University I met Dr. Mark James who suggested that I consider applying for Ph.D. position in the Pathobiological Science Department at Louisiana State University. I listened to his advice and the following fall was enrolled and found a new mentor, Dr. Kevin Macaluso. I am indebted to Dr. Macaluso, not only for the training his laboratory has provided me, but more importantly, for teaching me how to be an independent researcher. He taught me that to be successful, you must not only do good work in the lab, but you must also publish papers, write grants, and network and present at meetings. With the strong foundation he provided, I feel that I have been given the tools I need to continue to have a successful and productive career.

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ABSTRACT

Rickettsia felis is a gram-negative bacterium predominantly described in the cat flea, *Ctenocephalides felis*. Since first described in 1990 in a commercial cat flea colony in the United States, *R. felis* has been detected in numerous arthropod species in 28 countries around the world. Additionally, as the etiologic agent of flea-borne rickettsiosis, *R. felis* can cause disease in humans, with patients presenting with clinical symptoms typical of rickettsial diseases including: fever, headache, and myalgia. Transmission of *R. felis* within flea colonies is predominantly via vertical (transovarial and transstadial) transmission and mechanisms of horizontal transmission are undescribed. Studies are needed to describe both arthropod and vertebrate determinants of *R. felis* horizontal transmission. Here we describe the development of both arthropod and vertebrate models of *R. felis* infection and use the tools of molecular biology to characterize *R. felis* infection in both models. We first characterized *R. felis*-infection in a naturally *R. felis*-infected cat flea colony and observed that the prevalence of *R. felis*-infection within a cat flea colony is dynamic with an inverse relationship between *R. felis*-infection density and prevalence of *R. felis*-infection in the colony. Also, over the flea lifespan, *R. felis* infection remains steady with little fluctuation during the onset of flea bloodmeal acquisition and oogenesis. After characterizing *R. felis* replication in naturally infected fleas, we developed a biological assay to infect naïve fleas. This is the first demonstration of oral acquisition and persistent *R. felis*-infection of fleas fed an *R. felis*-infected bloodmeal. Lastly, we describe the initial results of a murine model of *R. felis* infection. In this model, *R. felis* efficiently disseminated in the mouse and is detectable in several tissues including the spleen and liver for up to 14-days post-inoculation. Elucidation of both arthropod and vertebrate determinant for *R. felis* transmission is necessary to understand the ecology of *R. felis* in nature.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1. General Overview of the Genus R*ickettsia*

Rickettsia (*Rickettsiales*: *Rickettsiaceae*) are small (0.3-0.5 μm in diameter and 0.8-2 μm

in length) bacilli-shaped, gram negative, obligate intracellular bacteria with no flagella (Raoult

and Roux 1997, Azad and Beard 1998, Perlman et al. 2006). The family *Rickettsiaceae* includes

two major genera of bacteria responsible for a number of zoonoses: *Orientia* (e.g.

O. tsutsugamushi) and *Rickettsia* (e.g. *R. rickettsii*) (Figure 1.1) (Boone et al. 2001). Species

within the genus *Rickettsia* are classically divided into three groups: (1) the spotted fever group

(SFG), (2) the typhus group (TG) and (3) the ancestral group (AG) (Boone et al. 2001).

Placement of *Rickettsia spp*. into various groups was originally based upon arthropod vector and

disease clinical presentation. The SFG-rickettsiae primarily utilize acarine vectors such as ticks

and mites, while the TG primarily utilizes insect vectors such as lice and fleas. Differentiation of

species within these two groups is also based upon intracellular positions, optimal growth

temperatures, percent G+C DNA contents, clinical features, epidemiology, and antigenic

characteristics (Weiss et al. 1984, Raoult and Roux 1997). A list of species from the genus

Rickettsia, along with their associated arthropod vector(s), geographic distribution, mode of

transmission, and associated disease, are provided in Table 1.1.

Recently, a new organization of the genus *Rickettsia* was proposed by arranging the

genus into four groups: ancestral group-AG (e.g. *R. bellii*), typhus group-TG (*R. typhi*),

transitional group-TRG (e.g. *R. felis*) and spotted fever group-SFG (e.g. *R. rickettsii*) (Gillespie

et al. 2007). This newly proposed organization of rickettsial species was heavily based on

comparison of major conserved gene sequences (e.g. *ompB*, *gltA*) within the genus (Figure 2.1).

Diseases caused by species in the genus *Rickettsia* are referred to generally as

ORDER	Rickettsiales					
FAMILY	Rickettsiaceae		Anaplasmataceae			
GENUS	Rickettsia	Orientia	Anaplasma	Ehrlichia	Wolbachia	Neorickettsia
SPECIES	R. typhi	O. tsutsugamushi	A. bovis	E. canis	W. pipientis	N. risticii
	R. prowazekii		A. marginale	E. chaffeensis	W. persica	N. sennetsu
	R. canadensis		A. phagocytophilum	E. ewingii	W. melophagi	N. helminthaeca
	R. bellii		"A. platys"	E. muris		
	R. australis			E. ruminantium		
	R. akari					
	R. felis					
	R. helvetica					
	R. massiliae					
	R. montanensis					
	R. rhipicephali					
	R. aeschlimannii					
	R. japonica					
	R. sibirica					
	R. parkeri					
	R. conorii					
	R. africae					
	R. rickettsii					
	R. slovaca					
	R. honei					

Figure 1.1: Taxonomic position of the Genus *Rickettsia*. The order Rickettsiales is divided into two families: *Rickettsiaceae* and *Anaplasmataceae*. Both genera of bacteria within the family *Rickettsiaceae*, *Rickettsia* and *Orientia*, contain species known to cause human disease.

Table 1.1: Members of the genus *Rickettsia*. A partial list of species of the genus *Rickettsia*, along with their associated disease, primary arthropod vector, geographic distribution, and mode of transmission.

Agent	Disease	Arthropod Vector	Geographic Distribuition	Mode of Transmission
R. rickettsii	Rocky Mountain spotted fever	*Dermacentor variabilis*; *D. andersoni*	United States	Tick bite
R. massiliae	n/a	*Rhipicephalus sanguineus, Rh.turanicus, Rh. muhsamae, Rh. lunulatus, Rh. sulcatus*	Italy	Tick bite
R. akari	Rickettsialpox	*Liponyssoides sanguineus*	Worldwide	Mite bite
R. africae	African tick-bite fever	*Amblyomma hebraeum, Am. variegatum*	Southern Africa	Tick bite
R. felis	Flea-bourne rickettsiosis	*Ctenocephalides felis*	Worldwide	not known
R. conori	Mediterranean spotted fever; Astrakhan fever, Israeli spotted fever; Indian tick typhus	*Rh. sanguineus, Rh. pumilio*	Asia,Europe, Africa	Tick bite
R. australis	Queensland tick typhus	*Ixodes holocyclus, I. tasmani*	Australia	Tick bite
R. parkerii		*Am. maculatum. Am. americanum, Am. triste*	South America	Tick bite
R. honei	Flinders Island spotted fever	*Aponomma hydrosauri, Am. cajennense, I. granulatus*	Australia	Tick bite
Thai tick typhus rickettsia TT-118	Thai tick typhus	*Ixodes* or *Rhipicephalus spp*.	Thailand	Tick bite
R. aeschlimannii	n/a	*Hyalomma marginatum marginatum, H. m. rufipes, Rh. Appendiculatus*	Tunisia, Morocco	Tick bite
Rickettsia siberica	North Asian tick typhus	*Dermacentor nuttalli*	Russia	Tick bite
Rickettsia typhi	Murine (endemic) typhus	*Xenopsylla cheopis; Ctenocephalides felis*	Worldwide	Flea bite
Rickettsia prowazekii	Epidemic typhus; Louse-borne typhus	*Pediculus humanus*; *Neohaematopinus sciuropteri*		Infected louse feces
	Brill-Zinsser diseas	none		Recrudescent infection

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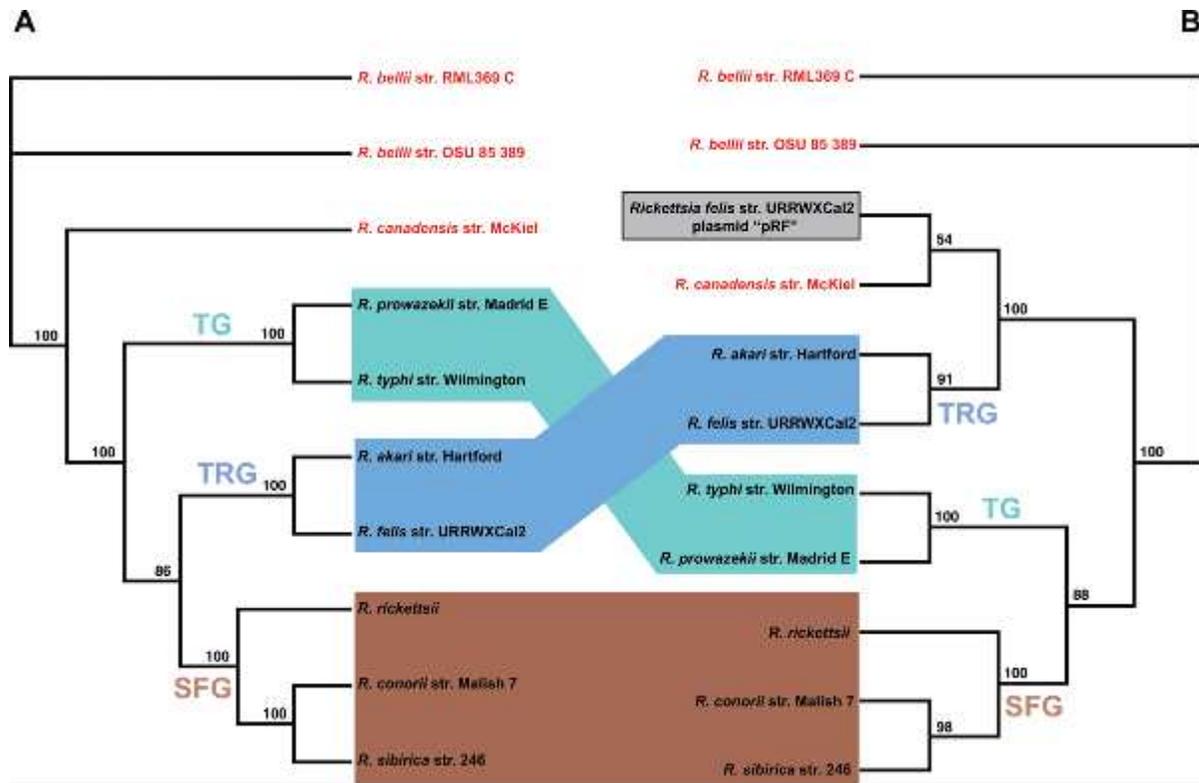
Figure 1.2: Reorganization of the genus *Rickettsia* (Gillespie et al. 2007). Depiction of the reorganization of the genus *Rickettsia* estimated from chromosomal proteins (A) and from the chromosome and plasmids of *R. felis* (B). A=ancestral group; TG= typhus group; TRG= transitional group; and SFG = spotted fever group. Figure from Gillespie et al. 2007.

"rickettsioses". As rickettsioses are zoonoses, their geographic distribution is limited by the geographic range of their vector species (Schriefer et al. 1994a). Factors that may support a zoonotic and vector-borne relationship may include: (a) characteristics of vertebrate reservoirs; (b) arthropod vectors required for primary maintenance; (c) human demographics within urban environment; and, (d) high human population density (Comer et al. 2001). Transmission of pathogenic bacteria to mammalian hosts/reservoirs often utilizes blood-feeding arthropod vectors such as ticks, fleas, and mites (Azad et al. 1992). Although a number of bacteria are associated with blood-feeding arthropods, members of the genus *Rickettsia* are recognized for their unique relationship with the vector.

Many rickettsial species are maintained in nature by vertical (transstadial and transovarial) transmission within their vector species. The epidemiology of most *Rickettsia* spp., and other vector-borne bacteria that are primarily maintained by transovarial and transtadial transmission, is largely determined by the geographic range of their vector species (Burgdorfer 1963, Farhang-Azad et al. 1985). Outbreaks of rickettsioses are highly focal in nature due to dependence on the vector. Bacterial species that are primarily maintained vertically within the vector often employ the vector species as a reservoir host as well (Comer et al. 2001). For example, *R. rickettsii*, *R. akari*, and *R. typhi*, are maintained by vertical transmission within *Dermacentor andersoni* (tick), *Liponyssoides sanguineus* (mite) and *Xenopsylla cheopis* (flea), respectively, all arthropods that are competent horizontal transmission vectors as well.

Rickettsioses generally cause acute febrile diseases frequently accompanied by rash, myalgia, and headache; but may also include a number of other clinical manifestations specific to the infecting rickettsial species (Zavala-Velazquez et al. 2002). Serologic-based methods have traditionally been used to diagnose rickettsioses, however, due to the high degree of antibody cross-reactivity between rickettsial species, specific diagnosis is not always possible. More

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recently, molecular diagnostic methods, because of their increased specificity and sensitivity, are being used to diagnose rickettsioses. Specific methods used to identify rickettsial infections include: direct or indirect immunofluorescence; enzyme-linked immunosorbent assays (ELISAs) with monoclonal or polyclonal antibodies; recovery of agents from vectors by culture in embryonated eggs or tissue culture cells; experimental infections in laboratory animals; polymerase chain reaction (PCR) alone or with dot hybridization; and, restriction fragment-length polymorphism analysis (RFLP). Of the above, methods involving PCR that employ specific primers, are the most sensitive and specific of these assays (Webb et al. 1990).

Because of the non-specific clinical symptoms common with rickettsial infections, many cases are misdiagnosed or go undiagnosed. If diagnosed, most rickettsial diseases can be effectively treated with antibiotics including, doxycycline, rifampin, and fluoroquinolones (Rolain et al. 1998, Rolain et al. 2002). Successful measures to control and prevent rickettsial diseases employ strategies to control or limit exposure to their arthropods vector.

1.2. Initial Characterization of *Rickettsia felis*

Initially discovered in a colony of cat fleas (*Ctenocephalides felis felis*) in 1990 by electron microscopy, *R. felis* was originally named ELB agent after the Elward Laboratory cat flea colony (EL Laboratory, Soquel, CA) (Adams et al. 1990). In 1996, the ELB agent was proposed to be a distinct rickettsial species, and the name "*Rickettsia felis*" was proposed (Higgins et al. 1996). Characterization and molecular analysis of *Rickettsia*-specific genes (e.g. *gltA*, 17-kDa protein gene, *ompB* etc.) aided in validating *R. felis* as a distinct rickettsial species (Azad et al. 1992, Radulovic et al. 1995b, Higgins et al. 1996, Bouyer et al. 2001). Although initial attempts to produce a sustained culture of either the ELB or the LSU strains of *R. felis* failed (Radulovic et al. 1995b, Bouyer et al. 2001), later attempts to isolate and propagate *R. felis* from cat fleas maintained by Flea Data, Inc. (Freeville, NY) proved successful (Raoult et al.

2001). The Flea Data, Inc. isolate was designated strain Marseille-URRWXCal$_2$ (also reported

as strain California 2 (Ogata et al. 2005)) and is the current type strain for *R. felis* (LaScola et al.

2002).

Unlike the majority of rickettsial species that were characterized by serologic means,

R. felis was originally characterized and its taxonomic position determined by molecular means

(PCR amplification and comparison of genus-specific genes) (Azad et al. 1992). The initial

placement of *R. felis* into the SFG was complicated because the *R. felis* 16S rDNA gene

sequence, along with several other gene sequences, resembled SFG *Rickettsia* (Roux and Raoult

1995, Higgins et al. 1996). However, biologically and clinically, *R. felis* resembled TG

Rickettsia including in: choice of vector species; cross-reactivity with *R. typhi* sera; cell lysis and

plaque formation; and a clinical presentation more similar to murine typhus (Schriefer et al.

1994a). Attempted amplification of the rickettsial outer membrane protein A (*ompA*) gene, a

gene common to all SFG rickettsial species, from *R. felis* was also unsuccessful (Azad et al.

1992). Because *R. felis* more closely resembled TG *Rickettsia* in many aspects of its biology,

several specific studies were conducted to confirm *R. felis* as a separate rickettsial species and

not a strain of *R. typhi* including: (1) propagation of the organism in cell culture to study; (2)

growth parameters; (3) cytopathology; (4) characterization by SDS-PAGE and immunoassays;

and, (5) sequenced gene comparisons to other rickettsial species (Higgins et al. 1996). Also,

studies utilizing PCR amplification and RFLP analysis confirmed unique *R. felis* 17-kDa gene

sequence and digestion patterns compared to *R. rickettsii, R. conorii,* and *R. typhi* (Azad et al.

1992, Schriefer et al. 1994a). Combined, these studies verified that *R. felis* was a distinct

rickettsial species.

Bouyer *et al*. (2001) sequenced additional genes for phylogenetic analysis of *R. felis*.

Although results of this molecular characterization of several *R. felis* genes placed *R. felis* in the

SFG, the inability to cultivate *R. felis* (strain *Ctenocephalides felis*-LSU[T]) did not satisfy the

requirements for type strain designation (Bouyer et al. 2001). A definitive culture of *R. felis* was

achieved in 2002 in African green monkey kidney cells (Vero) and *Xenopus* tadpole cells (XTC-

2) maintained at 28°C (optimal) and 32°C, and *R. felis* (strain Marseille-URRWFXCal$_2$[T]) was

named as the reference strain for *R. felis* (LaScola et al. 2002). Interestingly, the American Type

Tissue Culture deposit of *R. felis* (Marseille-URRWFXCal$_2$[T]) is not available at this time

(Macaluso; Personal communication). Recently, successful isolation of *R. felis* in arthropod

cells lines has been achieved (Horta et al. 2006, Pornwiroon et al. 2006).

1.2.1. Phylogenetics

      The original classification and phylogenic arrangement of the genus *Rickettsia* was

determined by serological studies. Since the original organization, conserved genera-specific

genes have been sequenced, compared among available *Rickettsia* species, and used to construct

phylogenies based upon gene differences and their likely evolution. Unlike most rickettsial

species, the initial description and classification of *R. felis* was based on molecular evidence

(Azad et al. 1992, Higgins et al. 1996, Bouyer et al. 2001) and was later followed by serologic-

based studies (LaScola et al. 2002). Early predictions based on certain gene sequences identified

R. felis in the SFG, however the disease it caused and the vector it utilized more closely fit

among the TG. Initial molecular-based classification of *R. felis* was utilized in large part due to

the difficulties in cultivating this organism. Although immediate phylogenic classification based

on serologic studies was delayed, a definitive culture was eventually established and serologic

classification by serotyping and antigenic classification were completed also placing *R. felis* in

the SFG (Raoult et al. 2001, LaScola et al. 2002, Fang and Raoult 2003).

1.2.1.a. Serologic-Based Classification

      A series of murine monoclonal and polyclonal antisera against *R. felis* and other TG and

SFG rickettsiae were used to serologically and antigenically characterize *R. felis* (Fang and Raoult 2003). This study identified the presence of two predominant heat-resistant proteins: 30-kDa and 60-kDa; and, after reacting mouse sera with antibodies to *R. felis*, five major bands were produced (120-, 60-, 30-, 17-kDa proteins and LPS-like antigen). Of these bands, the 120-, 60-, 30- and 17-kDa bands were sensitive to proteinase K digestion. Serotype analysis using microimmunofluorescence (MIF) assays indicated: that *R. felis* clustered with the SFG *Rickettsia*; cross-reacted with all SFG species evaluated (no or low cross-reactivity observed with TG species); and based upon specificity difference values was clustered close to *R. australis*, *R. akari* and *R. montanensis*. In this study, eight *R. felis*-specific monoclonal antibodies were also created, potentially, to be commercially developed to diagnose and differentiate *R. felis* infection from other rickettsial infections. The results of this study confirmed placement of *R. felis* in the SFG (Dumler et al. 2001).

1.2.1.b. Molecular-Based Classification

Although molecular-based phylogenies are more specific than serologic-based phylogenies, caution must be exercised when interpreting data based on only a single gene. Two critical points to consider when constructing and analyzing phylogenies based on gene sequences are: (1) choice of gene (it is necessary to use highly conserved genes to elucidate true divergences between species); and, (2) the basis of overall organization of species in a genus on a large number of different genes (Anderson and Tzianabos 1989). As the phylogenic placement of *R. felis* may vary depending on the gene being compared, multiple genes should be employed in taxonomic tree construction to get a true assessment of *R. felis* phylogenetic placement among other rickettsial species (Gillespie et al. 2007).

Phylogenetic analysis and taxonomic placement of *R. felis* has been examined using several genes, some of which are common to all rickettsiae and others that are specific to the

SFG. Examples of genus-specific genes common to both the SFG and TG include: 135-kDa outer membrane protein B gene (*ompB*); 17-kDa lipoprotein gene; 16S rRNA gene; citrate synthase gene (*gltA*); DNA-directed RNA polymerase gene (*rpoB*); S-adenosylmethionine synthetase gene (*metK*); signal recognition particle-docking protein gene (*ftsY*); DNA polymerase I gene (*polA*); and DNA polymerase III alpha chain gene (*dnaE*) (Azad et al. 1992, Roux and Raoult 1995, Stothard and Fuerst 1995, Roux et al. 1997, Andersson et al. 1999, Bouyer et al. 2001, Raoult et al. 2001). One example of a SFG-specific gene is *ompA*. Alignment of most *R. felis* genes identifies *R. felis* in or near a clade with *R. akari* and *R. australis*. Following is a brief description of commonly sequenced rickettsial genes and the taxonomic placement of *R. felis* for each gene sequence relative to other rickettsial species.

Citrate synthase gene (*gltA*): The citrate synthase gene is one of the most common genes used to differentiate *Rickettsia* species from other bacterial species. Analysis of the citrate synthase gene groups *R. felis* near *R. akari* and *R. australis* (Bouyer et al. 2001).

16S rRNA gene: The 16S rRNA gene was initially chosen to help characterize *R. felis* as a separate rickettsial species because of its stability as a housekeeping gene which makes it under less pressure from host immune responses and other environmental stresses. Analysis of this gene identified *R. felis* in a clade with *R. akari* and *R. australis* (Higgins et al. 1996).

17-kDa protein gene: The 17-kDa antigen found in all species of *Rickettsia* is an immunologically important surface protein and is one of the most characterized loci in any rickettsial genome (Anderson et al. 1987, Anderson et al. 1988, Anderson and Tzianabos 1989). Only bacteria in the genus *Rickettsia* are known to possess the 17-kDa antigen gene; and areas of divergence present in this gene between rickettsial species aided in the formation of the SFG and TG (Anderson and Tzianabos 1989). The conservation level of the 17-kDa antigen gene has been studied between rickettsial species using immunoblot analysis and comparison of

nucleotide alignment; both of which predict high homology between *Rickettsia*, especially in regions involved in controlling gene expression (Anderson and Tzianabos 1989). Because the 17-kDa protein gene is genus-specific, it is commonly used as a diagnostic tool to determine rickettsial infections, after which other genes (e.g. *ompB, ompA*) or techniques (e.g. RFLP) are used to determine the specific rickettsial species (Anderson and Tzianabos 1989). The presence of this gene has also been used to identify new rickettsial species; and its presence in the bacterial species originally designated ELB (now *R. felis*) supported placement of bacteria in the genus *Rickettsia* (Anderson and Tzianabos 1989, Tzianabos et al. 1989). Comparison of *R. felis* 17-kDa gene sequence with eight other rickettsial species, including members from both the SFG and TG, identified *R. felis* as different from all other sequenced strains, which was further supported by *R. felis gltA* RFLP analysis indicating that *R. felis* was a distinct rickettsial species in the SFG (Azad et al. 1992).

OmpA protein gene (*ompA*): The 190-kDa outer membrane antigen protein gene (*ompA*) is the major gene that defines the SFG. The immunodominant OmpA protein is believed to have a critical role in SFG rickettsial pathogenesis (e.g. cell adhesion and invasion) (Li and Walker 1998, Crocquet-Valdes et al. 2001). Antigenic variation in the conserved regions of this gene that flank tandemly repeated sequences, are useful in differentiating *Rickettsia* species and strains within the SFG (Gilmore, Jr. 1993, Zavala-Castro et al. 2005). Initially, *R. felis* was considered to be more closely related to the TG because experiments testing for the presence of this gene were negative (Azad et al. 1992). However, *R. felis* was later determined to indeed contain and transcribe a truncated version of this gene (Bouyer et al. 2001, Zavala-Castro et al. 2005). Further studies on the truncated *R. felis ompA* gene characterized it as a split gene that contained the peptide sequence for insertion into the inner membrane, but lacked the peptide sequence for transport to the outer membrane (Zavala-Castro et al. 2005). Also, because many

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stop codons are encoded within the gene that interrupt the reading frame, *ompA* was

hypothesized to be undergoing a degradation process to become a pseudogene perhaps as a result

of: long-term laboratory maintenance in fleas where the protein might not require the function

needed in the mammalian host; evolutionary adaptation to their environment; or, evolutionary

natural genetic heterogeneity among strains (Zavala-Castro et al. 2005).

Organization of *R. felis* into the SFG has been validated in both serologic and molecular

studies, however, a reorganization of *R. felis* within the genus *Rickettsia* has been proposed,

based on phylogenetic analysis of a number of conserved genus-specific genes and the presence

of a plasmid a plasmid in *R. felis* (Figure 1.2) (Gillespie et al. 2007). In this new arrangement

rickettsiae are organized into one of four groups: the ancestral group-AG (e.g. *R. bellii* and

R. canadensis), the typhus group-TG (e.g. *R. typhi* and *R. prowazekii*), the transitional group-

TRG (e.g. *R. felis* and *R. akari*) or the spotted-fever group-SFG (e.g. *R. rickettsii*, *R. sibirica*).

Based on this reorganization, *R. felis* would transfer into the TRG as results of several sequences

analyses places it before the core SFG-rickettsiae and after the TG-rickettsiae, similar to

R. akari.

1.2.2. Genome

Eleven genomes from species in the genus *Rickettsia* are currently available: two from

the TG, *R. prowazekii* (Andersson et al. 1998) and *R. typhi* (McLeod et al. 2004); seven from the

SFG, *R.* conorii (Ogata et al. 2001), *R. sibirica* (Malek et al. 2004), *R. rickettsii* (GenBank

accession no. AADJ01000001) (Ellison et al. 2008), *R. akari* (GenBank accession no.

NC_009881), *R. felis* (Ogata et al. 2005), *R. africae* (GenBank accession no. AAUY01000001),

and *R. massiliae* (Blanc et al. 2007); two from the AG, *R. bellii* (Ogata et al. 2006) and

R. canadensis (GenBank accession no. NC_009879). To date *R. felis* has the largest genome of

any sequenced *Rickettsia* sp. with *R. bellii* being a close second. Based on the currently

available genomes the following rickettsial species also contain plasmids: *R. felis*,

R. monacensis, *R. helvetica*, *R. peacockii*, *R. amblyommii*, *R. massiliae*, and *R. hoogstraalii* (Ogata et al. 2005, Baldridge et al. 2008).

Before the entire genome was available, individual *R. felis* genes were sequenced and compared to other rickettsial species. The first *R. felis* gene examined was the 16S rDNA gene which was sequenced and compared to 16S rDNA sequences of *R. rickettsii* and *R. typhi* to help confirm identity and determine its relative phylogenic placement (Schriefer et al. 1994a). The results of the sequence comparison revealed that *R. felis* was more closely related to the *R. rickettsii* than to *R. typhi*, differing at 9/1,407 and 24/1,407 bases, respectively, and examination of 17-kDa protein sequence to *R. rickettsii* revealed variation at only 6/80 amino acids (Azad et al. 1992). Sequencing of the *R. felis* genome revealed several unique features not common to other previously sequenced rickettsial genomes including putative genes for a conjugative pili (Ogata et al. 2005). *R. felis* was found to have one circular chromosome 1,485,148-bp long; and two circular plasmids: pRF and pRFδ, 62,829 and 39,263-bp, respectively. Pornwiroon et al. (2006), was also unable to detect a second plasmid upon screening of their *R. felis* (strain LSU). Additionally, after re-examination and re-annotation of the putative *R. felis* plasmid(s), Gillespie et al. (2007) determined *R. felis* contained only one plasmid: pRF. However, recent studies have demonstrated that plasmid number differs by *R. felis* strain, with some strains having no plasmids and others having two (Fournier et al. 2008).

Variable presence and number of plasmid(s) have also been observed among species in other genera of obligate intracellular bacteria (e.g. *Chlamydiaceae spp*.) (Palmer and Falkow 1986, Pickett et al. 2005). Several theories were proposed as to the maintenance of a plasmid in the genome of *R. felis* and include: potential function as a conjugative pilus; location of resistance genes; presence of bacteriocins; degrative properties; and, encoding of virulence

factors (Gillespie et al. 2007). Based on the pRF plasmid annotated gene sequence, the most

likely theory would be that this plasmid serves as a virulence plasmid, due to the number of

virulence gene homologs encoded on it (Gillespie et al. 2007). Examples of virulence gene

homologs found on the pRF plasmid include: ANK and TPR protein motif gene; surface antigen

gene; patatin homolog (phospholipase A2 activity, hemolytic activity), a chitinase (breakdown of

vector exoskeleton), ecotin (protease inhibitor), and a hyaluronidase gene (spreading factor). An

origin of replication and replication termination regions were also proposed (Gillespie et al.

2007). Acquistion of some of these virulence gene homologs were suggested to be the result of

horizontal gene transfer within the α-proteobacteria (Gillespie et al. 2007).

R. felis, thus far, has the highest number of gene families, and highest numbers of genes

within most gene families, than any other sequenced rickettsial species (Ogata et al. 2005).

These gene families include: surface cell antigens (*sca*); global metabolism regulators (*spoT*);

transposases; and, genes encoding protein-protein interaction motifs (e.g. ankyrin (ANK) repeats

and tetratricopeptide repeats (TRPs)). *R. felis* contains nine intact (*sca1-5, 8, 9, 12, 13*) and four

fragmented (*sca0, 7, 10, 11*) *sca* genes. Products of *sca* genes are commonly used as major

antigenic and genetic determinants to differentiate between rickettsial species. *R. felis* contains

14 spoT (*spoT1-13, 15*) paralogs that are classified into two groups according to their putative

function(s) in (p)ppGpp (guanosine tetra- and pentaphosphates) hydrolase and synthetase

activities. Protein-protein interaction motifs identified in the genome of *R. felis* include genes

encoding 22 ankyrin-repeat-containing proteins (function in transcription, cell cycle regulation,

and cell-to-cell signaling (Mosavi et al. 2002)), and 11 TPR-containing proteins (function in

protein-protein interaction (Blatch and Lassle 1999)).

Additional unique features of *R. felis* genome include: a putative toxin-antitoxin system

composed of 16 toxin and 14 antitoxin genes; antibiotic resistance gene homologs (including:

streptomycin; a class C β-lactamase, AmpC; a class D β-lactamase; penicillin acylase and an ABC-type multidrug transport-system protein); and a RickA homolog (involved in actin-based motility) (Ogata et al. 2005).

1.2.3. Cultivation

R. felis can be cultivated in a number of cell lines including: African green monkey kidney cells, Vero; murine fibroblast cells, L929; *Xenopus* tadpole cells, XTC-2; *Aedes albopictus* derived cells, C6/36; *Anopheles gambiae* cells, Sua5B; *Aedes albopictus* cells, Aa23; and, *Ixodes scapularis* derived cells, ISE6 (Table 1.2). Below is a description of *R. felis* cultivation in each of the different cell lines with special attention given to culture conditions.

1.2.3.a. Vero Cells

As the ELB agent, *R. felis* was initially cultivated by Radulovic et al. (1995b) in Vero (*Cercopithecus aethiops*) cells by inoculation of cell monolayers with homogenized suspensions of *R. felis*-infected *C. felis*, and incubated at 32°C and 35°C. The infection rate of ELB agent in Vero cells 11 days post-inoculation was 90-95%. Examination of infection-induced morphology of host cells by ELB agent revealed that infected cells were induced to form round cytopathic foci and plaques at day 11 post-infection and distinct macroscopic plaques with a reticular pattern at day 21 post-infection. This pattern of infection-induced host cell morphology changes along with growth pattern in Vero cells and delayed plaque formation was stated to more strongly resemble TG-rickettsiae then SFG-rickettsiae. The results of this study indicated that ELB agent can successfully adapt from insect cells to mammalian cells in terms of entry, survival and exit as determined by light microscopy, differential staining, and indirect immunofluorescent assays (IFA), however, the original isolate from this study was unsustainable.

In 2002, La Scola et al. definitively cultured *R. felis* in Vero cells, but demonstrated that

Table 1.2. Described cell culture systems for *R. felis* studies. *R. felis* can be propagated in a variety of both vertebrate and invertebrate cell lines. Strain (isolate) of *R. felis* and optimal culture temperature are indicated for each cell line.

	Cell line	Type of host cell	Temperature	*R. felis* strain	Reference
Vertebrate	Vero	African green monkey kidney	34°C	n.a.	Radulovic et al. 1995
			28-32°C	Marseille-URRWFXCal$_2$	Raoult et al. 2001
	L929	Murine fibroblast	34°C	n.a.	Radulovic et al. 1995
			34°C	LSU	Sakamoto et al. 2007
	XTC-2	Toad tadpole (*Xenopus laevis*)	28°C	Marseille-URRWFXCal$_2$	Raoult et al. 2001
Invertebrate	ISE6	Tick (*Ixodes scapularis*)	32°C	LSU	Pornwiroon et al. 2006
	C6/36	Mosquito (*Aedes albopictus*)	25°C	Pedreira	Horta et al. 2006
	Aa23	Mosquito (*Aedes albopictus*)	RT	LSU	Sakamoto et al. 2007
	Sua5B	Mosquito (*Anopheles gambiae*)	RT	LSU	Sakamoto et al. 2007

RT = room temperature

n.a. = not available

R. felis is temperature sensitive, growing optimally at 28°C, moderately at 32°C, and not

growing in temperature ≥32°C (LaScola et al. 2002). Cytoplamsmic foci and plaque formation

were observed in Vero cells incubated at the lower temperatures.

1.2.2.b. L929 Cells

As the ELB agent, *R. felis* was also initially cultivated in L929 (murine fibroblast) cells

by inoculation of cell monolayers with homogenized suspensions of *R. felis*-infected *C. felis*, and

incubated at 32°C and 35°C (Radulovic et al. 1995b). The infection rate of ELB agent in L929

cells 11 days post-inoculation was 65-70%, demonstrating that *R. felis* grows preferentially better

in Vero cells (Radulovic et al. 1995b). Work by La Scola et al. 2002, however could not

reproduce a successful culture of *R. felis* in this cell line with the same strain of *R. felis* (LaScola

et al. 2002). Recently, *R. felis* has been cultured in L929 cells, maintained in Dulbecco's

modified Eagle's medium supplemented with 5% fetal bovine serum and grown at 34°C

(Sakamoto and Azad 2007).

1.2.3.c. XTC-2 Cells

XTC-2 (*Xenopus laevis*) is an amphibian-derived cell line, where *R. felis* can be

successfully cultivated at 28°C (Raoult et al. 2001, LaScola et al. 2002). This cell line was

chosen because of it lower cultivation temperature, as *R. felis* was demonstrated to be

temperature sensitive. Using the *R. felis* Marseille-URRWFXCal$_2$^{T} strain, *R. felis*-infected

XTC-2 monolayers required 14 days for initial isolation, while subsequent passages required six

days for *R. felis* to infect 90% of cells. Cytoplasmic foci and plaque formation were observed in

XTC-2. *R. felis* was determined to grow most rapidly at 28°C in this cell line and died at

temperatures ≥32°C, and *R. felis* infected-XTC-2 cells grown at 28°C had twice the infection rate

of *R. felis*-infected Vero cells grown at 32°C.

1.2.3.d. C6/36 Cells

The first insect-derived cell line *R. felis* was cultivated in was the C6/36 (*Aedes albopictus*) cell line (Horta et al. 2006). In this study, *R. felis* was isolated from naturally infected cat fleas collected from dogs in Brazil. Fleas were externally disinfected, triturated and inoculated onto a monolayer of C6/36 cells. After several passages, infection of *R. felis* in C6/36 cells was established. At an incubation temperature of 25°C, C6/36 cells become confluently infected (90-100%) with *R. felis* within 15-20 days. Once again, *R. felis* was found to preferentially grow at a lower temperature (25°C) when compared with other rickettsial species that are cultivated *in vitro* at 30°C or higher (Weiss et al. 1984). To verify identity of the cultivated bacteria, PC*R* amplification of the 17-kDa protein gene, *gltA* and *ompB* were performed with affirmative results for *R. felis*.

1.2.3.e. ISE6 Cells

Recently, *R. felis* has been cultivated in an *Ixodes scapularis* (ISE6) tick-derived cell line (Pornwiroon et al. 2006). This cell line was chosen by the researchers because of its permissive nature in acquiring *Rickettsia* spp. infections. *R. felis* was successfully cultivated in this cell line at an incubation temperature of 32°C and 90% of cells were infected with *R. felis* within 14 days. *R. felis* was found to induce some cytopathic effects in the host cells including increased vacuolization, but minimal cell lysis was observed.

1.2.3.f. Aa23 Cells

With success in culturing other obligate intracellular bacteria, the *Aedes albopictus* cell line, Aa23, was examined for its ability to sustain *R. felis* infection (Sakamoto and Azad 2007). *R. felis* was successfully cultured in Aa23 cells, close to 100% infection by seven days, at room temperature and infected cells were observed to clump together, lyse and release large numbers of extracellular *R. felis*.

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<u>1.2.3.g. Sua5B Cells</u>

As with Aa23 cells, the *Anopheles gambiae* cell line, Sua5B, has also been used to culture obligate intracellular bacteria, and thus was examined for its ability to sustain *R. felis* infection (Sakamoto and Azad 2007). Similar to Aa23, *R. felis*-infected Sua5B cells were readily infected (close to 100% infection by seven days), clumped, lysed, and released large numbers of extracellular *R. felis*.

1.3. Ecology and Epidemiology of *Rickettsia felis*

<u>1.3.1 Infection in Arthropods</u>

The cat flea serves as the primary vector and reservoir of *R. felis*. Consequently, most of our current understanding of *R. felis* infection in arthropods comes from the *R. felis*-infected cat flea model. The distribution of *R. felis* within fleas has been examined using microscopic (transmission electron microscopy, TEM) and molecular methods (PCR). The initial characterization of *R. felis* morphology was accomplished by Adams *et al.* 1990 (Adams et al. 1990). Utilizing TEM, flea midgut epithelial cells contained *R. felis* located in the cytoplasm of the host cell, with dense cytoplasm, and surrounded by a clear space occasionally containing intracytoplasmic vacuoles (Bouyer et al. 2001, Pornwiroon et al. 2006). Inside the vector cell, rickettsiae were found to be located near the endoplasmic reticulum (Pornwiroon et al. 2006). Additional specific tissues in which *R. felis* has been identified in adult cat fleas include the muscle cells, fat body, tracheal matrix, ovaries, epithelial sheath of testes, and salivary glands (Adams et al. 1990, Bouyer et al. 2001, Macaluso et al. 2008). Within the epithelial cells of the salivary glands, *R. felis* were typically free in the cytoplasm often surrounded by a "halo" (cleared cytoplasmic contents), consistent with previous ultrastructural descriptions of *R. felis* in tick-derived cell culture (Pornwiroon et al. 2006) and in other flea tissues (Adams et al. 1990). The wide dissemination of *R. felis* within the flea host suggests a close association between fleas

and the bacteria; however, a correlation between rickettsial distribution in flea tissues and the distinct transmission route has not been determined.

Several qPCR assays have been developed for use as a diagnostic tool and to quantify rickettsiae in samples. In an antibiotic susceptibility study, replication of multiple isolates of *R. felis* were measured in XTC-2 cells treated with various antibiotics; however, assay details and *R. felis* load calculation methods in this study were not clear (Rolain et al. 2002). In another study that utilized qPCR, a probe-based method targeting a portion of the *ompB* gene was able to differentiate between *R. felis* and *R. typhi* infected fleas and had an assay sensitivity of 1 copy/µl (determined using serial dilutions of a plasmid containing a portion the *ompB* gene) (Henry et al. 2007). Comparison of crude (boiled flea lysate) versus kit-based DNA extraction methods determined crude-extraction of DNA was insensitive resulting in limited detection of *Rickettsia* (Henry et al. 2007). Additional studies examining molecular interactions between *R. felis* and the arthropod host will provide valuable information to decipher *R. felis* biology and epidemiology.

The presence of *R. felis* in commercial and institutional flea colonies has been extensively studied (Table 1.3). After the initial identification of *R. felis* in the Elward Laboratory cat flea colony, a 1999 survey examining the prevalence of *R. felis*-infection in commercial and institutional colonies demonstrated that *R. felis*-infection was wide-spread in controlled cat flea colonies (Higgins et al. 1994). In addition to cat fleas, *R. felis* has been detected molecularly in a panoply of blood-feeding arthropods worldwide, in 28 countries spanning five continents (Table 1.4). The use of the cat flea as the primary *R. felis* vector is concerning from a public health perspective because of the cat fleas' habit of indiscriminate host selection (Azad et al. 1992). In contrast to other described rickettsial species, *R. felis* is unique as it may potentially infect both insect and acarine hosts and has been detected in both ticks and mites. The prevalence of

Table 1.3. Prevalence of *R. felis* infection in commercial and institutional cat flea colonies.
R. felis-infection has been documented at a varying prevalence in several commercial and institutional cat flea colonies.

Flea Colony	Year assessed	*R. felis* prevalence	Reference
Ag Research Consultants*	1994	70%	Higgins et al. 1994
American Biological	1990	0%	Adams et al. 1990
Supply	1992	0%	Azad et al. 1992
Elward Labs (EL)	1992	100%	Azad et al. 1992
	1994	83%	Higgins et al. 1994
	2007	0%	Pornwiroon et al. 2007
Flea Data Inc.	2001	20-100%**	Raoult et al. 2001
Heska Corporation	1998	0%	Noden et al. 1998
	2008	0%	Macaluso et al. 2008
Louisiana State University	1994	86%	Higgins et al. 1994
(LSU)*	2000	65%	Wedincamp et al. 2000
	2002	65%	Wedincamp et al. 2002
	2007	94%	Pornwiroon et al. 2007
	2007	100%	Henry et al. 2007
	2008	96%, 67%, 35%	Reif et al. 2008
Merck and Co. Inc.*	1994	66%	Higgins et al. 1994
Nu-Era Research Farms	1994	83%	Higgins et al. 1994
Professional Laboratory	1994	50%	Higgins et al. 1994
Research Service (PLRS)*	2007	16%	Pornwiroon et al. 2007
Texas A&M University**	1994	43%	Higgins et al. 1994
University of California	1990	0%	Adams et al. 1990
University of Maryland at	1992	0%	Azad et al. 1992
Baltimore (UMAB)			
USDA, MAVERL**	1994	93%	Higgins et al. 1994

*Created from or supplemented with originally infected EL Lab fleas

**Potentially contaminated with originally infected EL Lab fleas

Table 1.4. Geographic distribution of *R. felis* in wild-caught arthropods. *R. felis* has been detected by molecular methods in numerous species of arthropods, recovered from mammalian hosts in several countries around the world. The prevalence of *R. felis*-infection in wild-caught fleas is variable.

Country	Surveyed mammalian host(s) with (+) *R. felis* vector	Surveyed vector (+) for *R. felis*	Prevalence of *R. felis*-infection	Reference
Algeria	Rodents and hedgehogs	*Archaeopsylla erinacei and C. canis*	100%	Bitan et al. 2006
Afghanistan	Gerbils	rodent fleas	9%	Marie et al. 2006
Argentina	Dogs	*C. felis*	22.6%	Nava et al. 2008
Australia	Dogs and cats	*C. felis and Echidnophaga gallinacea*	n.d.	Schloderer et al. 2006
Brazil	Dogs and cats	*Ctenocephalides spp.*	n.d.	Oliveira et al. 2002
	Dogs	*C. felis*	28-80%	Horta et al. 2005
	Dogs and horses	*Ctenocephalides sp. R. sanguineus and Amblyomma cajennense*	n.d.	Cardoso et al. 2006
	Dogs	*C. felis and C. canis*	36% *C. felis*, 19.1% *C. canis*	Horta et al. 2006
	Opossums, dogs and cats	*C. felis and Polygenis atopus*	41% (*C. felis*), 3-8% (*P. atopus*)	Horta et al. 2007
	Dogs and horses	*Riphicephalus sanguineus and C. felis*	n.d.	Oliveira et al. 2008
Canada	Cats	*C. felis*	18%	Kamrani et al. 2008
Chile	Cats	*C. felis*	70%	Labruna et al. 2007
Croatia	Sheep and goats	*Haemaphysalis sulcata*	20-26%	Duh et al. 2006
Cyprus	Rats	*C. felis*	5.60%	Psaroulaki et al. 2006
Democratic Republic of Congo	Collected off host	*P. irritans, E. gallinacea, Xenopsylla brasiliensis, Tunga penetrans*	10.72%	Sackal et al. 2008
Ethiopia	n.d.	Fleas	n.d.	Raoult et al. 2001[£]
France	Cats	*C. felis*	8.10%	Rolain et al. 2003
	Dogs and cats	*C. felis, C. canis, Archeopsylla erinacei*	17% (Cf), 27%(Cc), 100% (Ae)	Gilles et al. 2008b
Gabon	Monkey	*C. felis*	n.d.	Rolain et al. 2005
Germany	Dogs and cats	*C. felis, Archeopsylla erinacei*	100% *A. erinacei*, 9% *C. felis*	Gilles et al. 2008
Indonesia	Rodents and shrews	*Xenopsylla cheopis*	n.d.	Jiang et al. 2006
Israel	Cats	*C. felis*	1-13%	Bauer et al. 2006
Japan	None (collected by flagging)	*Ixodes ovatus, Haemaphysalis flava, H. kitasatoe*	n.d.	Ishikura et al. 2003
Mexico	Dogs	*C. felis*	n.d.	Zavala-Velazquez et al. 2002
New Zealand	Dogs and cats	*C. felis*	15%	Kelly et al. 2004
Peru	Dogs	*C. felis*	n.d.	Blair et al. 2004
Portugal	Rodent and hedgehog	*Archaeopsylla erinacei and Ctenophtalmus sp.*	3.50%	de Sousa et al. 2006
South Korea	Wild rodents	Chigger mites	n.d.	Choi et al. 2007
Spain	Dog	*n/a*	40%	Perez-Arellano et al. 2005
	Dogs and cats	*C. felis*	n.d.	Marquez et al. 2002
	Dogs and cats	*C. felis*	n.d.	Marquez et al. 2006
	Dogs and cats	*C. felis and C. canis*	28.40%	Blanco et al. 2006
Taiwan	Rodents	Mesostigmata mite	n.d.	Tsui et al. 2007
		C. felis	18.80%	Tsai et al. 2009
Thailand	Dogs and ferret-badger	*C. felis and C. canis*	4.40%	Parola et al. 2003
United Kingdom	Dogs and cats	*C. felis*	6-12%	Kenny et al. 2003
	Dogs and cats	*C. felis*	9-21%	Shaw et al. 2004

United States	Opossums	*C. felis*	1.80%	Williams et al. 1992
	Opossums	*C. felis*	3.70%	Schriefer et al. 1994
	Dog	*Pulex irritans*	n.d.	Azad et al. 1997
	Opossums	*C. felis*	3.8% in 1993 and 2.1% in 1998	Boostrom et al. 2002
	Rodents	*Anomiopsyllus nudata*	0.80%	Stevenson et al. 2005
	Brown pelican	*Carios capensis*	1.50%	Reeves et al. 2006
	Cats	*C. felis*	n.d.	Hawley et al. 2007
	Mice	*Xenopsylla cheopis*	24.80%	Eremeeva et al. 2008
Uruguay	Dogs and cats	*C. felis and C. canis*	41%	Venzal et al. 2006

n.d. = not determined

R. felis-infection in surveyed wild-caught arthropods ranges from 0.8% to 100%, depending on species and geographic location, but typically is less than 25%. To assess vector competence, additional studies will be required to discern the biological significance of *R. felis* infection in these various arthropod hosts.

1.3.2. Transmission Routes of *Rickettsia felis*

Global dissemination of *C. felis* is contributing to the vast distribution of *R. felis*. Although *R. felis* has been identified molecularly in a number of arthropod species, the cat flea is currently the only arthropod associated with biological transmission of *R. felis*. Maintenance of *R. felis* in the environment is most likely a function of stable vertical transmission, via transstadial and transovarial transmission within cat flea populations (Azad et al. 1992, Wedincamp, Jr. and Foil 2002). Mechanisms of possible *R. felis* transmission routes were postulated with the discovery of *R. felis* in flea reproductive tissue (Azad et al. 1992), because other rickettsial species had been shown to be vertically transmitted (Farhang-Azad et al. 1985, Azad and Beard 1998). Initial reports describing *R. felis* vertical transmission within flea populations employed PCR to detect *R. felis* in freshly deposited cat flea eggs, demonstrating that *R. felis* could be transovarially transmitted, a finding that correlated with the high prevalence (~90%) in the Elward Laboratory flea colony (Azad et al. 1992). A subsequent study examined the prevalence of *R. felis* in newly emerged unfed adult cat fleas from eight colonies in the USA and identified *R. felis* infection in all colonies, with prevalence ranging from 43-93% (Higgins et al. 1994). The authors attributed high *R. felis*-infection prevalence to efficient vertical and horizontal transmission. Because newly emerged, unfed adult fleas were examined, acquisition of *R. felis* via vertical transmission is the most reasonable conclusion, as horizontal routes of transmission were never definitively demonstrated.

The most comprehensive *R. felis* vertical transmission study was performed by

Wedincamp et al. (Wedincamp, Jr. and Foil 2002), in which the efficiency of *R. felis* vertical

transmission in cat fleas over twelve generations without the aid of an infectious bloodmeal or

vertebrate host was described. It was reported that *R. felis*-infection prevalence waned from 65%

to 2.5% by the twelfth generation in fleas fed bovine blood using an *in vitro* feeding system

compared to the steady 65% infection prevalence for cat-fed fleas. Although it is plausible that

in the cat-fed colony *R. felis*-infection prevalence was boosted by occasional rickettsemias,

uninfected fleas fed on these same cats did not acquire infection. *R. felis* transmission also was

not observed by copulation or direct contact between infected and uninfected fleas. With the

exception of vertical transmission among colonized cat fleas, biological transmission of *R. felis*

by other arthropods has not been described; *R. typhi* and *R. felis* are the only pathogenic

rickettsial species transovarially transmitted in arthropods other than ticks or mites (Azad et al.

1992).

Horizontal transmission of viable *R. felis* during juvenile or adult flea lifecycle stages has

not been demonstrated; however, the potential for horizontal transmission between arthropod and

vertebrate or arthropod and arthropod is likely. Observed by ultrastructural studies in the

salivary glands of cat fleas (Macaluso et al. 2008), saliva produced during bloodfeeding may

provide one route of *R. felis* transmission to a susceptible vertebrate host. Evidence for

rickettsiae transmission from flea to host via salivary secretion is supported by PCR

amplification of *R. felis* DNA in the blood of laboratory cats exposed to *R. felis*-infected cat fleas

and their subsequent seroconversion (Wedincamp, Jr. and Foil 2000). Containment of fleas in a

feeding capsule ensured that cat hosts did not become exposed to infection during grooming

procedures (e.g. ingesting infected fleas or flea feces), suggesting horizontal transmission via

flea feeding occured. Although cats were positive for *R. felis* DNA and antibodies to *R. felis*,

these methods do not address the viability of *R. felis* in this mammalian host. Additional studies

examining veterinary clinic cats infested with *R. felis*-infected fleas were unable to amplify

R. felis DNA, but could detect antibodies to *R. felis* indicating possible past infection or exposure

(Hawley et al. 2007, Bayliss et al. 2009). Transmission of rickettsiae in saliva during

bloodfeeding has been demonstrated with other rickettsial species (reviewed in Azad and Beard

1998) and recovery of *R. felis* from an infected mammalian host with subsequent infection of a

competent vector like the cat flea on an infected host have yet to be realized.

Co-feeding between *R. felis*-infected and uninfected fleas or other susceptible arthropods

poses another possible route of *R. felis* horizontal transmission. In this scenario, an infected flea

would be able to transmit *R. felis* in saliva or by regurgitation of rickettsiae to an uninfected flea

feeding nearby. Pathogen transmission via co-feeding has been described for numerous

arthropods, including *Ixodes scapularis*, in the transmission of *Borrelia burgdorferi* (Patrican

1997) and in *Culex* species with the transmission of the West Nile virus (McGee et al. 2007).

Rickettsial species, including *R. massiliae* in *Rhipicephalus turanicus* and *R. rickettsii* in

Dermacentor andersoni, have also been documented to employ co-feeding as a transmission

strategy (Philip 1959, Matsumoto et al. 2005). In one study, *Rickettsia*-free fleas allowed to feed

with *R. felis*-infected fleas for five days on an artificial feeding system the uninfected fleas did

not acquire infection (Wedincamp, Jr. and Foil 2002); however, the concentration of rickettsiae

in the shared meal and the susceptibility of the uninfected fleas to infection was not determined.

Although likelihood of *R. felis* transmission or maintenance is diminished, additional studies

examining the potential of co-feeding transmission are needed.

Larval feeding also may be another potential avenue for *R. felis* horizontal transmission.

Cat flea larvae feed on adult flea feces as their main source of nutrition. In addition to their

regular diet, cat flea larvae lead cannibalistic lives preying on eggs and other larvae (Lawrence

and Foil 2000, Hsu et al. 2002) all of which can lead to a greater success during larval and adult

maturation (Lawrence and Foil 2000). Feeding on *R. felis*-infected feces and/or cannibalism of *R. felis*-infected eggs/larvae may facilitate *R. felis* transmission to uninfected fleas. Initial studies using uninfected larvae allowed to feed on PCR-positive *R. felis*-infected feces, eggs, and earlier instar larvae were unsuccessful in acquiring *R. felis*; however, *R. felis* viability and persistence in flea feces and infective dose had not been assessed (Wedincamp, Jr. and Foil 2002).

Transmission of rickettsiae to vertebrates via infected feces is a common transmission strategy employed by the TG *Rickettsia*. The predominant mode of *R. typhi* transmission is through fleas feeding on rickettsemic vertebrate hosts and the subsequent shedding of infectious *R. typhi* in their feces for up to ten days (Azad and Beard 1998).   In addition to *R. typhi* being horizontally transmitted to a vertebrate host via infected flea feces, vertical transmission within flea populations has also been described, although at a lower rate (Farhang-Azad et al. 1985). The ability of fleas to transmit *R. typhi* both horizontally and vertically suggests similar mechanisms of transmission for *R. felis*. While the ability of fleas to acquire *R. felis* during blood feeding and then shed viable *R. felis* in their feces has not been fully examined, *in vitro* studies have demonstrated a infectious extracellular state of *R. felis* (Sunyakumthorn et al. 2008). The increased availability of more sensitive detection methods will allow for detection of low-level transmission by other postulated transmission routes.

Along with *Rickettsia* species, numerous bacterial endosymbionts such as *Wolbachia* sp. and *Spiroplasma* sp., have been described in domestic and wild-caught fleas (Gorham et al. 2003, Murrell et al. 2003, Pornwiroon et al. 2007, Jones et al. 2008). Abundant endosymbionts in arthropod populations may regulate the ability of *R. felis* to colonize through either similar tissue tropism or nutrient competition. Interference phenomenon, the establishment of a one species of *Rickettsia* inhibiting the transovarial transmission of a second *Rickettsia* species (Burgdorfer 1988, Macaluso et al. 2002), and niche competition have been described.

Subsequently, an examination of *R. felis* and *R. typhi* co-infection prevalence, where *R. felis*-infected cat fleas were fed blood containing *R. typhi* for nine days, demonstrated the ability of cat fleas to acquire dual infection; however, infection rates were at a lower prevalence than in either single infection, indicating that cat fleas previously infected with *R. felis* may interfere with the establishment of *R. typhi* (Noden et al. 1998). Complete inhibition of *R. typhi* infection in *R. felis*-infected fleas was not observed in all fleas, and whether or not *R. felis* is able to inhibit vertical transmission of *R. typhi* to flea progeny or inhibit transmission to a susceptible vertebrate host is not known. During the initial description of *R. felis* (ELB agent), *Wolbachia* were described in the ovaries of cat fleas (Adams et al. 1990). Since then, *Wolbachia* species have been described in several species of fleas (Gorham et al. 2003, Rolain et al. 2003, Dittmar and Whiting 2004, Luchetti et al. 2004) with possible interactions between *R. felis* and *Wolbachia* species especially interesting as they occupy many of the same host cells (niches) and likely compete for similar host resources. In insects, the influence of microbial interactions on *Wolbachia* abundance has been demonstrated (Bordenstein et al. 2006, Goto et al. 2006). Specifically in fleas, assessment of microbiota in the *R. felis*-free Elward Laboratory cat flea colony demonstrated *Wolbachia* to be the predominant bacteria, compared to decreased detection of *Wolbachia* in the LSU colony with an *R. felis* prevalence of ~94% (Pornwiroon et al. 2007). In addition to *Wolbachia*, *R. felis*-infected arthropods have also been infected with either *Bartonella clarridgeiae* (Rolain et al. 2003), *Bartonella henselae* (Shaw et al. 2004), *Bartonella quintana* (Rolain et al. 2003, Marie et al. 2006), and *Haemoplasma sp*. (Shaw et al. 2004) or *Spiroplasma*, *Stenotrophomonas*, and *Staphylococcus* (Pornwiroon et al. 2007). Occupying the same cells or organs in the arthropod host means *R. felis* must be able to contend with other vertically maintained endosymbionts and microbiota acquired during feeding (Pornwiroon et al. 2007). For example, *Spiroplasma*, negatively affected the population of *Wolbachia* during co-

infection of *Drosophila melanogaster* (Goto et al., 2006). The biological impact of the interspecific relationship of co-infecting rickettsial and/or other bacterial species in the flea host requires further examination.

Bacterial species, such as *Wolbachia* and *Spiroplasma*, are readily able to manipulate their host's biology (e.g. cytoplasmic incompatibility, male-killing, feminization) to facilitate their transmission (Werren 1997, Dobson et al. 1999, Fry et al. 2004, Montenegro et al. 2006, Duron et al. 2008). The fitness cost associated with *R. felis* infection of fleas is not clear and needs to be examined (Pornwiroon et al. 2007) as other pathogenic *Rickettsia*, such as *R. rickettsii* and *R. prowazekii*, have been shown to negatively impact the fitness of their host arthropod (Snyder and White 1945, Burgdorfer and Brinton 1975, Niebylski et al. 1999). Like *Wolbachia* and *Spiroplasma*, other insect-specific *Rickettsia* species can negatively impact the fitness of their arthropod hosts, such as the rickettsial species in the two-spotted lady beetle, *Adalia bipunctata* which is associated with male embryo mortality (Werren et al. 1994, Perlman et al. 2006). The ability of *R. felis* to influence the biology of a respective vector may delineate which arthropod species are competent vectors (Pornwiroon et al. 2007). Further studies examining *R. felis* interactions with other microbiota in the arthropods vector are needed.

1.3.3. Mammalian Reservoirs

Although there have been multiple reports trying to confirm the presence of *R. fe*lis in mammalian reservoirs, a definitive mammalian reservoir is still undescribed. As *R. felis* is distributed worldwide and may utilize several vector species, it is likely that maintenance of *R. felis* in nature requires a mammalian host, which may also vary geographically. Serologic-based investigations have tried to define the prevalence and incidence of *R. felis*-infection in specific populations of domestic and wild animals. Several peri-domestic species associated with the cat flea vector have been implicated, including: cats (Higgins et al. 1996, Boostrom et

al. 2002, Case et al. 2006, Labruna et al. 2007); dogs (Richter et al. 2002, Oteo et al. 2006); and, opossums (Williams et al. 1992, Schriefer et al. 1994b, Boostrom et al. 2002, Bayliss et al. 2009), all of which have been found seropositive for *R. felis*-infection outside of laboratory experiments (Table 1.5).

Although *R. felis* has been detected molecularly in several mammalian species, all *R. felis* recovery attempts from vertebrate hosts have been unsuccessful. Definitive proof of infection, "gold standard", of any pathogen, is direct culture from an infected host; however, several factors such as: low pathogen concentration, presence in only specific tissues, non-optimal culture conditions, and waxing/waning of infection can impede recovery. Because of these potential issues, indirect methods of diagnosing infections have been developed and can employ both serologic and molecular techniques. Boostrom et al. (2002) conducted a survey of flea-infested opossums in a murine typhus endemic area using serologic (IFA) and molecular (PCR) assays to detect *R. felis* infection in both the potential mammalian reservoir (the opossum) and fleas. PCR assays utilized the rickettsial 17-kDa protein gene and positive samples were processed by RFLP (*Alu* I digestion) to determine the rickettsial species. Total rickettsial seroprevalence among opossums was determined to be 25.5%. IFA of flea midgut smears revealed 22% of fleas positive for *R. felis* infection and 8% positive for *R. typhi* infection. Results of PCR analysis of 17-kDa protein gene revealed that 2.6% of fleas were infected with a *Rickettsia sp.*, from which RFLP analysis confirmed that 21% and 79% of fleas were infected with *R. typhi* and *R. felis*, respectively. These results suggest a role for opossums as mammalian reservoirs for *R. felis*.

As the primary species of flea that infests cats in the United States, a study was conducted to determine the prevalence of *R. felis* infection in cat fleas recovered from infested cats (Hawley et al. 2007). Cats exposed to *R. felis* via naturally infected *R. felis*-infected

Table 1.5. Diagnosis of *R. felis* infection in mammals. Several sero-surveys of mammals with or without *R. felis*-infected fleas have been conducted to identify potential mammalian reservoirs of *R. felis*.

Country	Mammalian host(s) positive for *R. felis* (% positive)	On-host vector (+) for *R. felis* - infection	Tissue Tested	Diagnostic Test	Reference
Chile	Cat (73%)	*C. felis*	Serum	IFA	Labruna et al. 2007
Germany	Dog (n.a.)	n/a	Serum	MIF, Western blot	Richter et al. 2002
Peru	Rat (n.a.)	n/a	Blood	n/a	Blair et al. 2004[£]
Spain	Dog (n.a.)	n/a	Serum	PCR (*gltA* , ompA, ompB)	Oteo et al. 2006
Taiwan	Cat	*C. felis*	Serum	IFA	Tsai et al. 2009
USA	Opossums (30%)	*C. felis*	Serum	DFA	Williams et al. 1992
	Opossums (33%)	*C. felis*	Blood	PCR (17kDa, *gltA*), IFA	Schriefer et al. 1994
	Cat (8%)	n/a	Serum	n/a	Higgins et al. 1996[£]
	Cat (81)*	*C. felis*	Serum	Serology, PCR	Wedincamp et al. 2000
	Cat (100)*	*C. felis*	Serum	IFA	Wedincamp 2002
	Opossums (33% 1993 and 22% in 1998)	*C. felis*	Serum	IFA	Boostrom et al. 2002
	Cat (<15%)	n/a	Serum	IFA	Boostrom et al. 2002[£]
	Cat (11%)	n/a	Serum	IFA	Case et al. 2006
	Cat (n.a.)	*C. felis*	Blood	PCR (*gltA* , *ompB*)	Hawley 2007
	Cat (3.9%)	n/a	Serum	IFA	Bayliss et al. 2008

*Infested under experimental conditions

fleas were examined using both serologic (IFA) and molecular (PCR) assays to detect *R. felis* infection. *R. felis* was detected in 64% of fleas collected from the cats, however all cats (cat blood) tested negative for *R. felis* infection by PCR and IFA. Although *R. felis* was not detected in the cat blood tested, cats should not automatically be ruled out as potential mammalian reservoirs of *R. felis*. Other studies have demonstrated the ability of cats to develop a *R. felis*-seropositive status (Wedincamp, Jr. and Foil 2002). Therefore before ruling out a particular mammalian species as a potential *R. felis*-reservoir, multiple factors should first be considered including: the duration of exposure to feeding *R. felis*-infected fleas, health status of the animal, and predilection site of *R. felis* for the sampled tissue site.

Additional studies are necessary to determine if mammals are capable of being infected with *R. felis* and serving as an infection source for vectors. Development of animal models of *R. felis*-infection would address several of these questions and allow in-depth examination of the biology of *R. felis* in mammalian hosts, including predilection sites of *R. felis*, infection kinetics, and host immune response. These results could elucidate valuable information concerning the prevention and treatment of *R. felis* infections in human and animals and could be extrapolated to the prevention and treatment of other rickettsial and/or flea-borne diseases.

<u>1.3.4. Human Infection</u>

In nature, *R. felis* was first identified in cat fleas collected from opossums in California near foci of murine typhus infections (Williams et al. 1992). Because *R. felis* overlapped in areas with *R. typhi*, a retrospective study was performed in Texas on patients initially diagnosed with murine typhus to either verify a murine typhus diagnosis or identify a misdiagnosed *R. felis* infection (Schriefer et al. 1994a). In the Texas study, five patients initially diagnosed with murine typhus were examined by PCR screening, RFLP, and southern hybridization for *R. felis* infection. Tests confirmed that one of the five examined patients was positive for *R. felis*

infection and this case was recognized as the first human case of *R. felis* infection (Schriefer et al. 1994a).

Since then, human cases of *R. felis*-infection have been reported in 12 countries worldwide (Figure 1.3) including: Spain (Perez-Arellano et al. 2005, Bernabeu-Wittel et al. 2006, Nogueras et al. 2006, Oteo et al. 2006), Germany (Richter et al. 2002), France (Raoult et al. 2001), Brazil (Raoult et al. 2001, Galvao et al. 2006), Mexico (Zavala-Velazquez et al. 2000, Galvao et al. 2006, Zavala-Velazquez et al. 2006), Thailand (Parola et al. 2003), Taiwan , South Korea (Choi et al. 2005), Laos (Phongmany et al. 2006), Egypt (Parker et al. 2007) and Tunisia (Znazen et al. 2006) (Table 1.6). Interestingly, only in Tunisia and Egypt was there a human case of *R. felis*-infection reported without additional reports of *R. felis*-positive arthropods. Serosurveys to detect *R. felis*-infection in humans have also been conducted. In Spain, serum samples tested by IFA to survey the past exposure of two populations of people to *R. felis* reported up to 7.1% of people had antibodies to *R. felis* (Bernabeu-Wittel et al. 2006, Nogueras et al. 2006). Misdiagnosis of *R. felis*-infection as another rickettsial infection, such as murine typhus or Mediterranean spotted fever is also common (Schriefer et al. 1994a, Raoult et al. 2001), implying a higher prevalence of *R. felis* infection in areas where multiple rickettsial species overlap.

The clinical disease caused by *R. felis*-infection has been designated several names including: flea-borne spotted fever, cat flea typhus, and *R. felis* rickettsiosis. Clinical manifestations of *R. felis*-infection are similar to symptoms caused by other rickettsial organisms and can range in severity (Table 1.6). Typical symptoms can include fever, rash, headache, myalgia, and eschar at the bite site (Brouqui et al. 2007). More severe symptoms can result from visceral (abdominal pain, nausea, vomiting and diarrhea) and neurologic (photophobia and hearing loss) involvement (Zavala-Velazquez et al. 2000, Galvao et al. 2006). The variable
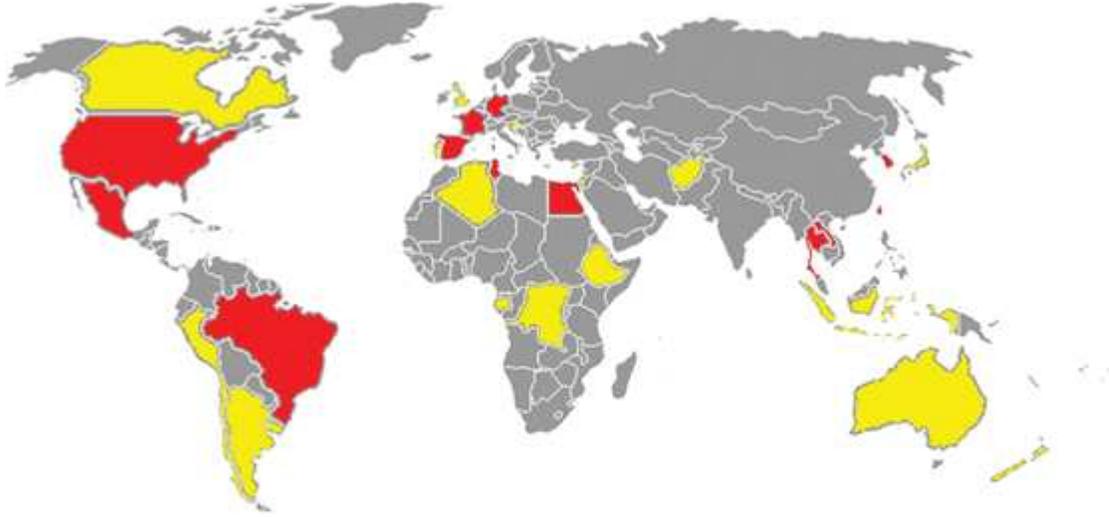
Figure 1.3. Reported distribution of *R. felis*. *R. felis* has been detected molecularly and serologically in several countries around the world. In some countries *R. felis* has been detected in arthropods only (YELLOW), while in other countries, *R. felis* has both been detected in arthropods and human cases of *R. felis* rickettsiosis have been reported (RED). Tunisa and Egypt are the only countries where a human case has been reported, but *R. felis* has not been reported in arthropods.

Table 1.6. Human cases and reported clinical manifestations of flea-borne rickettsiosis.
Several cases of *R. felis* rickettsiosis have been reported around the world. Reported clinical manifestations range from mild (e.g. fever, headache and myaliga) to severe (e.g. visceral and cerebral involvement).

Country	No. Cases	Rash	Fatigue	Acute Fever	Acute Headache	Acute Arthralgia	Acute Myalgia	Other	Reference
Brazil	2	2/2	n.d.	2/2	n.d.	n.d.	n.d.	stupor (2/2), coma (1/2), thrombocytopenia (1/2), vomiting (2/2), elevated liver enzymes (1/2)	Raoult et al. 2001
Egypt	1	n.d.	n.d.	1/1	n.d.	n.d.	n.d.	n.d.	Parker et al. 2007
France	2	2/2	n.d.	2/2	n.d.	n.d.	n.d.	n.d.	Raoult et al. 2001
Germany	1	1/1	1/1	1/1	1/1	n.d.	n.d.	splenomegaly, elevated liver enzymes	Richter et al. 2002
Laos	1	n.d.	n.d.	1/1	n.d.	n.d.	n.d.	n.d.	Phongmany et al. 2006
Mexico	3	3/3	3/3	2/3	2/3	1/3	2/3	nuchal pain (1/3), leucopenia (1/3), leucocytosis (1/3), anemia (1/3), thrombocytosis (1/3), abdominal pain (2/3), nausea (1/3) vomiting (1/3), diarrhea (1/3), photophobia (2/3), hearing loss (1/3), conjunctivitis (1/3)	Zavala-Velazquez et al. 2000
	1	0/1	n.d.	1/1	n.d.	n.d.	n.d.	skin lesions, cough, pulmonary infiltrates	Zavala-Velazquez et al. 2006
South Korea	3	n.d.	n.d.	3/3	n.d.	n.d.	n.d.	n.d.	Choi et al. 2005
Spain	5	0/5	n.d.	5/5	4/5	4/5	4/5	dry cough (3/5), abdominal pain (1/5), elevated liver enzymes (5/5), conjunctivitis (1/5)	Perez-Arellano et al. 2005
	2	1/2	n.d.	2/2	n.d.	2/2	n.d.	malaise (2/2), elevated liver enzymes (2/2)	Oteo 2006
Taiwan	1	0/1	1/1	1/1	1/1	0/1	n.d.	chills, pyuria, acute polyneuropathy	Tsai et al. 2008
Thailand	1	0/1	n.d.	1/1	1/1	n.d.	n.d.	chills, hepato-splenomegaly, leukopenia, vomiting	Parola et al. 2003
Tunisia	8	8/8	n.d.	8/8	n.d.	n.d.	n.d.	peripheral adenopathy (2/8), interstitial pneumopathy (1/8)	Znazen et al. 2006
USA	1	0/1	n.d.	1/1	n.d.	n.d.	n.d.	n.d.	Schriefer et al. 1994

presentation of clinical disease can make diagnosis difficult and refinement of the full spectrum

of clinical disease associated with *R. felis*-infection will expedite accurate diagnoses.

Development of a *R. felis*-disease animal model would allow for examination of *R. felis*

pathogenesis, clarifying clinical aspects of *R. felis*-infection.

1.4. Diagnosis of *Rickettsia felis* Infection in Vertebrate and Invertebrate Hosts

Detection of *R. felis* infection can be accomplished by serological or molecular diagnosis

(Table 1.7). In humans and animals, rickettsial infection is routinely diagnosed using serologic

methods that employ the human or animals' antibody response to rickettsial antigens. The most

common methods of serologic diagnosis include: microimmunofluorescent assay (MIF), direct

immunofluorescent assay (DFA), indirect immunofluorescent assay (IFA), enzyme-linked

immunosorbent assay (ELISA), Western blotting and cross-adsorption assays. Multiple

serologic tests are often required to confirm diagnosis the specific infecting rickettsiae because

cross-reactivity between rickettsial species is common. *R. felis* has been detected serologically

in humans and domestic animals; however, diagnosis via these methods can be a challenge due

to cross-reactivity with other rickettsial species, variable cut-off titer values, and reagent

availability. Further compounding the issues of accurate diagnosis is the clinical similarity of

many rickettsial diseases. In areas where *R. felis*-infected arthropods (specifically infected fleas)

were identified, several retrospective studies, employing serological diagnosis, have been

conducted to determine *R. felis*-infection prevalence of among local human and animal

populations (Bernabeu-Wittel et al. 2006, Nogueras et al. 2006, Pinter et al. 2008). Ideally

serological results should be verified by molecular tests, although this is not always possible,

especially when looking for evidence of past infections.

In general, molecular diagnosis of rickettsial infection offers greater sensitivity and

specificity. Molecular detection usually involves PCR assays to first determine rickettsial

Table 1.7. Assays used to diagnose cases of flea-borne rickettsiosis. Both molecular and serological assays have been used to identify human cases of flea-borne rickettsioses.

Country	# Cases	Diagnostic Method	Tissue Tested	Reference
Brazil	2	MIF, nested PCR (*gltA*)	Serum	Raoult et al. 2001
	1	Diagnostic methods not mentioned	n/a	Galvao et al. 2006
Egypt	1	PCR (17kDa)	Blood	Parker et al. 2007
France	2	MIF	Serum	Raoult et al. 2001
Germany	1	MIF, Western blot, nested PCR (PS120 protein gene)	Serum	Richter et al. 2002
Laos	1	MIF, Western blot, cross-adsorption	Serum	Phongmany et al. 2006
Mexico	3	Skin biopsy, PCR (17kDa), serological test	Serum, skin	Zavala-Velazquez et al. 2000
	1	IFA, PCR (17kDa)	Whole blood	Zavala-Velazquez et al. 2006
	2	Diagnostic methods not provided	n/a	Galvao et al. 2006
Spain	5	MIF, Western blot, cross adsorption	Serum	Perez-Arellano et al. 2005
	2	PCR (*gltA*, *ompA*, *ompB*)	Serum	Oteo et al. 2006
	33	IFA	Serum	Bernabeu-Wittel et al. 2006
	7	IFA	Serum	Nogueras et al. 2006
South Korea	3	Multiplex-nested PCR (*rompB*, *gltA*), sequencing and RFLP	Serum	Choi et al. 2005
Taiwan		qPCR (*groEL*, *17kDa*, *ompB*), IFA	Whole blood	Tsai et al. 2008
Thailand	1	IFA, Western blot, cross adsorption	Serum	Parola et al. 2003
Tunisia	8	IFA, Western blot, cross adsorption	Serum	Znazen et al. 2006
USA	1	PCR (17kDa), RFLP, Southern blot	Whole blood	Schriefer et al. 1994
	3	ELISA	Serum	Wiggers et al. 2005

infection, followed by restriction fragment-length polymorphism (RFLP) analysis, dot-blot

hybridization, or sequencing of rickettsial genes to identify the specific infecting rickettsial

species. Genes commonly used for rickettsial detection include the *Rickettsia* genus specific 17-

kDa antigen gene, the 16S rRNA gene, the citrate synthase gene (*gltA*), *ompB*, and *ompA* (this

gene is truncated in *R. felis*). Molecular detection is common in arthropod surveys of *R. felis*-

infection and assessment of individual arthropods can help elucidate the prevalence of *R. felis* in

specific area. For example, in a survey of fleas collected from rodents and a hedgehog in

Portugal, *R. felis* was detected and differentiated from *R. typhi* in *A. erinacei* and *Ctenophtalmus*

species by amplification and sequencing of the *gltA* and *ompB* genes (DeSousa et al. 2006).

Likewise, in Israel, multiple genotypes of *R. felis* were identified in cat fleas by detection and

sequencing of the 17-kDa antigen, *gltA*, *ompA* and *ompB* genes (Bitam et al. 2006), which

demonstrates the specificity of molecular assays in differentiating not only rickettsial species, but

also in differentiating between strains of rickettsial species.

Molecular detection assays have also been utilized to describe *R. felis* infection in

mammals and humans. In a survey of opossums infested with *R. felis*-infected cat fleas, blood

samples were collected and sequencing of amplified *gltA* and 17kDa gene products demonstrated

R. felis infection (Schriefer et al. 1994b). In Germany, *R. felis*-infection was diagnosed in a

person and their dog by amplification from serum samples and sequencing of the *R. felis* PS120

protein gene (Richter et al. 2002). More sensitive molecular tools have been employed to

identify specific rickettsial infections. Development of qPCR assays able to detect as little as

one to ten specific rickettsial gene copies, allow for detection of low-level *R. felis*-infections

(Henry et al. 2007). The development of species specific probes allows for the immediate

differentiation and diagnosis of specific rickettsial infection (Rozmajzl et al. 2006, Henry et al.

2007). Although molecular assays may be more sensitive and specific, limits in machine and

reagent availability are common. Molecular assays also only detect current rickettsial infections whereas serological based assays can diagnose evidence of past infections. Currently, there is no standard protocol for physicians to diagnose *R. felis*-infection in patients, and despite circumstantial evidence of infection, *R. felis* has not been isolated from a human case of infection.

1.5. Prevention, Control, and Treatment

1.5.1. Prevention and Control

As with most vector-borne pathogens, control of the vector species often leads to efficient control of the pathogen. Because cat fleas are described as the primary vector and reservoir of *R. felis*, and a vertebrate reservoir remains unknown, flea control should be the focus of attention when considering ways to reduce/eliminate cases of *R. felis* rickettsiosis in humans and animals. Prevention of *R. felis* infection is most easily achieved through: avoidance of fleas; prevention of flea infestations on companion animals; and, avoidance of high risk occupations (e.g. veterinarians, farmers, animal shelter employees). Persons with high risk occupations should regularly practice good flea control measures, such as treating pets and routine cleaning.

Flea control can be achieved through both non-chemical and chemical means. Non-chemical based flea control may include routine vacuuming and cleaning in residences where flea infestations are a problem. Any bedding or clothing that may be suspected of harboring any flea lifecycle stage should either be disposed of or thoroughly cleaned with hot, soapy water.

Chemical control of flea infestations is a multi-million dollar a year business geared toward the prevention/reduction of flea infestations on pets and domestic animals. Examples of chemically based flea control products include: anti-flea systems such as Frontline™ or Advantage™ to prevent flea infestation of animals and flea bombs to control/eliminate heavy infestations in homes.

<u>1.5.2. Treatment of *Rickettsia felis* Rickettsiosis</u>

Patients diagnosed with *R. felis* rickettsiosis, can successfully be treated with doxycycline (200 mg/day) for 10 days (Oteo et al. 2006). Pregnant women and children should not be given doxycycline or fluoroquinolones, and in these cases physicians should consider macrolide compounds or investigate other alternatives (Rolain et al. 2002). Also, not all treated patients may develop a seropositive state (IgG antibodies) (Oteo et al. 2006).

In one study, plaque and dye-uptake assays along with qPCR were used to evaluate the antibiotic susceptibility of *R. felis*, *R. conorii*, and *R. typhi* to: amoxicillin, ciprofloxacin, doxycycline, erythromycin, gentamicin, levofloxacin, ofloxacin, rifampin, SXT, telithromysin, and thiamphenicol (Rolain et al. 2002). Immunofluorecence staining assays (Ives et al. 1997), were also employed because *R. felis* has not been shown to cause much cytopathic effects in cell cultures. Results indicated that *R. felis* is resistant to gentamicin, amoxicillin, and trimethoprim-sulfamethoxazole; poorly susceptibly to erythromycin; and, susceptible to doxycyline, rifampicin, thiamphenicol, tellthromycin and fluoroquinolones (Rolain et al. 2002). Additional studies have shown that *R. felis* is resistant to erythromycin, unlike other rickettsial species, for which this is a successful drug (Rolain et al. 1998, LaScola et al. 2002, Kenny et al. 2003). The *R. felis* resistance to gentamicin, amoxicillin and trimethoprim-sulfamethoxazole and susceptibility to doxycyline, rifampicin, thiamphenicol and fluoroquinolones has been confirmed in other studies (Radulovic et al. 1995a, LaScola et al. 2002, Kenny et al. 2003).

Antibiotic resistance homologs have been identified in the genome of *R. felis* including: streptomycin; a class C β-lactamase, AmpC; a class D β-lactamase; penicillin acylase and an ABC-type multidrug transport-system protein (Ogata et al. 2005).

1.6. Summary

Identification of arthropods and mammals susceptible to stable infection with *R. felis* can

serve as a platform for developing methods to study *R. felis*-host interactions. *R. felis*-host interactions can be examined using both *in vitro* and *in vivo* systems. Several cell culture systems for *in vitro* examination of *R. felis* have been established and provide a valuable tool for examining *R. felis* biology. The development of bioassays employing arthropods and mammals capable of being infected with *R. felis* are crucial to study *R. felis* biology *in vivo*. Established cat flea colonies infected with *R. felis*, such as the LSU *C. felis* colony, provide a valuable tool to study *in vivo* the biology (infection kinetics, tissue tropism, etc.) of *R. felis* within its primary host and vector. Within the flea, host the interspecific relationship of *R. felis* with other flea bacterial endosymbionts that may affect the establishment or transmissibility of *R. felis*, and vector competence of individual fleas, can also be addressed. As of yet, no *in vivo* mammalian model of *R. felis*-infection has been established. Establishment of an animal model will facilitate a more accurate understanding of *R. felis*, defining pathogenesis in both animals and humans and delineating the transmission of *R. felis* from arthropod to animal, and vice versa. Identification of key molecules in both the arthropod and mammalian host that aid in establishment and maintenance of *R. felis* infection and subsequent transmission could aid in the development of transmission control strategies within vector populations.

Despite being transmitted by a cosmopolitan vector and listed as an emerging infectious disease, our current understanding of *R. felis* biology is incomplete with several issues that require attention. Towards understanding *R. felis* transmission mechanisms the following areas need to be addressed: identification of competent arthropod vectors and vertebrate reservoirs; identification of alternate *R. felis* transmission routes; development of sensitive and specific detection assays; and, refinement of the definition of clinical disease. Every year there are new reports of arthropod, animal, and human cases of *R. felis* infection occurring in countries. Determination of the key species involved in transmission will allow for the identification of

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relationships and patterns of *R. felis*-infection and will help pinpoint what and where control measures should be implemented. Several tools are currently at our disposal including *R. felis*-infected commercial/institutional flea colonies, cell culture systems capable of supporting *R. felis* growth, and genome and proteome descriptions, to facilitate research towards delineating the biology, epidemiology, and ecology of *R. felis*.

Despite stable vertical transmission in laboratory settings, the occurrence of *R. felis* in numerous species of fleas and ticks in nature suggests that horizontal transmission occurs. Deciphering the role of horizontal transmission in the maintenance of *R. felis* and defining the determinants of *R. felis*-infection in vertebrate and invertebrate hosts are essential. The broad hypothesis of this dissertation is that horizontal transmission is a necessary maintenance strategy of *R. felis* in nature that is influenced by: persistence in the vector; the ability of naïve vectors to become infected; and, dissemination and replication in the vertebrate host. The specific objectives of this dissertation were to: (1) examine *R. felis*-infection dynamics in the flea vector at the population and individual flea level; (2) determine ability of fleas to horizontally acquire *R. felis*-infection; and, (3) assess *R. felis* infection in a vertebrate model.

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CHAPTER 2
PREVALENCE AND INFECTION LOAD DYNAMICS OF *RICKETTSIA FELIS* IN ACTIVELY FEEDING FLEAS[1]

2.1. Introduction

Rickettsia felis is an arthropod-associated intracellular, gram-negative bacterium with a worldwide distribution (Azad et al. 1997). Molecular and serological evidence suggests *R. felis* is infectious for humans, causing a disease similar to murine typhus (Schriefer et al. 1994), although direct evidence of horizontal transmission of *R. felis* from arthropods to humans has not been demonstrated. Several studies have detected *R. felis* DNA in a diverse range of flea species of which *Ctenocephalides felis*, the cat flea, is considered the primary vector (Adams et al. 1990). Laboratory studies have confirmed *R. felis*-infection in *C. felis* populations is predominantly maintained by transstadial and transovarial transmission (Azad et al. 1992, Wedincamp, Jr. and Foil 2002). Stable vertical transmission of *R. felis* has been examined in colonized fleas and the reported prevalence of *R. felis* in commercial and institutional *C. felis* colonies ranged from 43-100% (Azad et al. 1997, Wedincamp, Jr. and Foil 2000, Wedincamp, Jr. and Foil 2002, Henry et al. 2007). Within an isolated colony, such as the *C. felis* colony at Louisiana State University (LSU), prevalence of *R. felis* is variable with studies reporting a 65% and 100% prevalence of *R. felis* in 1999 (Wedincamp, Jr. and Foil 2000) and 2007 (Henry et al. 2007), respectively. Vertical transmission of *R. felis* persists in *C. felis* for at least 12 generations without the aid of an *R. felis*-infected bloodmeal; however, over successive generations prevalence wanes to low levels (<10%) (Wedincamp, Jr. and Foil 2002). The specific mechanisms by which *R. felis* prevalence fluctuates within a *C. felis* colony are unknown, but in

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nature, the prevalence of *R. felis* in a flea population is likely amplified by fleas feeding on *R. felis*-infected mammalian hosts (Williams et al. 1992).

Ultrastructural analyses have been used to characterize *R. felis* within the flea host, as well as in cell lines (Adams et al. 1990, Pornwiroon et al. 2006, Macaluso et al. 2008). Using transmission electron microscopy, the presence of *R. felis* has been demonstrated in *C. felis* midgut epithelial cells, ovaries, salivary glands, and muscle (Adams et al. 1990, Macaluso et al. 2008). Additionally, molecular detection of *R. felis* has utilized traditional and quantitative real-time PCR (qPCR), typically targeting a portion of the genus-common 17-kDa antigen gene, for field sample diagnostics (Rolain et al. 2002, Stevenson et al. 2005, Henry et al. 2007). While qPCR has been utilized to assess the replication kinetics of *R. felis in vitro* (Rolain et al. 2002), *in vivo* (arthropod and vertebrate) models of infection, for the purpose of examining the infection kinetics of *R. felis*, have not been examined. Development of such models will further elucidate the mechanisms for maintenance of *R. felis*, specifically in the flea populations that serve as vectors and reservoirs, and broaden our understanding of the ecology of *R. felis*.

Recent analysis of rickettsial replication in a *Rickettsia*/tick model (*Rickettsia amblyommii*/*Amblyomma americanum*) utilizing qPCR demonstrated a generalized dissemination of rickettsiae and, notably, a steady state level of rickettsial infection during relatively long (7-14 day) tick feeding periods (Zanetti et al. 2008). While the direct regulators of rickettsial replication are under investigation in the slow-feeding tick host, the kinetics of rickettsial replication within the flea has not yet been examined. After locating a suitable host, cat fleas immediately begin feeding. Within 24 to 36 hr after initiating bloodfeeding, female cat fleas begin laying eggs and reach peak egg production (40 to 50 eggs/day) in 4 to 9 days (Dryden 1989, Dryden and Rust 1994, Rust and Dryden 1997). Feeding adult fleas can live as long as

100 days, however, fleas that have initiated feeding and are removed from the host usually die

within 24 to 48 hr (Rust and Dryden 1997). Similar to other *Rickettsia*/arthropod relationships,

R. felis is maintained via vertical transmission in the LSU colony of *C. felis*; however, rickettsial

response (replication) to physiological events related to flea reproduction is unknown. The

hypothesis to be tested in this study is that within colonized fleas, the intimate relationship

between *R. felis* and *C. felis* allows for coordination of rickettsial replication and the

metabolically active periods during flea bloodmeal acquisition and oogenesis. In the present

study, the replication kinetics of *R. felis* in the cat-fed, LSU *C. felis* colony were investigated by

developing a qPCR assay to detect and quantify *R. felis* in actively feeding cat fleas. Likewise,

the influence of flea gender and *R. felis* prevalence within the colony on rickettsial load in cat

fleas during bloodmeal acquisition was examined. A sensitive and specific method of detection,

qPCR, allowed for accurate detection and quantification of *R. felis* in *C. felis*, including samples

with low levels of infection. Assessment of *R. felis*-infection prevalence and infection density

dynamics will help elucidate the epidemiology of *R. felis*, an emerging infectious pathogen.

2.2. Materials and Methods

2.2.1. Fleas and Cats

Unfed *C. felis* were obtained from a colony maintained at LSU (Baton Rouge, LA). For

the past 20 years, this colony has been persistently infected at a varying prevalence with *R. felis*

(Foil et al. 1998). At the Louisiana State University School of Veterinary Medicine (LSU-SVM)

unfed newly-emerged, adult *C. felis* (mixed sex) were allowed to feed on 2 year-old, short-hair,

flea-naïve, specific pathogen free cats (Harlan, Indianapolis, IN) as previously described by Foil

et al. (1998). All cats were housed individually with 12:12 light:dark cycles at 20-22.2°C and

40-60% relative humidity. Pre-study blood collected from all cats was negative for *Rickettsia* by

PCR. Briefly, fleas were contained in a capsule secured to the shaved lateral thorax of a cat and were able to feed through the fine mesh of the capsule. Before each trial (Day 0), colony *R. felis*-infection prevalence, infection load, and infection density of a minimum of nine individual fleas were assessed. Animal use in this research was conducted in accordance with protocols approved by the LSU Institutional Animal Care and Use Committee, and an approved protocol (#08044) is on file in the office of the Division of Laboratory Animal Medicine at LSU-SVM.

2.2.2. Experimental Design

Three separate trials were conducted over the course of one year. Each trial was initiated approximately six months from the start of the previous trial. All fleas were drawn from the same colony, therefore, fleas in subsequent trials were descendants of the previous trial as the colony was not supplemented with outside fleas. The dynamic *R. felis*-infection prevalence in the LSU *C. felis* colony did not allow trials to be exact replicates of one another.

In each trial, capsules containing ~100, unfed adult (mixed sex) fleas were secured to an individual cat. Approximately ten fleas were randomly selected and removed from the capsule for nine consecutive days. After collection, all flea samples were immediately frozen at -20°C until further processing. Every three days, fleas remaining in the study were transferred to a new capsule and returned to the cat.

2.2.3. DNA Isolation

Fleas recovered daily from the cat host were individually sexed, assigned sample numbers, placed in microcentrifuge tubes and homogenized with sterile plastic pestles. Genomic DNA (gDNA) was extracted using either QIAGEN DNeasy Tissue Kit (Chatsworth, CA) according to the manufacturer's instructions and eluted in 50 μl Buffer AE (Trial 1), or using a modified version of the HotSHOT DNA extraction protocol (Trials 2 and 3) as described next

(Truett et al. 2000). Briefly, individual fleas were placed in microcentrifuge tubes partially submerged in liquid nitrogen and pulverized using sterile plastic pestles. Ground samples were incubated at 95°C for 45 min in 30 μl alkaline lysis reagent (25 mM NaOH, 0.2 mM disodium EDTA, pH of 12), cooled to 4°C for 5 min and mixed with 30 μl of neutralizing reagent (40mM Tris-HCl, pH of 5). All gDNA preparations were stored at -20°C.

2.2.4. Rickettsial Detection and Identification by PCR and Sequencing

R. felis infection in flea samples was detected by PCR amplification of a small portion of the *R. felis* 17-kDa antigen gene using primers (*Rf*17.153 5'-AGGACAGCTTGTCGGAGTAGG-3'and *Rf*17.309 5'-ACGCCATTCTACGCTAGTGC-3') derived from the complete genome of *R. felis* available on GenBank (Accession #: CP000053). A portion of *C. felis* 18S rDNA, amplified by primers (*Cf*18S 5'-TGCTCACCGTTTGACTTGG-3' and *Cf*18S 5'-GTTTCTCAGGCTCCCTCTCC-3') derived from an available partial sequence of *C. felis* 18S rDNA in GenBank (Accession #: AF136859), was used as a control to check the integrity of the template DNA. All primers used for standard PCR and qPCR were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). PCR products were amplified using isolated gDNA (individual flea lysates) or water (negative control) as template, gene-specific primers and 2x PCR Master Mix (Promega, Madison, WI). PCR conditions were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 seconds (s), annealing at 60°C for 45 s, extension at 72°C for 1 min, and a final extension at 72°C for 7 min. Amplified PCR products were visualized by electrophoresis on ethidium bromide stained 2.0% agarose gels. To verify identity, representative PCR products from each trial were cloned into pCR4-TOPO vectors (Invitrogen, Carlsbad, CA) and sequenced as previously described (Pornwiroon et al. 2007).

2.2.5. Southern Blot Analysis

A Southern blot was conducted to estimate the *C. felis* 18S rDNA copy number. Southern blot was performed as previously described by Horigane et al. (Horigane et al. 2007). Briefly, gDNA was extracted from *Rickettsia*-uninfected, unfed adult cat fleas (Heska Corporation, Loveland, CO) using Qiagen DNeasy DNA Extraction Kit and digested with five restriction enzymes (6 μg gDNA/enzyme): *Eag* I, *Pst* I, *Xba* I, *Xho* I (New England BioLabs, Ipswich, MA), and *Eco*R I (Promega, Madison, WI). DNA was separated on a 0.8% TAE gel and transferred to a positively charged nylon membrane (Amersham Biosciences, Piscataway, NJ) with 20X SSC, and fixed by UV. *C. felis* 18S rDNA was identified using the probe, *Cf*18SP, that was constructed based on a portion of the *C. felis* 18S rDNA sequence and amplified with primers *Cf* 18S$_{for}$ and *Cf* 18S$_{rev}$. The probe was labeled with PCR DIG Labeling Mix (Roche, Indianapolis, IN) according to the manufacturer's protocol. Anti-digoxigenin-AP (Fab fragment, Roche) and CDP Star (New England Bio Labs) were used for detection.

2.2.6. Construction of Internal-Control Plasmid for Quantitative Real-Time PCR

To quantify copies of *R. felis* and *C. felis* genes in flea samples, serial dilutions of a plasmid containing single-copy portions of both the *R. felis* 17-kDa antigen gene and *C. felis* 18S rDNA were used to generate a standard curve. The plasmid was constructed by PCR amplification of a 157-base pair (bp) fragment of *R. felis* 17-kDa antigen gene and a 179-bp fragment of *C. felis* 18S rDNA with *Rf*17.153$_{for}$-*Rf*17.309$_{rev}$ and *Cf*18S$_{for}$-*Cf*18S$_{rev}$ primers, respectively. Each amplicon was cloned into to a pCR4-TOPO vector and sequenced to confirm identity as previously described (Pornwiroon et al. 2007). Both amplicons were PCR amplified using a gene-specific primer and either a M13-forward or M13-reverse primer, digested with *Eco*R I and ligated together. The ligation product was amplified by PCR with primer pair

*Rf*17.153$_{for}$ and *Cf*18S$_{rev}$, cloned and sequenced. The resulting plasmid, pCR4-TOPO-*Rf*17kDa+*Cf*18SrDNA, served as a standard template. The minimum detection limit for pCR4-TOPO-*Rf*17kDa+*Cf*18SrDNA was 10 copies.

2.2.7. Quantitative Real-Time PCR

For each gene, qPCR components and template that included 2x iTaq SYBR Green Supermix (BioRad, Hercules, CA); 100 nM of each primer; DNase/RNase-free water; and 5 µl of gDNA template (samples), water (negative control), or serial 10-fold dilutions (1×10^8 to 10 copies) of pCR4-TOPO-*Rf*17kDa+*Cf*18SrDNA were pre-mixed in 35 µl volumes in 96-well plates and aliquoted in triplicate 10 µl reactions on 384-well plates. The qPCR was performed with an ABI 7900HT unit (Applied Biosystems, Foster City, CA) using conditions previously described (Zanetti et al. 2008). Results were analyzed with ABI 7900HT sequence detection system (SDS v2.3) software. The specificity of the assay was verified and the expected single peak for the internal-control plasmid and positive gDNA samples, but not in the water (negative control) samples, was identified in the dissociation curve. Additionally, representative qPCR products from each trial were verified by gel analysis to confirm the specificity of the reaction and cloned and sequenced to confirm identity (data not shown). Trial *R. felis*-infection prevalence in the *C. felis* colony was quantified as the percent of fleas positive for *R. felis* infection out of the total number examined per trial. *R. felis*-infection load was quantified as the copy number of *Rf*17kDa per individual flea lysate. *R. felis*-infection density was quantified as the ratio of log transformed *Rf*17kDa and log transformed *Cf*18S rDNA copy numbers (*Rf*17kDa/*Cf*18S) per individual flea.

2.2.8. Statistical Analysis

Rickettsial load in fleas and the ratio of *Rf*17kDa/*Cf*18S were assessed after the

logarithmic transformation of the quantity of the genes of interest (*Rf*17kDa and *Cf*18S).

Analysis of variance, (SAS statistical package, Version 9.1.3, GLM procedure ANOVA, Cary,

NC) was performed to examine potential differences between rickettsial load in fleas and ratio of

*Rf*17kDa/*Cf*18S copy number over the study period; when overall significance was found,

Tukey's honestly significant difference (HSD) post hoc test was used to examine pairwise

differences of means of main effects. Pairwise *t*-tests of least square means were performed to

determine any interaction effects between trial, gender, and experimental day for rickettsial

infection load and ratio of *Rf*17kDa/*Cf*18S. An *F*-test was used for general comparisons of

grouped means. For all comparisons, a *P*-value of < 0.05 was considered significantly different.

2.3. Results

2.3.1. Prevalence of *R. felis* in LSU *C. felis* Colony

The specificity of *R. felis*-infection of *C. felis* (LSU colony) was confirmed by

sequencing a portion of the 17-kDa antigen gene from a representative subset of fleas positive

for *Rickettsia spp*. infection. Obtained sequences were compared to those in GenBank and all

samples sequenced demonstrated a 100% identity to *R. felis* (accession number CP000053).

The prevalence of *R. felis*-infection in individual *C. felis* lysates was assessed by qPCR

using primers that amplified a portion of the 17-kDa antigen gene. Trial prevalence and the daily

prevalence range for each trial are listed in Table 2.1. *R. felis*-infection prevalence was greatest

during Trial 1 and decreased 23% and 61% in Trials 2 and 3, respectively. When prevalence was

assessed separately for male and female fleas, *R. felis* infection was gender-independent.

2.3.2. Quantification of *Rf17*kDa and *Cf*18S Gene Copies During Flea Feeding

To determine *R. felis*-infection load, fleas were individually titrated and the total number

of *R. felis* (17-kDa copy number) in each individual flea sample was determined by qPCR.

Table 2.1. Trial *R. felis*-infection prevalence and daily range of cat fleas positive for *R. felis*-infection. In this study each trial was conducted ~6 months from the previous trial, therefore allowing *R. felis*-infection prevalence to be assessed over time.

Trial	Trial prevalence	Daily range of cat fleas positive for *R. felis* infection
1	96%	89-100%
2	73%	40-90%
3	35%	10-60%
Mean of Trials	68%	46-83%

Serial dilutions of pCR4-TOPO-*Rf*17kDa+*Cf*18SrDNA were used to generate a standard curve

and extrapolate *R. felis* quantities per individual flea lysate (Table 2.2a). The range of detected

*Rf*17kDa was 3.1×10^1 to 3.74×10^5 copies per reaction, corresponding to a 1.3×10^3 to 1.6×10^7

total *Rf*17kDa load per flea lysate. The mean quantities of *R. felis* at each time point for

individual flea samples in each trial are presented in Figure 2.1. Among all trials, fleas had

significantly different *R. felis*-infection loads; increasing 4.75-fold during the course of the study

between fleas in Trial 1 and Trial 3. Trial 3 fleas had the greatest *R. felis*-infection loads (mean

load ± SEM across all time points was $7.88 \times 10^6 \pm 5.43 \times 10^5$) followed by Trial 2 ($2.54 \times 10^6 \pm$

1.40×10^5) and Trial 1 ($1.66 \times 10^6 \pm 1.62 \times 10^5$). Combining data from all trials, when flea gender

was considered, female fleas had significantly greater mean *R. felis*-infection loads than males.

However, at individual time points within a trial or across trials there were few significant

differences in *R. felis*-infection loads between male and female fleas.

When the mean daily *R. felis*-infection loads for each trial, and for all trials combined, were

compared, significant variability in *R. felis*-infection load was observed by day compared to the

starting point at Day 0 within Trials 1 and 2 (Figure 2.1). No significant variability in daily

R. felis-infection load was observed in Trial 3, when compared to the Day 0 infection load.

Analyses of the mean daily *R. felis* infection load combining all Trials demonstrated that there

was no single day when *R. felis*-infection load was consistently greater within the study period.

Likewise, although variability in *R. felis*-infection load was observed in some trials, there was no

consistent pattern of increased or decreased rickettsial infection across all trials. Therefore, a

consistent *R. felis*-infection load during flea feeding was observed as there was no significant

correlation between *R. felis* replication and flea feeding or oviposition events.

      To estimate flea sample size, *R. felis*-infected and -uninfected *C. felis* were individually

Table 2.2a. Copy number of *Rf*17kDa in individual flea lysates

Trial	Exp. day	Total no. of fleas surveyed (no. *R.felis*-infected/no. uninfected)	1	2	3	4	5	6	7	8	9	10	Daily mean ±SEM *R. felis*-infection load
Trial 1	0	9 (8/1)	4.79E+05	5.85E+06	2.47E+06	9.20E+06	1.97E+06	1.60E+06	1.45E+06	-	5.20E+06		3.53E+06 ± 1.05E+06
	1	10 (10/0)	2.41E+06	3.86E+06	7.11E+05	1.88E+06	9.93E+05	4.69E+06	4.76E+06	2.94E+05	2.08E+06	7.97E+05	2.25E+06 ± 5.26E+05
	2	10 (10/0)	3.21E+06	2.40E+06	1.28E+06	1.62E+06	7.01E+05	8.42E+05	4.40E+06	1.52E+06	2.61E+06	1.69E+06	2.03E+06 ± 3.62E+05
	3	10 (9/1)	9.58E+05	1.73E+06	4.94E+05	1.81E+05	1.79E+06	2.11E+06	1.07E+06	7.33E+05	-	7.61E+05	1.09E+06 ± 2.17E+05
	4	10 (9/1)	3.65E+05	1.84E+06	1.82E+06	6.10E+05	-	8.20E+05	1.82E+06	1.34E+06	1.31E+06	1.54E+06	1.27E+06 ± 1.85E+05
	5	10 (9/1)	4.75E+05	6.05E+05	1.05E+06	-	3.31E+05	8.55E+05	8.10E+05	2.51E+05	6.86E+04	1.32E+05	5.08E+06 ± 1.14E+05
	6	10 (10/0)	1.49E+05	1.77E+05	1.07E+05	9.49E+05	1.97E+06	1.11E+06	1.31E+06	1.52E+06	7.00E+05	4.04E+03	7.99E+05 ± 2.16E+05
	7	10 (10/0)	2.15E+06	7.82E+05	1.04E+06	3.10E+05	2.05E+06	2.28E+06	4.94E+03	1.06E+06	3.01E+06	3.79E+06	1.65E+06 ± 3.83E+05
	8	10 (10/0)	3.26E+05	5.83E+04	1.93E+06	1.28E+06	1.77E+04	6.12E+05	1.67E+05	1.40E+06	2.19E+06	1.10E+06	9.08E+05 ± 2.49E+05
	9	10 (10/0)	7.19E+05	1.58E+06	4.56E+05	1.79E+06	2.94E+06	5.08E+06	6.11E+06	1.76E+06	3.94E+06	1.12E+06	2.55E+06 ± 6.06E+05
												Mean *R. felis*-Infection Load	**1.66E+06 ± 1.62E+05**
Trial 2	0	40 (35/5)	2.29E+06	2.35E+06	2.76E+06	2.25E+06	1.16E+06	2.45E+06	2.53E+06	2.74E+06	1.45E+06	3.48E+06	2.98E+06 ± 3.33E+05
			2.34E+06	2.59E+06	1.42E+06	1.58E+06	2.18E+06	1.28E+06	3.02E+06	-	3.13E+06	2.25E+06	
			-	3.00E+06	1.84E+06	2.79E+06	2.90E+06	1.33E+06	3.17E+06	-	2.24E+06	3.62E+06	
			3.35E+06	8.67E+06	5.59E+06	2.90E+06	6.08E+06	-	1.30E+03	-	6.62E+06	6.99E+06	
	1	10 (8/2)	2.58E+06	1.36E+06	1.78E+06	2.90E+06	2.79E+06	3.38E+06	2.27E+06	-	2.37E+06	-	2.43E+06 ± 2.26E+05
	2	10 (6/4)	2.97E+06	-	3.01E+06	1.64E+06	2.99E+06	2.83E+06	-	3.15E+06	-	-	2.76E+06 ± 2.29E+05
	3	12 (10/2)	-	2.28E+06	1.38E+06	1.39E+06	2.01E+06	1.98E+06	-	2.67E+06	4.67E+06	2.60E+06	2.43E+06 ± 2.95E+05
			2.52E+06	2.79E+06									
	4	10 (6/4)	1.59E+06	3.64E+06	-	2.81E+06	1.70E+06	4.40E+06	3.45E+06	-	-	-	2.93E+06 ± 4.57E+05
	5	10 (9/1)	2.21E+06	2.58E+06	3.40E+06	2.82E+06	3.24E+06	-	2.52E+06	8.78E+05	3.43E+06	2.61E+06	2.63E+06 ± 2.61E+05
	6	10 (6/4)	4.67E+06	-	2.34E+06	-	9.58E+05	-	1.23E+06	2.60E+06	-	1.03E+06	2.14E+06 ± 5.80E+05
	7	10 (5/5)	-	2.01E+06	2.80E+06	-	-	-	1.36E+06	2.38E+06	-	3.67E+06	2.44E+06 ± 3.86E+05
	8	10 (8/2)	1.93E+06	4.33E+06	-	5.27E+06	2.33E+06	4.55E+06	1.94E+06	-	1.35E+06	3.60E+06	3.16E+06 ± 5.16E+05
	9	10 (4/6)	2.59E+06	-	2.97E+06	-	-	-	-	3.14E+06	-	2.32E+03	1.47E+06 ± 7.64E+05
												Mean *R. felis*-Infection Load	**2.54E+06 ± 1.40E+05**
Trial 3	0	27 (9/18)	-	-	-	-	-	-	-	1.03E+07	1.10E+07	1.44E+07	9.43E+06 ± 1.12E+06
			-	-	1.23E+07	-	-	1.05E+07	1.03E+07	-	-	-	
			-	-	-	4.23E+06	5.21E+06	6.61E+06	-	-	-	-	
	1	10 (5/5)	6.72E+06	-	9.19E+06	5.86E+06	-	8.94E+06	-	5.86E+06	-	-	7.31E+06 ± 7.33E+05
	2	10 (5/5)	5.53E+06	-	5.14E+06	5.32E+06	-	-	-	-	8.19E+06	7.72E+06	6.38E+06 ± 6.51E+05
	3	10 (1/9)	3.68E+06	-	-	-	-	-	-	-	-	-	3.68E+06
	4	10 (6/4)	3.65E+06	4.32E+06	9.11E+06	6.33E+06	6.22E+06	6.44E+06	-	-	-	-	6.01E+06 ± 7.81E+05
	5	10 (4/6)	-	-	1.01E+07	1.11E+07	-	-	-	-	2.57E+06	4.55E+06	7.09E+06 ± 4.17E+06
	6	10 (2/8)	-	-	1.33E+07	-	-	1.29E+07	-	-	-	-	1.31E+07 ± 2.18E+05
	7	10 (4/6)	-	-	-	-	8.36E+06	1.57E+07	-	1.46E+07	5.28E+06	-	1.10E+07 ± 2.50E+06
	8	10 (3/7)	-	-	-	-	4.64E+06	-	-	-	2.69E+06	6.32E+06	4.55E+06 ± 1.05E+06
	9	10 (2/8)	-	1.23E+07	-	8.16E+06	-	-	-	-	-	-	1.02E+07 ± 2.08E+06
												Mean *R. felis*-Infection Load	**7.88E+06 ± 5.43E+05**

Table 2.2b. Copy number of *Cf*18S in individual flea lysates (shaded boxes represent infected fleas)

Trial	Exp. Day	Total no. of fleas surveyed (no. *R.felis*-infected/no. uninfected)	*Cf*18S count for individual flea lysate samples										Daily mean ±SEM *Cf*18S count for *R. felis*-infected fleas	Daily mean ±SEM *Cf*18S count for uninfected fleas
			1	2	3	4	5	6	7	8	9	10		
Trial 1	0	9 (8/1)	5.28E+07	2.21E+08	2.90E+08	3.98E+08	2.65E+08	3.34E+08	1.53E+08	1.41E+08	1.36E+08		2.31E+08 ± 4.01E+07	1.41E+08
	1	10 (10/0)	5.52E+08	2.72E+08	1.16E+08	6.87E+07	9.97E+07	6.45E+08	8.39E+08	7.71E+07	4.25E+08	1.59E+08	3.25E+08 ± 2.75E+07	n/a
	2	10 (10/0)	8.56E+08	4.07E+08	4.80E+08	2.82E+08	3.78E+08	1.24E+08	1.25E+08	2.79E+08	3.80E+08	5.97E+08	3.91E+08 ± 2.19E+07	n/a
	3	10 (9/1)	3.60E+08	2.03E+08	1.12E+08	2.59E+07	1.73E+08	8.68E+08	9.31E+08	1.61E+08	4.80E+08	4.16E+08	3.61E+08 ± 1.09E+08	4.80E+08
	4	10 (9/1)	2.17E+08	3.65E+08	6.40E+08	4.24E+08	3.01E+08	8.80E+08	5.64E+08	8.76E+07	1.38E+08	1.37E+08	3.84E+08 ± 8.99E+07	3.01E+08
	5	10 (9/1)	8.55E+08	1.93E+08	1.94E+08	2.56E+08	3.87E+07	2.05E+08	3.08E+08	2.80E+08	2.26E+07	6.93E+07	2.41E+08 ± 8.39E+07	2.56E+07
	6	10 (10/0)	2.72E+07	1.01E+08	5.21E+07	2.72E+08	4.83E+08	1.06E+08	2.82E+08	1.99E+08	1.21E+08	2.14E+08	1.86E+08 ± 4.30E+07	n/a
	7	10 (10/0)	3.33E+08	9.23E+07	5.60E+07	5.34E+07	1.56E+08	2.71E+08	2.11E+08	3.08E+08	5.26E+08	8.91E+08	2.90E+08 ± 8.12E+07	n/a
	8	10 (10/0)	7.59E+07	2.57E+08	2.59E+08	2.34E+08	1.22E+08	1.07E+08	2.12E+07	4.65E+07	2.84E+08	1.90E+08	1.60E+08 ± 3.06E+07	n/a
	9	10 (10/0)	1.01E+08	1.46E+08	1.02E+08	2.07E+08	2.16E+08	3.61E+08	7.33E+08	3.73E+08	4.26E+08	1.31E+08	2.80E+08 ± 6.28E+07	n/a
												Mean *Cf*18S Count	**2.85E+08 ± 2.35E+07**	**2.37E+08 ± 9.89E+07**
Trial 2	0	40 (35/5)	2.25E+08	1.10E+08	2.27E+08	2.40E+08	9.02E+07	1.97E+08	2.56E+08	4.41E+08	1.16E+08	4.44E+08	3.94E+08 ± 8.46E+07	5.03E+08 ± 2.52E08
			6.49E+07	2.00E+08	1.03E+08	1.42E+08	7.82E+07	1.22E+08	2.37E+08	3.70E+08	3.01E+08	1.80E+08		
			8.39E+07	2.50E+08	6.42E+07	3.09E+08	3.90E+08	9.02E+07	5.02E+08	1.13E+08	2.33E+08	3.40E+08		
			1.98E+08	1.76E+09	1.22E+09	7.32E+08	2.54E+09	4.81E+08	4.04E+08	1.47E+09	5.28E+08	4.40E+08		
	1	10 (8/2)	1.71E+08	6.92E+07	1.13E+08	2.13E+08	2.72E+08	2.31E+08	1.92E+08	1.38E+08	1.82E+08	1.88E+08	2.90E+08 ± 8.12E+07	1.63E+08 ±2.49E+07
	2	10 (6/4)	1.78E+08	1.96E+08	1.20E+08	2.68E+08	4.65E+08	3.88E+08	1.70E+08	2.61E+08	2.57E+08	3.41E+08	2.90E+08 ± 8.12E+07	2.41E+08 ± 3.79E+08
	3	12 (10/2)	1.39E+08	2.47E+08	1.30E+08	1.15E+08	2.34E+08	1.38E+08	4.11E+08	3.35E+08	6.08E+08	1.84E+08	2.91E+08 ± 5.76E+07	2.75E+08 ± 1.36E+08
			6.04E+08	3.17E+08										
	4	10 (6/4)	3.84E+08	4.12E+08	1.67E+08	5.13E+08	2.50E+08	3.94E+08	4.77E+08	3.29E+08	3.56E+08	4.22E+08	4.5E+08 ± 3.72E+07	3.18E+08 ± 5.42E+07
	5	10 (9/1)	6.94E+08	4.27E+08	2.92E+08	3.81E+08	2.33E+08	2.05E+08	3.59E+08	1.14E+08	3.64E+08	3.33E+08	3.55E+08 ± 5.25E+07	2.05E+08
	6	10 (6/4)	4.40E+08	4.18E+08	1.48E+08	1.71E+08	1.83E+08	1.69E+08	1.35E+08	2.79E+08	1.49E+08	1.81E+08	2.28E+08 ± 4.72E+07	2.27E+08 ± 6.39E+07
	7	10 (5/5)	3.22E+08	3.81E+08	5.31E+08	1.47E+08	1.87E+08	2.16E+08	1.77E+08	3.20E+08	7.82E+08	4.41E+08	3.70E+08 ± 5.95E+07	3.31E+08 ± 1.16E+08
	8	10 (8/2)	3.77E+08	5.68E+08	2.33E+08	3.57E+08	1.66E+08	2.57E+08	3.21E+08	6.60E+07	1.12E+08	1.20E+08	2.85E+08 ± 5.46E+07	1.50E+08 ± 8.37E+07
	9	10 (4/6)	1.96E+08	2.65E+07	2.36E+08	4.52E+08	2.05E+08	2.40E+08	2.01E+08	1.42E+08	3.25E+08	2.01E+08	1.94E+08 ± 1.94E+07	2.42E+08 ± 5.79E+07
												Mean *Cf*18S Count	**2.98E+08 ± 3.29E+07**	**2.65E+08 ± 4.18E+07**
Trial 3	0	27 (9/18)	1.12E+09	7.00E+08	7.14E+08	8.28E+08	1.04E+09	9.31E+08	5.17E+08	8.51E+08	5.57E+08	1.16E+09	6.57E+08 ± 9.01E+07	7.66E+08 ± 7.29E+07
			8.78E+08	1.29E+09	6.74E+08	8.22E+08	1.11E+09	5.71E+08	6.96E+08	1.22E+09	6.31E+08	4.40E+08		
			3.52E+08	4.19E+08	3.99E+08	3.96E+08	5.24E+08	4.78E+08	3.73E+08					
	1	10 (5/5)	4.61E+08	8.47E+08	4.69E+08	3.83E+08	4.78E+08	2.85E+08	2.38E+08	1.56E+08	4.08E+08	2.15E+08	3.51E+08 ± 5.90E+07	4.37E+08 ± 1.14E+08
	2	10 (5/5)	5.22E+08	1.30E+09	3.24E+08	4.21E+08	7.77E+08	5.54E+08	9.64E+08	2.64E+08	4.73E+08	6.77E+08	4.83E+08 ± 5.84E+07	7.71E+08 ± 1.76E+08
	3	10 (1/9)	5.21E+08	6.00E+08	8.31E+08	8.59E+08	3.52E+08	3.08E+08	3.99E+08	9.06E+08	5.96E+08	4.75E+08	5.21E+08	5.92E+08 ± 7.60E+07
	4	10 (6/4)	8.55E+08	5.67E+08	7.56E+08	7.29E+08	8.96E+08	7.80E+08	6.07E+08	5.76E+08	6.19E+08	6.72E+08	7.64E+08 ± 4.70E+07	6.19E+8 ± 1.99E+07
	5	10 (4/6)	2.28E+09	1.33E+09	2.91E+09	1.27E+09	1.88E+09	1.29E+09	5.07E+08	2.42E+08	2.39E+08	3.55E+08	1.19E+09 ± 6.17E+08	1.26E+09 ± 3.18E+08
	6	10 (2/8)	2.61E+09	4.03E+08	1.36E+09	1.88E+09	1.23E+09	9.19E+08	1.11E+09	9.36E+08	7.15E+08	6.50E+08	1.14E+09 ± 2.21E+08	1.19E+09 ± 2.57E+08
	7	10 (4/6)	7.49E+08	1.37E+09	7.63E+08	6.92E+08	7.42E+08	1.22E+09	1.53E+09	1.15E+09	2.81E+08	6.63E+08	8.48E+08 ± 2.16E+08	9.61E+08 ± 1.68E+08
	8	10 (3/7)	5.60E+08	9.10E+08	1.21E+09	7.41E+08	9.34E+08	1.28E+09	5.52E+08	3.23E+08	3.73E+08	8.32E+08	7.13E+08 ± 1.72E+08	7.96E+08 ± 1.41E+08
	9	10 (2/8)	1.63E+09	1.07E+09	1.44E+09	1.28E+09	1.15E+09	1.52E+09	2.00E+09	4.78E+08	8.50E+08	4.15E+08	1.18E+09 ± 1.05E+08	1.19E+09 ± 2.00E+08
												Mean *Cf*18S Count	**7.85E+08 ± 7.34E+07**	**8.58E+08 ± 5.64E+07**
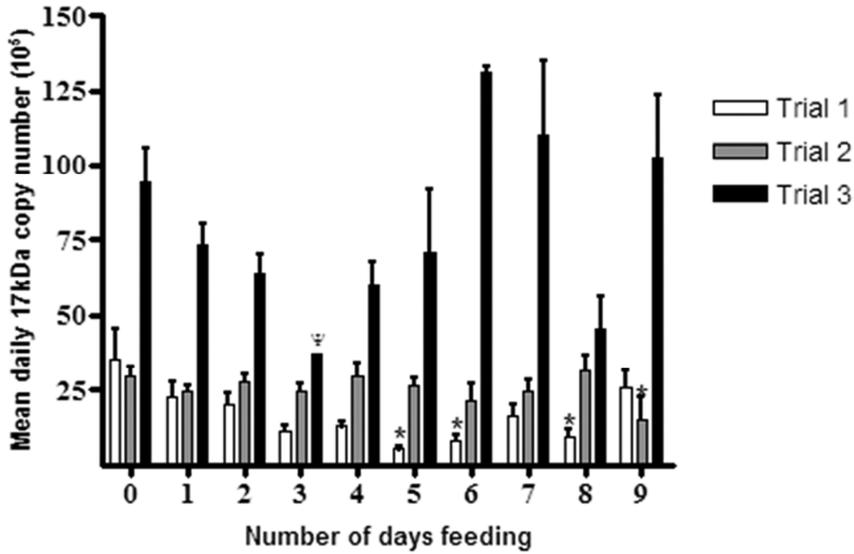
Figure 2.1. *R. felis*-**infection load in individual** *C. felis* **lysates.** For nine consecutive days, 10 fleas were randomly selected and removed from the feeding capsule situated on a cat host. In *Rickettsia* positive fleas, qPCR was used to quantify the mean (± SEM) *R. felis*-infection load. Results are presented as the mean daily *R. felis*-infection load (17-kDa antigen gene copy number) of individual, whole flea lysates during flea bloodmeal acquisition. Within each trial significant differences from Day 0 (unfed fleas) are marked with an asterisk. $^{\Psi}$In Trial 3, Day 3 only one flea was positive for *R. felis* infection.

titrated and the *Cf*18S rDNA copy number within a single flea was determined by qPCR. Serial

dilutions of pCR4-TOPO-*Rf*17kDa+*Cf*18SrDNA were used to generate a standard curve and

extrapolate *Cf*18S rDNA copy number per individual *C. felis* lysate (Table 2.2b). Within

individual trials, mean *Cf*18S copy numbers did significantly vary during the feeding period on

some days, however there was no single day where *Cf*18S copy numbers were consistently

different. For both *R. felis*-infected and -uninfected fleas, the mean *Cf*18S copy numbers

increased with each subsequent Trial, with a significant 2.6-fold increase in mean *Cf*18S copy

numbers between Trials 2 and 3. When data from all three trials were combined, significantly

greater *Cf*18S copy numbers were observed in female *C. felis* compared to male *C. felis*.

Likewise, uninfected *C. felis* had significantly greater mean *Cf*18S copy numbers compared to

R. felis-infected *C. felis*.

2.3.3. *R. felis*-Infection Load in *C. felis* Expressed as a Ratio of *Rf*17kDa/*Cf*18S

　　　　R. felis-infection of *C. felis* was further assessed as a ratio of *R. felis* and *C. felis* genes.

Examination of the published genome demonstrated that the *R. felis* 17-kDa antigen gene is a

single-copy gene (Ogata et al. 2005). In this study, the copy number of *C. felis* 18S rDNA was

estimated by Southern blot analysis. *C. felis* gDNA was digested with 5 restriction enzymes all

resulting in the presence of a single band of variable size (dependent on enzyme used) (Figure

2.2). Although each enzyme produced a single band indicating that the *C. felis* 18S rDNA gene

may be single-copy, a tandem arrangement of multiple gene copies may not be discernable using

Southern blot analysis alone.

　　　　For individual flea lysates positive for *R. felis*, *R. felis*-infection density was examined.

Assessed by qPCR, copies of *Rf*17kDa and *Cf*18S were determined relative to their positions in

the standard curve and extrapolated to the individual flea lysate. To determine *R. felis*-infection
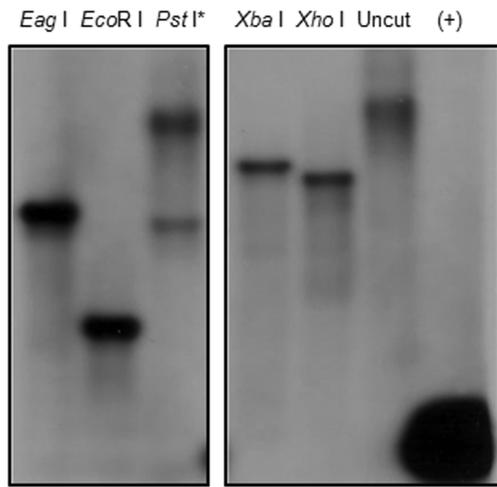
Figure 2.2. Estimation of *C. felis* 18S rDNA copy number using southern blot. *C. felis* genomic DNA (6 μg/enzyme) was digested with *Eag* I, *Eco*R I, *Pst* I, *Xba* I and *Xho* I. Uncut gDNA served as a negative control and PCR product of a portion of *C. felis* 18S rDNA served as a positive control. Genomic DNA was hybridized with *Cf*18S, a 179-bp probe, to estimate the number of 18S rDNA gene copies in *C. felis*. Digestion with each enzyme results in a single digestion product. (*DNA was not completely digested by *Pst* I. Top band is uncut DNA.)

density Rf17kDa/Cf18S ratios for individual flea samples were calculated after the logarithmic transformation of the quantity of the genes of interest (Rf17kDa and Cf18S). The mean daily Rf17kDa/Cf18S ratios for each trial are presented in Figure 2.3.

The mean ($\pm$ SEM) trial Rf17kDa/Cf18S ratio increased significantly between subsequent trials from 0.72 ± 0.0064 to 0.75 ± 0.0059 to 0.78 ± 0.0032, in Trials 1-3, respectively. When flea gender was considered, female fleas in Trials 2 and 3 had significantly greater Rf17kDa/Cf18S ratios than female fleas in Trial 1, but were not significantly different from one another. Male fleas in Trials 1 and 2 did not have significantly different Rf17kDa/Cf18S ratios from one another, but both had significantly lower ratios than male fleas in Trial 3. Comparing female and male fleas within trials, males had significantly greater Rf17kDa/Cf18S ratios in Trial 1 than females, but in Trial 2 females had significantly greater Rf17kDa/Cf18S ratios than males. Due to the limited occurrence of *R. felis* infection in male fleas in Trial 3, gender-based ratio differences could not be statistically assessed.

In individual trials, significant differences in Rf17kDa/Cf18S ratios were observed on multiple days, however, among all trials no single day was consistently significantly different from Day 0. The mean daily Rf17kDa/Cf18S ratios for all trials were combined and compared, independent of flea gender, and no significant variability in Rf17kDa/Cf18S ratio was observed by day compared to the starting point at Day 0. Analyses of the mean daily Rf17kDa/Cf18S ratio for female fleas, combining and comparing all trials, identified a significantly lower ratio on Day 6 compared to Day 0, a trend similar to that observed for females in Trial 1, but not females in Trials 2 and 3. No significant differences in mean daily Rf17kDa/Cf18S ratios for all trials combined were observed for male fleas on any day compared. Additionally, a correlation between the ratio of Rf17kDa/Cf18S and flea bloodmeal acquisition was not observed. For
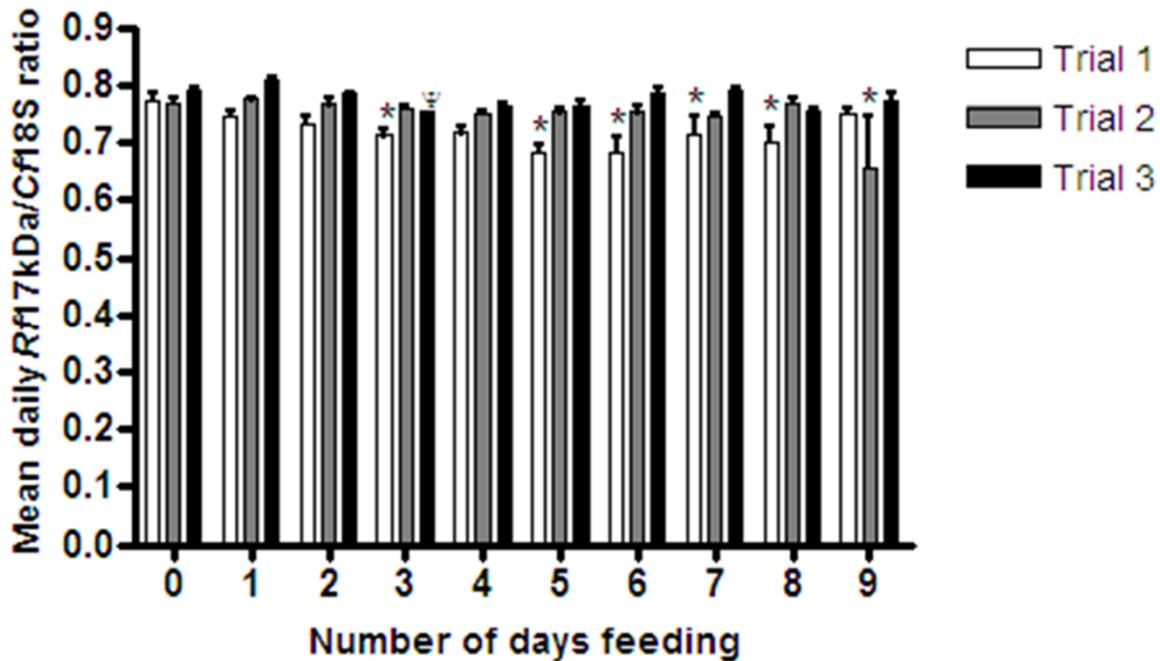
Figure 2.3. *R. felis*-**Infection Load in** *C. felis* **Expressed as a Ratio of** *R. felis* **and** *C. felis* **Genes.** For nine consecutive days 10 fleas were randomly selected and removed from the feeding capsule situated on a cat host. In *Rickettsia* positive fleas, *R. felis* infection was determined by quantifying the *R. felis* 17-kDa copy number. For *R. felis*-infected fleas, *C. felis* 18S rDNA copy number was quantified to serve as a comparison point for *R. felis* infection. *R. felis*-infection density was determined by logarithmically transforming and taking the ratio of *R. felis* 17-kDa and *C. felis* 18S rDNA (*Rf*17kDa/*Cf*18S) copy numbers and the mean (± SEM) daily *Rf*17kDa/*Cf*18S ratios for each trial were calculated. Within each trial significant differences from Day 0 (unfed fleas) are marked with an asterisk. [Ψ]In Trial 3, Day 3 only one flea was positive for *R. felis* infection. All trials have significantly higher ratios than the previous trial, indicating that rickettsial burdens are increasing in the LSU *C. felis* colony.

female fleas, during the time of peak oviposition (Day 6) there was a decrease in *Rf*17kDa/*Cf*18S

ratio. No distinct relationship was observed between *R. felis*-infection load and *C. felis* 18S copy

number. A consistent infection rate of *R. felis*, with minimal significant increases or decreases in

infection load is maintained during flea metabolically active periods.

2.4. Discussion

Both traditional and real-time PCR have been used to further characterize rickettsial

infection in fleas. Amplification of a portion of the gene encoding the rickettsial-common 17-

kDa antigen allowed for identification and subsequent differentiation by restriction fragment

length polymorphism of both *Rickettsia typhi* and *R. felis* in *C. felis* (Noden et al. 1998). In this

study, the presence of *R. felis* as the rickettsial species infecting the LSU *C. felis* flea colony was

confirmed by traditional PCR and gene sequence analysis. Real-time PCR has also been used for

quantitative assessment of *R. felis* replication and as a diagnostic tool to assess *R. felis* infection

in arthropod hosts. Replication of four isolates of *R. felis*, including the LSU strain, was

measured in XTC-2 cells treated with antibiotics; however, the techniques for establishing

rickettsial gene copy numbers were not provided and the qPCR assay utilized is not clear (Rolain

et al. 2002). More recently, a probe-based qPCR targeting rickettsial *ompB* for detection and

differentiation of *R. felis* and *R. typhi* in colonized and wild-caught *C. felis* was described (Henry

et al. 2007). It was demonstrated that crude extraction (boiled flea lysate) of DNA was an

insensitive procedure, compared to kit-based DNA extraction, resulting in limited detection of

Rickettsia. In the present report, qPCR was utilized to analyze the rickettsial load in individual

C. felis during flea feeding and oviposition. Differences in sensitivity between kit-based and

HotSHOT DNA recovery techniques utilized in the current study were undetectable. Also,

Henry *et al* (Henry et al. 2007) utilized serial dilutions of a plasmid containing a portion of the

ompB target sequence and determined the sensitivity of the assay to be 1 copy/μl; 10 times

greater sensitivity than the 17-kDa antigen gene target sequence and SYBR Green assay in the

current study. Although there is increased sensitivity in the probe-based assay compared to the

SYBR Green assay used in the current study, the large rickettsial load in LSU fleas does not

require detection of low numbers of *R. felis*. Examining gene copy numbers by qPCR is limited

in differentiating between live/dead organisms; however, DNA analysis can still provide a

reasonable assessment of infection load in hosts that are naturally infected and/or utilized in the

vertical transmission of the organism. This study provides the first application of qPCR to

examine the kinetics of *R. felis* infection within its anautogenous vector and primary reservoir,

the cat flea.

Wide dissemination of vertically maintained bacteria is common in arthropods (Dobson

et al. 1999, Cheng et al. 2000). Within the flea, *R. felis* infects many types of tissues, including

the salivary glands (Adams et al. 1990, Macaluso et al. 2008); in this study, *R. felis* infection load

was examined at the whole individual flea level. Among the *R. felis*-infected fleas, a mean of

3.9×10^6 rickettsiae during feeding was identified. All trials were significantly different in their

mean *R. felis* infection load, with Trial 1 fleas and Trial 3 fleas having the lowest and highest

individual flea rickettsial loads, respectively. Regardless of whether infection load was

quantified as total load per flea or as a ratio of *R. felis* and *C. felis* genes, rickettsial load was not

definitively affected by flea bloodmeal acquisition or oogenesis, nor was there a consistent

R. felis replication pattern observed across all trials. The significant decrease in the mean trial

*Rf*17kDa/*Cf*18S ratio on Day 6 is skewed by the results of Trial 1, in which more fleas were

positive for *R. felis*-infection. A qPCR was utilized to examine spotted fever group rickettsiae

within the tick *A. americanum* and, similar to this study, a steady level of *R. amblyommii* load

associated with bloodmeal acquisition was identified when infection was assessed on an individual tissue basis (Zanetti et al. 2008). Therefore, in the two models examined, there appears to be a balance between rickettsial load and host size in actively feeding arthropods. Future studies examining *R. felis* infection within its flea vector and determination of infection density within specific tissues (*e.g.* salivary glands and ovaries) will further elucidate the biological interactions between rickettsiae and flea hosts.

In addition to *C. felis*, *R. felis* has been molecularly identified in numerous species of wild-caught fleas, including *C. canis* (Parola et al. 2003)*, Xenopsylla cheopis* (Jiang et al. 2006), *Archaeopsylla erinacei* (Bitam et al. 2006), *Spilopsyllus cuniculi* (Schloderer et al. 2006), *Echidnophaga gallinacean* (Schloderer et al. 2006), *Anomiopsylla nudata* (Stevenson et al. 2005); and, while it is likely that the prevalence of *R. felis* in a flea population is amplified by an infectious bloodmeal, the acquisition mechanisms and stability of transmission are not known. The prevalence of *R. felis* in wild-caught fleas has been reported to be 1-20%, which is typically lower than that observed in colonized fleas (on average >50%) (Azad et al. 1997, Boostrom et al. 2002, Zavala-Velazquez et al. 2002, Rolain et al. 2003, Bauer et al. 2006). The efficient replication and vertical transmission of *R. felis* within fleas may minimize the necessity of frequent mammalian infections to maintain *R. felis* in nature; however, experimental evidence indicates the transmission cycle of *R. felis* is more complex. In laboratory reared flea colonies, such as the LSU *C. felis* colony, the presence and dynamic prevalence of *R. felis* make it a valuable tool to examine *Rickettsia*/flea interactions (Boostrom et al. 2002, Pornwiroon et al. 2007). Wedincamp and Foil (2002) investigated the efficiency of *R. felis* vertical transmission in *C. felis* without the aid of an infectious bloodmeal and demonstrated fluctuating, but decreasing prevalence through twelve generations. During the last 15 years, several independent studies

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have identified the variable prevalence of *R. felis*, ranging from 43-100% (Higgins et al. 1994, Noden et al. 1998, Henry et al. 2007, Pornwiroon et al. 2007). The LSU *C. felis* colony is maintained solely on cat hosts, but the role of cat hosts as a source of infectious bloodmeal is unclear. Cats continuously fed on by *R. felis*-infected fleas seroconvert two to four months post-exposure (Wedincamp, Jr. and Foil 2000). *R. felis* DNA has been detected in cat blood (Wedincamp, Jr. and Foil 2000); however, recovery of *R. felis* from these cats has been unsuccessful thus far [K. R. Macaluso, unpublished data]. Each trial utilized a flea-naïve cat host, minimizing the possibility of cat-derived, immune-mediated rickettsial clearance in feeding fleas. Interestingly, even when fleas are fed only on live hosts, there is variance in *R. felis* prevalence between generations as demonstrated in the current study that utilized subsequent generations of fleas for each trial. After a substantial population loss followed by a population expansion in the LSU *C. felis* colony, the prevalence of *R. felis* in the flea population neared 100% (Pornwiroon et al. 2007). In individual fleas, 3.1×10^1 to 3.74×10^5 of *Rf*17kDa copies per reaction were observed by qPCR, with only four fleas having detectable *Rf*17kDa copy numbers (in total lysate) under 1.0×10^4. Although not proven in this study, the dramatic contrast of *Rf*17kDa copy numbers of these four fleas in comparison to the remaining *R. felis*-infected fleas may suggest a role for low-level horizontal acquisition of *R. felis* in these few fleas from feeding on a shared host or larval cannibalism. Under laboratory conditions with either natural or artificial hosts, the mechanisms of prevalence and infection load fluctuations are intriguing and require further study to assess if carriage of *R. felis* is beneficial to *C. felis*.

Alternatively, there may be microbial-dependent influence on *R. felis* prevalence, as intracellular *Wolbachia* spp. in the fleas have been identified (Pornwiroon et al. 2007). *Wolbachia* spp. infect many arthropods and are readily able to manipulate their arthropod host

(*e.g.* feminization, cytoplasmic incompatibility) with a potential to impact host fitness (Fry et al. 2004, Werren 1997, Dobson et al. 1999). While *Wolbachia* spp. have been identified in fleas, their impact on flea fitness and relationship with other flea microbiota, such as *R. felis*, have not been examined (Pornwiroon et al. 2007, Gorham et al. 2003). Not only can microbiota compete with one another for host resources, they can often manipulate their environment affecting arthropod-host fitness (Werren 1997, Fry et al. 2004). For example, vertical transmission of the tick-borne human rickettsial pathogens, *R. rickettsii*, *R. parkeri*, and *R. conorii,* are associated with decreased arthropod-host fitness, whereas vertical maintenance of other *Rickettsia* spp. do not impact tick fitness (Macaluso et al. 2001, Macaluso et al. 2002, Zanetti et al. 2008). Interaction and possible competition of vertically transmitted microbiota and their potential impact on host fitness is likely complex and needs to be scrutinized. Utilization of qPCR and laboratory models of *R. felis* transmission will further elucidate mechanisms of transmission in nature.

Although no clear replication pattern was observed in actively bloodfeeding and ovipositing fleas, there was an inverse correlation between colony *R. felis*-infection prevalence and *R. felis*-infection load in individual fleas. Specifically, as the prevalence of *R. felis* decreased in our flea colony from 96% in Trial 1 to 35% in Trial 3, the mean *R. felis*-infection load in individual fleas increased 4.75-fold (Figure 2.4). Quantification of increasing *R. felis*-infection was demonstrated at the whole flea level (counting *Rf*17kDa copy numbers) and verified again when assessing infection as a ratio of *Rickettsia* and flea genes. *C. felis* 18S rDNA copies also increased in both *R. felis*-infected and uninfected fleas across all trials. The ratio of *R. felis* and *C. felis* genes increases significantly from Trial 1 to Trial 3, demonstrating that the larger fleas (greater *Cf*18S quantities) in Trial 3 have an unproportionately greater *R. felis* burden
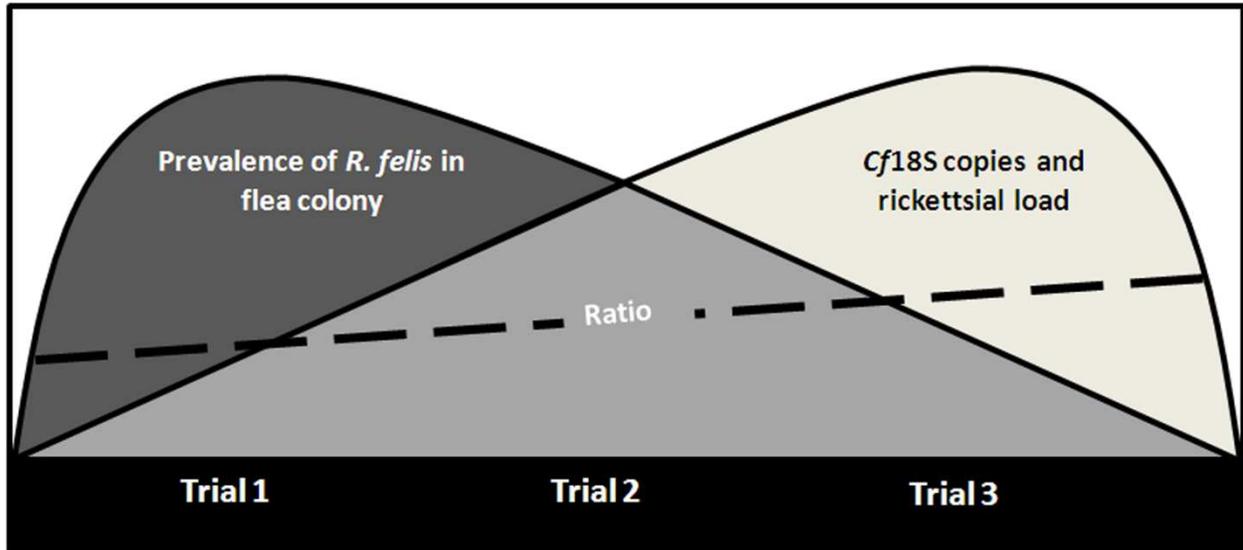
Figure 2.4. Experimental model depicting the relationship between *R. felis* and *C. felis*. Supported by the results of this study, *R. felis* prevalence and individual flea infection load in the LSU *C. felis* colony are inversely correlated. The ratio of *Rf*17kDa/*Cf*18SrDNA also increased significantly between Trial 1 and Trial 3 indicating fleas are infected at a greater density. Trials are situated within the model according to their individual results. As a population, fleas in Trial 1 had the highest prevalence of *R. felis* infection and the lowest mean individual *R. felis*-infection load. Conversely, fleas in Trial 3 had the lowest prevalence of *R. felis*-infection and the greatest mean individual *R. felis*-infection load. Trial 2 fleas represent a median demonstrating the progression of decreasing colony prevalence and increasing infection load from Trial 1 to Trial 3. The ratio of *R. felis* and *C. felis* genes increases significantly from Trial 1 to Trial 3, demonstrating that fleas in Trial 3 have a greater *R. felis* burden (higher *Rf*17kDa/*Cf*18S ratio) than fleas in Trial 1. These results indicate that at increased infection loads, *R. felis* may influence flea fitness to facilitate their own successful transmission to the next generation of fleas or to a susceptible mammalian host.

than the smaller fleas in Trial 1. These results verify the *R. felis*-infection load per individual

flea lysate findings, supporting that infection load is increasing beyond the expected proportional

increase relative to *C. felis* 18S rDNA copy number. Whether or not larger, more densely

infected fleas are more competent vectors for *R. felis* will be interesting to explore further.

Variable prevalence and infection density in *C. felis* may represent a *R. felis* ecological

maintenance strategy, in which waning prevalence signals, by an unknown mechanism, increased

infection burdens in individual fleas potentially facilitating more efficient transmission to

progeny (vertical transmission) or a reservoir host (horizontal transmission) in order to persist in

the flea population. This report is the first quantitative assessment of *R. felis* infection in fleas.

The data also suggests that the previously unrecognized horizontal transmission of *R. felis* occurs

among fleas and that prevalence of *R. felis* in the population is correlated to individual flea

rickettsial load. The results of this study will help elucidate the epidemiology of *R. felis*, an

emerging infectious pathogen, by demonstrating the dynamic prevalence and infection density

changes that occur within a flea population.

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CHAPTER 3
BLOODMEAL ACQUISITION OF *RICKETTSIA FELIS* BY CAT FLEAS

3.1. Introduction

Rickettsia felis is a gram-negative, obligate intracellular bacterium predominately

described in the cat flea, *Ctenocephalides felis*. *Rickettsia* traditionally have been divided into

two major groups: spotted fever group (SFG) and the typhus group (TG). As with early reports,

the placement of *R. felis* into either rickettsial group remains controversial and currently *R. felis*

is included in the newly formed Transitional Group (TGR) (Gillespie et al. 2007). Similar to

SFG *Rickettsia*, *R. felis* has been identified in the salivary glands of its arthropod vector

suggesting a role for transmission via salivary secretions; however, unlike most SFG *Rickettsia*,

R. felis utilizes an insect rather than an acarine vector. Insects are used as vectors for TG

Rickettsia and transmit rickettsiae vertebrates primarily by fecal contamination with subsequent

transmission to insects feeding on an infectious vertebrate bloodmeal.

Since the first description as the ELB agent in a commercial cat flea colony in 1990

(Adams et al. 1990), *R. felis* has been detected in numerous arthropod species worldwide

(reviewed in Chapter 1). Within cat fleas, *R. felis* causes a disseminated infection, described in

the midgut epithelial cells, muscle cells, fat body, tracheal matrix, ovaries, epithelial sheath of

testes and salivary glands of cat fleas (Adams et al. 1990, Macaluso et al. 2008). As specific

identification of disseminated infection in other arthropod species has not been examined, the

only currently recognized biological vector of *R. felis* is the cat flea.

R. felis-infection of vertebrates has also been documented. In humans, *R. felis* is the

etiologic agent of flea-borne rickettsiosis. Patients with *R. felis* rickettsiosis present with

symptoms similar to other rickettsial infections which include: fever, headache and fatigue

(Schriefer et al. 1994a, Zavala-Velazquez et al. 2000, Raoult et al. 2001, Znazen et al. 2006).

Peridomestic mammals that commonly serve as hosts for fleas including: dogs, cats, and opossums, have also been surveyed for *R. felis* infection to examine the prevalence of *R. felis*-infection in a population (Schriefer et al. 1994b, Richter et al. 2002, Labruna et al. 2007). Frequently, these surveys are in direct response to identification human cases of *R. felis* infection or in areas where *R. felis* has been detected in arthropods. Although serologic methods (e.g. IFA, Western blot) have traditionally been used to detect *R. felis* infection in arthropods and vertebrates, development of several molecular assays (e.g. quantitative real-time PCR – qPCR) offer greater detection sensitivity and specificity (Henry et al. 2007, Reif et al. 2008).

Laboratory and commercial cat flea colonies are currently utilized to study the transmission ecology and biology of *R. felis*. Utilizing these colonies, *R. felis* was observed to be predominantly maintained within cat flea cohorts via vertical (transovarial and transstadial) transmission as fleas reportedly remained infected over 12 generations without the aid of an infectious bloodmeal (Wedincamp, Jr. and Foil 2002). However, as generations of fleas developed a downward trend of *R. felis*-infection prevalence was observed, indicating a necessary role for horizontal transmission in maintaining *R. felis* in the flea colony population.

Additional evidence for horizontal *R. felis* transmission has come from both detection of *R. felis* in other blood-feeding arthropods and cases of *R. felis*-infection in vertebrates. Assessment of systemic *R. felis*-infection in the majority of *R. felis*-positive arthropod species has not been done; and detection of *R. felis* as the result of a recent *R. felis*-infected (not necessarily infectious to the arthropod) bloodmeal is more plausible than *R. felis* being able to infect a panoply of arthropod species. *R. felis* DNA has also been amplified from cats used as a bloodmeal source for *R. felis*-infected cat fleas (Wedincamp, Jr. and Foil 2000).

Although *R. felis* DNA has been detected in the blood of infected humans and animals, isolation of *R. felis* from a vertebrate has not been accomplished. Also, despite being described

in a multitude of potential arthropod vectors and vertebrate hosts, the specific acquisition of

R. felis by a susceptible arthropod vector from an infected bloodmeal has not demonstrated.

Because cat fleas are one natural vector, the hypothesis being tested in this study is that cat fleas

feeding on an *R. felis*-infected bloodmeal will be able to acquire the bacteria and develop a

persistent infection. Using an artificial flea feeding system the ability of fleas to horizontally

acquire *R. felis* infection via an infected bloodmeal was examined. Vertical transmission of

R. felis has been demonstrated to be an *R. felis* maintenance strategy in flea colonies; therefore

the potential of horizontally (bloodmeal) infected flea to transmit *R. felis* transovarially to their

offspring with subsequent transstadial transmission in flea progeny was also examined. A major

hindrance to control and prevention strategies, the transmission ecology of *R. felis* in nature still

remains unknown. Before the ecology of *R. felis* can be fully understood, questions concerning

the basic transmission mechanisms need to be answered. In the current study, *R. felis* acquisition

and persistent infection in previously uninfected cat fleas after exposure to an *R. felis*-infected

bloodmeal is described.

3.2. Materials and Methods

3.2.1. Source of Fleas

Newly emerged, unfed cat fleas (*Ctenocephalides felis* Bouche) were purchased from

Elward II (EL) (Soquel, CA). Cat fleas from EL Laboratory have been previously reported to be

free of *R. felis*-infection (Pornwiroon et al. 2007). Fleas were maintained using an artificial dog

(Wade and Georgi 1988) from FleaData® (Farmington, NY) and fed defibrinated bovine blood

(Rockland Immunochemicals, Inc, Gilbertsville, PA) alone, the same blood heat-inactivated,

doped with *R. felis*, or a combination of the above. Eggs, not separated from feces, were reared

to adults on sand with artificial diet (Lawrence and Foil 2002) in a 70% humidity incubator and

examined for presence of *R. felis*.

3.2.2. *Rickettsia*

Rickettsia felis (LSU), originally isolated from the Louisiana State University cat flea colony (Pornwiroon et al. 2006) was cultured in *Ixodes scapularis*-derived ISE6 cells. Cells were maintained in L15B growth medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) and 10% tryptose phosphate broth (Sigma, St. Louis, MO) at pH 6.8 to 7.0 in a humidified 5% CO_2 incubator at 32°C as previously described (Sunyakumthorn et al. 2008). Both short-form and long-form *R. felis* (Sunyakumthorn et al. 2008) were examined for their ability to infect fleas fed an *R. felis*-infected bloodmeal.

3.2.3. Artificial Bloodmeal Infection

For flea infection, half of a confluent T-75 flask of ISE6 cells >90% infected with *R. felis* was resuspended. In Trials 1 and 2, different preparations of *R. felis* in bloodmeals were examined for their ability to infect fleas exposed to *R. felis* via an infected bloodmeal and included: (i) *R. felis* in intact ISE6 cells; (ii) *R. felis* from lysed ISE6 cells (*R. felis*-infected ISE6 cells passed ten times through 23 ½ gauge needle); and, (iii) long-form *R. felis* (both intracellular and extracellular forms) (Sunyakumthorn et al. 2008). As a control, an additional group of fleas were fed uninfected, intact ISE6 cells. In Trial 3, three groups of approximately 125 mixed sex fleas were exposed to identical bloodmeal preparations of *R. felis* (approximately $5x10^9$ *R. felis*/bloodmeal preparation) in intact ISE6 cells. Also in Trial 3, a fourth group of 125 mixed-sex fleas were fed ISE6 cells to serve as a control. Exposure bloodmeals for fleas were prepared by centrifuging 1 ml of resuspended *R. felis*-infected ISE6 cultures (~5×10^9 *R. felis*) or ISE6 culture alone (control) for 10 min at 13×g speed in a microcentrifuge at 4°C. Culture medium was removed and the *R. felis*-infected or uninfected ISE6 cell pellet was resuspended in 600 μl of heat-inactivated (incubated at 56°C for 30 min), defibrinated bovine/calf blood. Fleas were allowed to freely feed on the *R. felis*-infected or uninfected bloodmeal for 24 h. After the

"exposure meal," fleas were maintained on defibrinated bovine blood (not heat-inactivated) which was replaced every 1 to 3 days for the duration of the experiment.

3.2.4. DNA Isolation

Fleas, sampled at designated time-points, were individually sexed, assigned sample numbers, placed in 0.5 ml microcentrifuge tubes, and pulverized with sterile plastic pestles in a liquid nitrogen bath. Genomic DNA (gDNA) was extracted using a modified version of the HotSHOT DNA extraction protocol (Truett et al. 2000). Briefly, individual, flea lysates were incubated at 95°C for 45 min in 20 µl alkaline lysis reagent (25 mM NaOH, 0.2 mM disodium EDTA, pH of 12), cooled to 4°C for 5 min and mixed with 20 µl of neutralizing reagent (40 mM Tris-HCl, pH of 5). All gDNA preparations were stored at -20°C. For flea feces, at designated time-points, approximately 50 mg of egg-free flea feces was collected and diluted in 200 µl of 1X PBS. Extraction of gDNA from feces was accomplished using the QIAGEN DNeasy Tissue Kit (Chatsworth, CA) according to the manufacturer's instructions for extraction of DNA from blood samples and eluted in 40 µl Buffer AE.

3.2.5. Rickettsial Detection and Quantification by Quantitative Real-Time PCR

Individual fleas were assessed for *R. felis* infection by qPCR amplification of a 157-bp portion of the *R. felis* 17-kDa antigen gene as previously described (Reif et al. 2008) and or the rickettsial outer membrane protein B (*ompB*) (Blair et al. 2004). Briefly, qPCR components and template that included 2x iTaq SYBR Green Supermix (BioRad, Hercules, CA); 100 nM of each primer; DNase/RNase-free water; and 5 µl of gDNA template (samples), water (negative control), or serial 10-fold dilutions ($1x10^8$ to 1 copy) of pCR4-TOPO-*Rf*17kDa+*Cf*18SrDNA were pre-mixed in 35 µl volumes in 96-well plates and aliquoted in triplicate 10 µl reactions on 384-well plates. The qPCR was performed with an ABI 7900HT unit (Applied Biosystems, Foster City, CA) at LSU-SVM using conditions previously described (Reif et al. 2008). Results

were analyzed with ABI 7900HT sequence detection system (SDS v2.3) software. The specificity of the assay was verified and the expected single peak for the internal control plasmid and positive gDNA samples, but not in the water (negative control) samples, was identified in the dissociation curve. Additionally, representative qPCR products from each trail were verified by gel analysis to confirm the specificity of the reaction and cloned and sequenced to confirm that fleas were infected with *R. felis*. For *R. felis*-positive flea samples a second assay was performed to determine *R. felis* infection density in individual fleas where both the *R. felis* 17-kDa and cat flea 18S rRNA genes were amplified and quantified by extrapolating the individual gene Ct values from serial dilutions of plasmid pCR4-TOPO-*Rf*17kDa+*Cf*18SrDNA, which contained single-copy portions of both genes as previously described (Reif et al. 2008). *R. felis*-infection density was quantified as the ratio of log transformed *Rf*17kDa and *Cf*18S rDNA copy-numbers (*Rf*17kDa/*Cf*18S) per individual flea.

3.2.6. Horizontal Transmission of *R. felis* Via Infectious Bloodmeal

Fleas exposed for 24 h to an *R. felis*-infected or uninfected bloodmeals were examined at weekly intervals, starting at 7-days post-exposure (dpe) for acquisition and persistence of *R. felis*-infection. At each collection point, ten fleas were randomly selected and assessed for *R. felis* infection. Genomic DNA (gDNA) was extracted from individual fleas and *R. felis* infection was determined by qPCR amplification of the rickettsial 17-kDa gene. As a quality control measure and to verify gDNA extraction was successful, *C. felis* 18S rDNA was also amplified from all samples and any sample negative for *C. felis* 18S rDNA was removed from the data set and not analyzed. For *R. felis*-positive fleas, an additional qPCR comparing the ratio of the rickettsial 17-kDa and *C. felis* 18S rDNA genes was performed to calculate the infection density of *R. felis* in individually infected fleas. In Trials 1 and 2, the success of *R. felis* infection was compared between different preparations of the *R. felis*-infected bloodmeal. In Trial 3,

variations in acquisition of *R. felis* infection were compared between three groups of fleas that were fed identical *R. felis*-infected bloodmeals.

3.2.7. Vertical Transmission of *R. felis* to Flea Progeny

In Trial 1, eggs of fleas exposed to an *R. felis*-infected bloodmeals were collected every 2 to 7 days (every time the feeding cage was changed) and reared to adults. Once reared to adult stage, gDNA from approximately 400 F1 progeny, collected over the duration of the trials, were examined for vertical transmission of *R. felis*-infection from parental fleas infected via an infectious bloodmeal to their progeny by qPCR amplification of the rickettsial 17-kDa gene.

3.2.8. Detection of *R. felis* in Cat Flea Feces

Flea feces were collected every 2 to 7 days after initial exposure to an *R. felis*-infected or uninfected bloodmeal in Trials 2 and 3. Fecal samples were examined for the presence or absence of *R. felis* gDNA by qPCR amplification of the rickettsial 17-kDa gene. In Trial 3, the weight of each fecal sample was standardized to approximately 50-mg to determine the amount of *R. felis* being shed in the feces. The viability of *R. felis* in flea feces, determined by the presence of *R. felis* 17-kDa transcripts, was also examined in Trial 3 at 9-, 21- and 28-dpe. Extraction of total RNA from flea feces was accomplished using Qiagen's RNeasy Mini Kit (Chatsworth, CA) according to the manufacturer's instructions for total RNA isolation from cells with the following modifications: approximately 50-mg of feces disrupted and homogenized with two stainless steel 4.77-mm bbs in 200 µl of Buffer RLT using a TissueLyser (Qiagen, Chatsworth, CA), for 1 min at 30 Hz. Samples were washed as directed with an extra wash in 500 µl Buffer RPE and eluted in 30 µl RNase-free water. RNA samples were DNaseI treated (Promega, Madison, WI) according to the manufacturer's instructions. From DNaseI-treated RNA samples *R. felis* 17-kDa gene-specific (same primers as above) cDNA was synthesized using SuperScript® First-Strand Synthesis System (Invitrogen, Carlsbad, CA). For all samples

no-RT controls were included to verify the absence of DNA contamination. *R. felis* viability was determined by qPCR amplification (as described above) of *R. felis* 17-kDa from prepared cDNA samples.

3.2.9. Statistical Analysis

Rickettsial load in fleas and the ratio of *Rf*17kDa/*Cf*18S were assessed after the logarithmic transformation of the quantity of the genes of interest (*Rf*17kDa and *Cf*18S). Analysis of variance, (SAS statistical package, Version 9.1.3, GLM procedure ANOVA, Cary, NC) was performed to examine potential differences between rickettsial load in fleas and ratio of *Rf*17kDa/*Cf*18S copy number over the study period; when overall significance was found, Tukey's honestly significant difference (HSD) post hoc test was used to examine pairwise differences of means of main effects. Pairwise *t*-tests of least square means were performed to determine any interaction effects between trial, gender, and experimental day for rickettsial infection load and ratio of *Rf*17kDa/*Cf*18S. An *F*-test was used for general comparisons of grouped means. For all comparisons, a *P*-value of < 0.05 was considered significantly different.

3.3. Results

3.3.1. Confirmation of *R. felis* Absence in EL Laboratory *C. felis* Colony

Previous studies have reported the EL cat flea colony to be *R. felis*-free (Pornwiroon et al. 2007). To verify absence of *R. felis*-infection in this colony, total gDNA was extracted from 20 newly-emerged unfed EL fleas (10 female and 10 male) and assessed for *R. felis*-infection by qPCR amplification of the rickettsial 17-kDa antigen and outer membrane protein B (*ompB*) genes. PCR amplification of the 17-kDa and *ompB* genes from *R. felis* maintained in cell culture and distilled water served as controls and yielded the expected positive and negative results, respectively. No rickettsial products were amplified from any flea sample, confirming previous reports (Pornwiroon et al. 2007) of the absence of *R. felis* in the EL *C. felis* colony.

3.3.2. Detection of Horizontally Transmitted *R. felis* Infection in Fleas Fed an *R. felis*-Infected Bloodmeal

In three trials, groups of fleas were fed an *R. felis*-infected or -uninfected bloodmeal for 24 h. After the 'exposure meal', in each trial, ten fleas from every group were collected weekly for as long as fleas were alive in a group or up to five weeks, and individually assessed for *R. felis* infection. The gDNA was extracted from individual fleas and *R. felis* infection was determined by qPCR amplification of a portion of the rickettsial 17-kDa antigen gene. The specificity of *R. felis*-infection in fleas was confirmed by sequencing a portion of the rickettsial 17-kDa gene from a representative subset of fleas positive for *R. felis* infection. All sequenced samples had a 100% identity to *R. felis* URRWXCal2, complete genome (GenBank accession number CP000053).

The incidence of *R. felis* infection in previously uninfected fleas was examined between the different *R. felis* bloodmeal preparations: *R. felis* in intact ISE6 cells; *R. felis* from lysed ISE6 cells; and, long-form *R. felis*. In two separate trials, all *R. felis*-infected bloodmeal preparations were capable of resulting in *R. felis*-infection in fleas, (Table 3.1). Fleas fed *R. felis*-infected ISE6 cells (intact cell prep) were observed to live the longest and acquired *R. felis*-infection at a 47% average incidence at individual collection points. *R. felis* gDNA was never detected in any fleas fed bloodmeals with uninfected ISE6 cells.

In a third trial, three groups of fleas were fed identical *R. felis*-infected bloodmeals of intact, *R. felis*-infected ISE6 cells (~5×10^9 *R. felis*/bloodmeal preparation) and assessed for variations in *R. felis* acquisition and infection. Although some variation in the incidence of *R. felis*-infection was observed between experimental groups, no individual group was significantly different from another and the mean incidence of *R. felis*-infection steadily increased from ~50% to 90% over four weeks (Figure 3.1). No significant difference was

observed in the survival of control versus *R. felis*-infected fleas; however, there were always

more fleas remaining in the *R. felis*-exposed groups than in the control groups.

3.3.3. Determination of *R. felis*-Infection Density in Fleas Infected Via an *R. felis*-Infected Bloodmeal

As *R. felis* was detected in fleas up to five weeks post-*R. felis* bloodmeal in Trials 1 and

2, *R. felis*-infection density in individual fleas was assessed in Trial 3 to determine if fleas were

gradually recovering from infection overtime, or whether *R. felis* was establishing a persistent or

increasing infection. In Trial 3 for *Rickettsia* positive fleas, *R. felis* infection was first

determined by quantifying the *R. felis* copy-number in individual fleas. Variations in *R. felis* 17-

kDa copy-number were compared between groups after the logarithmic transformation of the

mean number of *R. felis* 17-kDa copies per group at individual collection points. Between

replicate experimental groups, *R. felis* 17-kDa copy-number did not significantly differ between

groups at individual time points. When *R. felis* 17-kDa copy-number was compared by

collection time, a significant increase in *R. felis* 17-kDa copy-number was observed at 14-, 21-,

and 28-dpe, compared to 7-dpe. To serve as a comparison point, in *R. felis*-infected fleas, the cat

flea 18S rDNA gene also was amplified and quantified for individual flea samples. Variations in

C. felis 18S rDNA copy-number were compared between groups after the logarithmic

transformation of the mean number of *C. felis* 18S rDNA copies per group at individual

collection points. Between groups there was no significant difference in *C. felis* 18S copy-

number at any time point. Comparing *C. felis* 18S copy-number at individual collection points,

C. felis 18S copy-number was significantly increased at 14-, 21- and 28-dpe, compared to 7-dpe;

and was also significantly increased at 21- and 28-dpe, compared to 14-dpe. The density of

R. felis infection in individual fleas was determined by taking the ratio of the logarithmically

transformed *R. felis* 17-kDa and *C. felis* 18S rDNA copy-numbers and compared after taking the

Table 3.1. Comparison of *R. felis*-infected bloodmeal preparations on success of *R. felis*-infection in fleas. Groups of fleas were fed various preparations of *R. felis*-infected bloodmeals in order to determine the impact of infectious bloodmeal preparation on flea acquisition of *R. felis* infection. *R. felis*-infected bloodmeals were prepared by resuspending an *R. felis*/ISE6 pellet (ISE6 pellet for controls) in 600 µl of heat-inactivated bovine blood. Groups of fleas were allowed to feed for 24 h on the *R. felis*-infected bloodmeal and were subsequently maintained on defibrinated bovine blood (not heat-inactivated). Extracted gDNA from individual fleas (10 fleas/group) was assessed for *R. felis* infection, on a weekly basis for up to five weeks post-exposure bloodmeal, by qPCR amplification of the *R. felis* 17-kDa gene. For quality control purposes, *C. felis* 18S also was amplified from all samples and any sample where we were not able to amplify *C. felis* 18S was removed from analysis. On average, fleas exposed to intact *R. felis* survived the longest and had a 47% average incidence of *R. felis*-infection. (n.a. not available – all fleas in group dead)

	Group	Incidence of *R. felis* infection				
		7-dpe	13-dpe	21-dpe	28-dpe	35-dpe
	Control	0%	0%	n.a.	n.a.	n.a.
	Intact cell prep 1	55%	45%	50%	58%	86%
Trial 1	Lysed cell prep 1	18%	0%	n.a.	n.a.	n.a.
	Intact cell prep 2	60%	27%	20%	27%	n.a.
	Lysed cell prep 2	30%	29%	n.a.	n.a.	n.a.
	Long-form	30%	n.a.	n.a.	n.a.	n.a.
	Control	0%	0%	0%	0%	n.a.
	Intact cell prep 3	50%	10%	60%	50%	67%
Trial 2	Lysed cell prep 3	50%	30%	63%	n.a.	n.a.
	Intact cell prep 4	40%	50%	44%	n.a.	n.a.
	Lysed cell prep 4	50%	0%	20%	n.a.	n.a.
	Long-form	0%	0%	38%	50%	n.a.
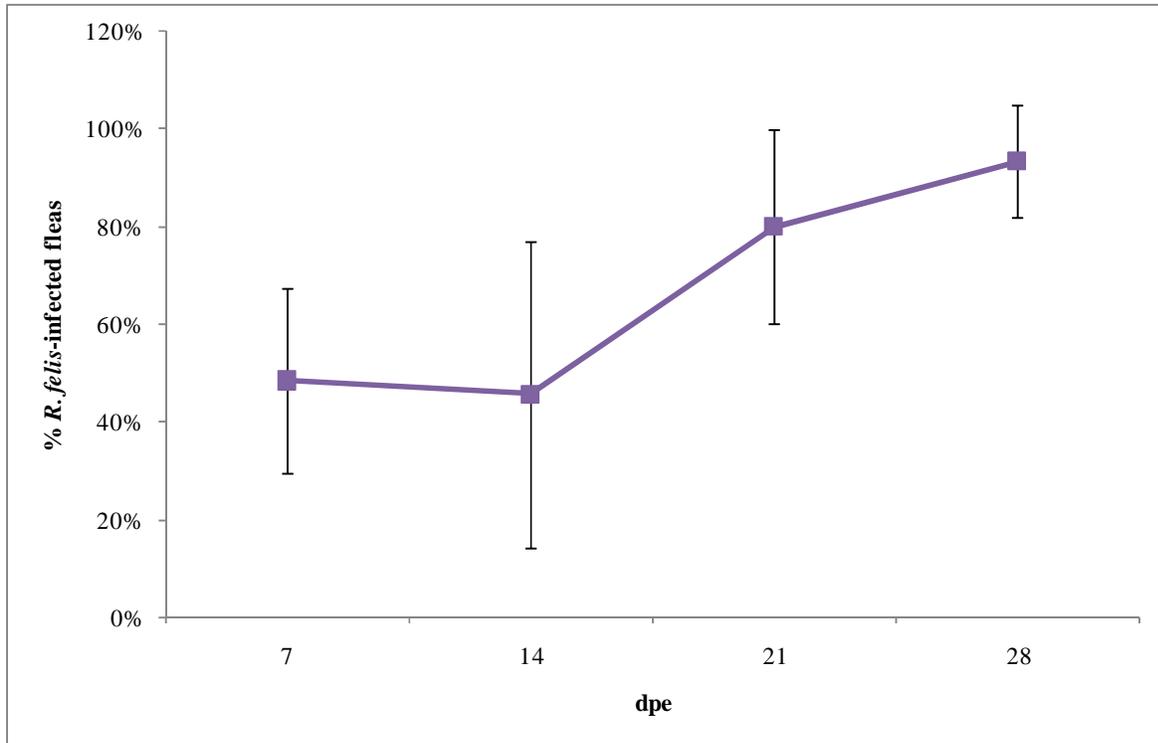
Figure 3.1. Incidence of *R. felis* infection in fleas fed an *R. felis*-infected bloodmeal. Acquisition of *R. felis*-infection in fleas was examined in three groups of fleas fed identical bloodmeals artificially infected with intact, *R. felis*-infected ISE6 cells and a control group fed a bloodmeal containing uninfected ISE6 cells only. The incidence of *R. felis* infection was assessed 7-, 14-, 21- and 28-dpe by randomly sampling ten fleas from each group. The gDNA was extracted from individual fleas and presence of *R. felis* gDNA was determined by qPCR amplification of the *R. felis* 17-kDa gene. Although the incidence of *R. felis* infection varied between experimental groups, group variation at individual time points was not significantly different. After 14-dpe a mean increase in the incidence of *R. felis*-infection was observed at each subsequent collection point. Rickettsial gDNA was never amplified from fleas in the control group.

mean (±SEM) Rf17kDa/Cf18S ratio for each collection point. Between groups there was no significant difference in the Rf17kDa/Cf18S ratio at any individual collection point. The mean logRf17kDa/logCf18S ratio was 0.50. Comparing *R. felis* infection density between collection points, a significant increase in the Rf17kDa/Cf18S ratio was observed between 7- and 14-dpe followed by a slight, but not significant, decrease at 21- and 28-dpe (Figure 3.2).

3.3.4. Detection of *R. felis* in Flea Feces

Total gDNA was extracted from samples of egg-free flea feces from each group in Trials 2 and assessed every 2 to 7 days (every time feeding cage was cleaned) for *R. felis* by qPCR amplification of the rickettsial 17-kDa gene. Not only was *R. felis* gDNA detected in the feces of fleas exposed to an *R. felis*-infected bloodmeal 2-dpe as expected, but fecal samples were positive for *R. felis* gDNA up to 25-dpe (Table 3.2). Detection of *R. felis* gDNA in flea groups administered different preparations of *R. felis*-infected bloodmeals was possible in all groups. The shedding pattern of individual fleas was not assessed in this study, only the presence or absence of detectable levels of *R. felis* gDNA in flea feces was examined. Feces from all control flea groups and environmental controls were consistently negative for *R. felis* DNA.

3.3.5. Quantification of *R. felis* in Flea Feces

In Trial 3, approximately 50-mg of egg-free feces was collected from each of three replicate groups every 2 to 7 days and examined for *R. felis* gDNA by qPCR for up to 28-dpe. The quantity of *R. felis* in fecal samples was determined by extrapolation of the *R. felis* 17-kDa copy-number against a standard curve. Variations in mean (±SD) *R. felis* 17-kDa copy-number was compared after taking the natural log of the total *R. felis* 17-kDa copy-number per sample. Over the course of the study, the presence of *R. felis* gDNA in flea feces varied; however, *R. felis* gDNA was detectable in feces up to 28-dpe. All feces for control group fleas as well as environmental controls were negative for *R. felis* gDNA. When fecal samples were standardized
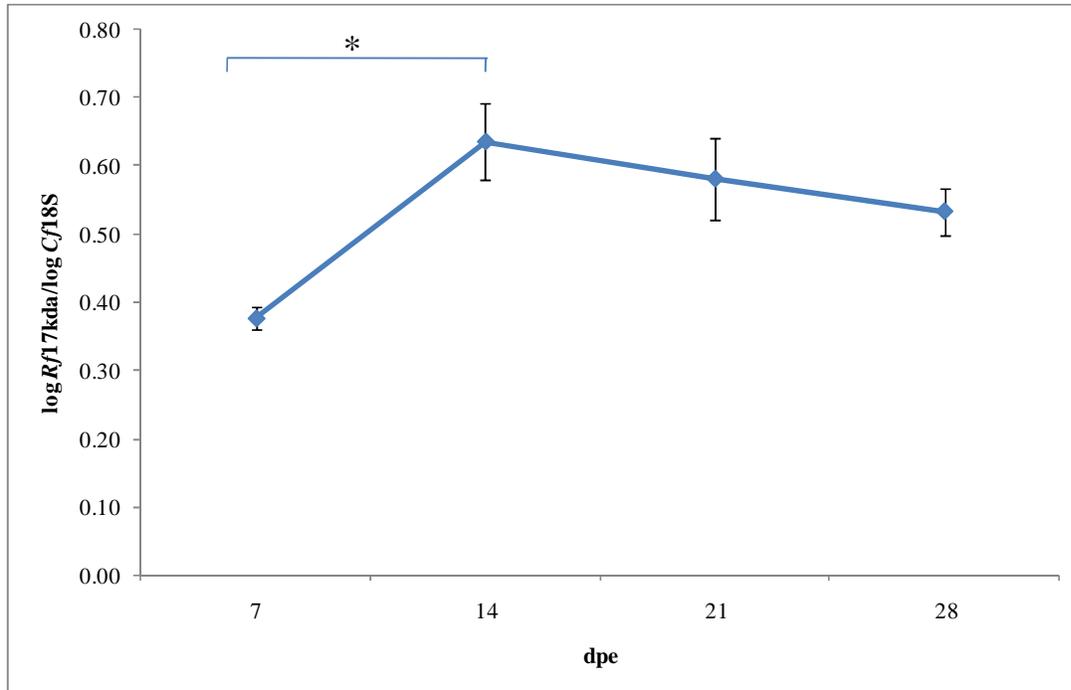
Figure 3.2. Assessment of *R. felis*-infection density in fleas fed an *R. felis*-infected bloodmeal based on ratio of *Rf*17kDa/*Cf*18S. In *Rickettsia* positive fleas, *R. felis* infection was determined by quantifying the *R. felis* 17-kDa copy number in individual fleas. To assess *R. felis*-infection density in fleas, the *C. felis* 18S rDNA copy number was quantified to serve as a comparison point. The density of *R. felis* infection in fleas was determined by taking the ratio of the logarithmically transformed *R. felis* 17-kDa and *C. felis* 18S rDNA copy numbers in individual flea samples and taking the mean (±SEM) *Rf*17kDa/*Cf*18S ratio for each collection point. Although there was some variation in *R. felis*-infection density between groups, all groups were similar to one another at individual collection points. Between 7-dpe and 14-dpe the *R. felis*-infection density in fleas significantly increased followed by a slight decrease at 21- and 28-dpe in mean *R. felis*-infection density. (*indicates significant difference)

Table 3.2. Detection of *R. felis* gDNA in feces of fleas fed different *R. felis*-infected bloodmeal preparations. Variations in the presence of *R. felis* gDNA in feces of fleas fed different *R. felis*-infected bloodmeal preparations was examined by qPCR amplification of the *R. felis* 17-kDa gene from gDNA extracts of flea fecal samples. *R. felis* gDNA was detected in all groups fleas fed *R. felis*-infected bloodmeals, independent of preparation, typically up to 21-dpe. *R. felis* gDNA was detected in feces of fleas fed *R. felis*-intact cell prep 3 for up to 25-dpe. *R. felis* gDNA was never detected in feces from any control group fleas. (n.a. = fecal samples not available)

	Detection (+/-) of *R. felis* gDNA in flea feces											
	1	3	5	7	9	12	14	17	21	25	28	35
Control	-	-	-	-	-	-	-	-	-	-	-	n.a.
Intact cell prep 3	+	+	+	+	+	+	+	+	+	+	n.a.	n.a.
Lysed cell prep 3	+	+	+	+	+	+	+	-	+	n.a.	n.a.	n.a.
Intact cell prep 4	+	+	+	+	-	+	+	+	+	n.a.	n.a.	n.a.
Lysed cell prep 4	+	+	+	+	+	+	+	-	+	n.a.	n.a.	n.a.
Long-form	+	+	+	+	+	+	+	+	+	-	n.a.	n.a.

by weight, the quantity of *R. felis* gDNA did not vary significantly among replicate groups at any collection point (Figure 3.3). Significant variations in *R. felis* 17-kDa copy-number in feces across collection time points were assessed by comparing the logarithmically transformed mean *R. felis* 17-kDa copy-number at individual collection points. Due to the limited amount of flea feces at 21- and 28-dpe, flea fecal samples were pooled between groups and therefore could not be statistically analyzed at these time points. After an initial significant decline in *R. felis* 17-kDa copies in flea feces between 2- and 4-dpe, the *R. felis* 17-kDa copy-number in samples increases overtime and is significantly greater at 28-dpe compared to 4-dpe (Figure 3.3).

The viability of *R. felis* in flea feces was determined by amplification of *R. felis* 17-kDa from cDNA synthesized of flea feces total RNA extracts. Rickettsial transcription was detected only in flea feces at 16-dpe. As with detection of *R. felis* gDNA in flea feces, the contribution of individual fleas to the presence of *R. felis* gDNA or RNA in flea fecal samples was not assessed in this study. All no-RT samples were negative for presence of *R. felis* gene products.

3.3.6. Detection of *R. felis* in Progeny

F1 progeny were collected over the course of all three trials and reared to adults. Approximately 400 individual F1 progeny from Trial 1 were examined by qPCR amplification of the rickettsial 17-kDa gene for vertically transmitted *R. felis* infections, however, no Fl fleas were positive for *R. felis*-infection.

3.4. Discussion

The horizontal transmission strategies employed by *R. felis* are not fully understood. Outside of the persistent transovarial and transstadial transmission observed in colonies of cat fleas, direct evidence for additional *R. felis* transmission strategies have only been speculated based on the detection of *R. felis* infection in vertebrates and the anatomical localization of *R. felis* in the flea. Specific transmission with subsequent infection of *R. felis* from arthropod to
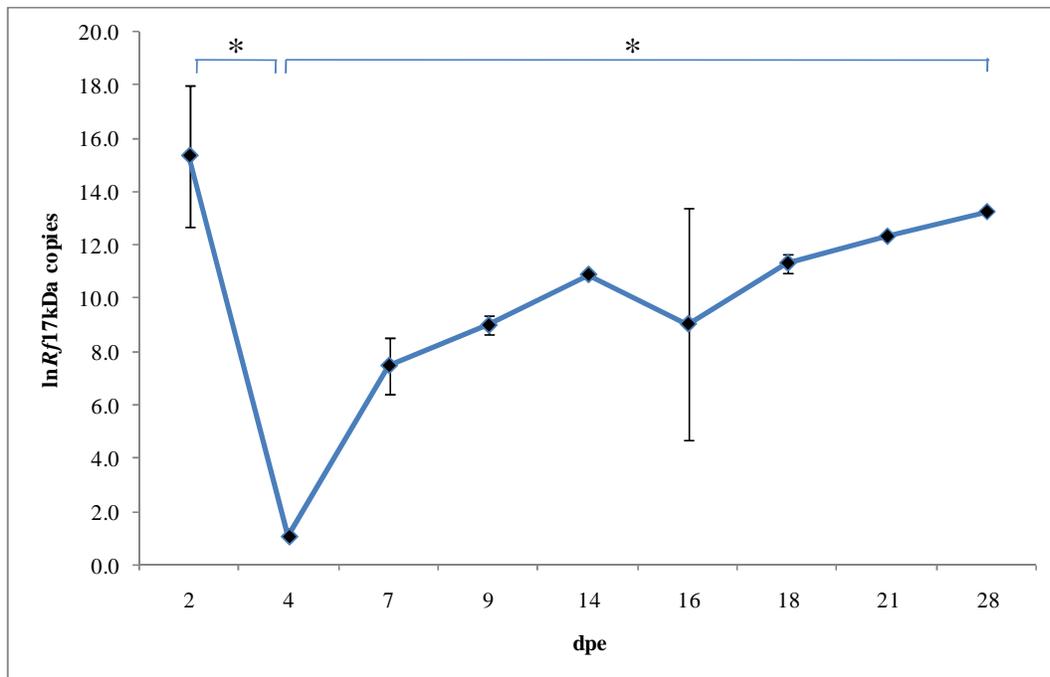
Figure 3.3. Determination of *R. felis* 17-kDa copy-number in flea fecal samples. Total gDNA was extracted from 50-mg egg-free fecal samples at 2, 4, 7, 9, 11, 14, 16, 18, 21- and 28-dpe from each of three replicate groups and assessed by qPCR for presence of *R. felis Rf*17kDa. The *Rf*17kDa copy-number was determined in samples by extrapolating the gene quantity against a standard curve and calculating the total *Rf*17kDa gene copies in 50-mg of sample. Variations in mean (±SD) *R. felis* 17-kDa copy number was compared over 28-dpe after taking the natural log of the total *R. felis* copy-number per sample. On 4- and 14-dpe, fecal samples from only one group were positive for *R. felis* gDNA. On 21- and 28-dpe fecal samples were pooled between groups due to a limited quantity of available feces as the number of fleas in each group decreased. The greatest *R. felis* 17-kDa copy-number was observed in flea feces at 2-dpe as expected after fleas had fed on *R. felis*-infected bloodmeals. Between 2- and 4-dpe *R. felis* 17-kDa copy-number significantly decreased likely as a result of fleas feeding on uninfected bloodmeals. Between 4- and 28-dpe *R. felis* 17-kDa copy-number had significantly increased, likely released from *Rickettsia*-infected midgut cells. (*indicates significant difference)

vertebrate, vertebrate to arthropod, or arthropod to arthropod (outside of vertical transmission),

has not been demonstrated. The absence of a described *R. felis* arthropod-vertebrate

transmission cycle has raised doubts as to whether or not arthropods are even able to acquire

R. felis infection from an infected vertebrate (Weinert et al. 2009), suggesting that the only

transmission route in the maintenance of *R. felis* in nature is vertical transmission between fleas.

The present study definitively demonstrates for the first time horizontal acquisition, with a

resulting persistent infection, of *R. felis* by an arthropod feeding on an infectious bloodmeal.

Previous studies have examined potential mechanisms and routes of *R. felis* horizontal

transmission. In one study, all progeny of previously uninfected fleas fed artificially *R. felis*-

infected blood for 2 to 3 days were negative for *R. felis*-infection (Wedincamp, Jr. and Foil

2002). In the current study, *R. felis* infection in fleas was detected for up to five weeks after

feeding on only a single *R. felis*-infected bloodmeal. Detection of *R. felis* in fleas for five weeks

indicates that fleas were not only able to acquire *R. felis* from an infected bloodmeal but were

able to develop a sustained infection likely for the remainder of their lives. Also in the current

study several preparations of *R. felis*-infected bloodmeals were compared. Each preparation

resulted in flea acquisition of *R. felis* infection; however, fleas fed preparations of intact, *R. felis*-

infected ISE6 cells lived the longest and had the highest average incidence of subsequent *R. felis*-

infection at individual collection points. In Trial 3, *R. felis* infection was quantified by counting

the number of *R. felis* 17-kDa copies per individual flea and *R. felis* infection density was further

examined by comparing the ratio of *R. felis* 17-kDa to *C. felis* 18S rDNA gene copies. Although

significantly more *R. felis* 17-kDa copies were observed at 14-, 21- and 28-dpe compared to 7-

dpe, a significant increase in *R. felis* infection density (*Rf*17kDa/*Cf*18S) was only observed

between 7- and 14-dpe. Fleas can be maintained on an artificial feeding system for a maximum

of six weeks, however the majority of fleas die between three and four weeks; thus, limiting the

duration of our studies and reducing sample numbers at the later collection points. The limited

number of male fleas at later collection points did not allow for comparison *R. felis* acquisition

between gender, and potential differences in gender *R. felis*-infection incidence need to be

examined in the future.

In the current study the incidence of *R. felis*-infected fleas increased over the course of

the trial. The observed increase in *R. felis*-infected fleas may be due to several factors including:

concentration of *R. felis*-infected fleas as a result of: death of uninfected flea; acquisition of new

infections via co-feeding or direct contact; or, detection of *R. felis* infection in fleas with

previously undetectable *R. felis* levels. Many rickettsial species have an unknown fitness cost to

their arthropod host (e.g. *R. bellii*); however, others are associated with a negative fitness cost

(e.g. *R. rickettsii*) to their arthropod host (Burgdorfer and Brinton 1975, Niebylski et al. 1999).

For example, *Rickettsia*-infected *Acyrthosiphon pisum* (pea-aphid) have a smaller fresh body

weight and lower total number of offspring than a *Rickettsia*-free strain (Sakurai et al. 2005).

Additional studies have described that the degree of negative fitness effects caused by *Rickettsia*

is also influenced by environmental factors (Gottlieb et al. 2006). In a study of *Rickettsia*

infection in white flies, the observed concentration of *Rickettsia* in the gut was hypothesized to

indicate some nutritional dependence on *Rickettsia* by the white fly (Gottlieb et al. 2006). Not

reported as frequently as other bacterial species (e.g. *Wolbachia* spp.), some rickettsial species

have also be documented to influence reproduction in their arthropod host (Hagimori et al.

2006). No fitness effect or reproductive manipulation has previously been observed in *R. felis*-

infected fleas (Wedincamp, Jr. and Foil 2002); although, it is interesting that in the present study

R. felis-infected fleas were more prevalent than uninfected fleas at the later collection time

points.

The increase in *R. felis* incidence observed in this current study, may be the result of

infection via co-feeding of *R. felis*-infected and uninfected fleas on the same bloodsource.

Transmission of pathogens between arthropods via co-feeding has been previously documented

in the transmission of several pathogens including, *Borrelia burgdorferi* in *Ixodes scapularis*

ticks (Patrican 1997); West Nile virus in *Culex* species (McGee et al. 2007); *Rickettsia massiliae*

in *Rhipicephalus turanicus* (Matsumoto et al. 2005); and, *Rickettsia rickettsii* in *Dermacentor*

andersoni (Philip 1959). Future studies examining the capacity of *R. felis*-infected fleas to

transmit *R. felis* infection to uninfected fleas via co-feeding are needed. Horizontal transmission

of *R. felis* via direct contact and/or sexual transmission has previously been examined where

uninfected and *R. felis*-infected fleas were placed together and mated; however, no transmission

of *R. felis* by contact or copulation was detected (Wedincamp, Jr. and Foil 2002). Also, progeny

of uninfected females mated to infected males were negative for *R. felis* (Wedincamp, Jr. and

Foil 2002), indicating that vertical transmission of *R. felis* is maternally inherited.

Finally, the increased incidence of *R. felis* in our study may be the result of detection of

R. felis-infection in fleas with previously undetectable levels. Although our qPCR assay is

sensitive enough to detect one-copy of the rickettsial 17-kDa gene, detection of *R. felis* infection

in fleas with only a few rickettsiae is difficult. As fleas were allowed to feed freely on the

R. felis-infected bloodmeal, the exact number of rickettsiae that each flea ingests is unknown and

likely varies between fleas. Future studies examining the minimum number of *R. felis* that need

to be ingested for fleas to acquire *R. felis*-infection are needed.

Although in the current study the model of *R. felis* acquisition utilizes an artificial system,

support for horizontal acquisition of *R. felis* via an infected bloodmeal may come from the

plethora of arthropod species from which *R. felis* has been detected. Most likely, detection of

R. felis in some arthropods, especially those collected off animals, may be the result of detection

of an *R. felis*-positive bloodmeal in the guts of these arthropods, rather than a truly infected

arthropod. A definitive description of *R. felis* infection in an arthropod resulting from an infected vertebrate bloodmeal has not been demonstrated. Previous studies that have attempted to demonstrate *R. felis* acquisition by an arthropod from an infected animal have not been successful. In one study, uninfected fleas were fed on *R. felis* sero-positive cats however, all fleas and their progeny were negative for *R. felis* infection (Wedincamp, Jr. and Foil 2002). A seropositive status of the cats in that study does not imply that these animals had an active infection, and perhaps at the time of flea feeding an active *R. felis* infection in the cats had already concluded. However, in another experiment on the vertical transmission, *R. felis* prevalence waned (63% to 2.5% over 12 generations) in fleas artificially fed uninfected bloodmeals, versus fleas fed on a cat host that retained a 65% *R. felis* infection prevalence (Wedincamp, Jr. and Foil 2002), supporting the likelihood of flea acquisition of *R. felis* via a bloodmeal.

Transmission of *R. felis* from an infected vertebrate to a susceptible arthropod vector likely requires a minimum threshold infection in the vertebrate (e.g. during periods of bacterimia) for a feeding arthropod to ingest a sufficient amount of bacteria to become infected. The number of ingested bacteria necessary to result in arthropod infection can also vary between bacterial species and strains. For example, *R. typhi* can only be acquired by feeding fleas during periods of rickettsemia (days 3-20 post-inoculation) (Farhang-Azad et al. 1983). The efficiency of *R. typhi* transmission requires only the ingestion of a few rickettsiae for the rat flea to become infected and subsequently able to transmit *R. typhi* to another vertebrate (Vaughan and Azad 1990). The high transmission efficiency of *R. typhi* to feeding fleas was attributed to several factors including the small size of the rickettsiae; lack of peritrophic membrane formation; rapid breakdown of blood cells; and rhythmic contraction and expansion of the midgut circulating the bloodmeal and increasing the likelihood of rickettsial-epithelial cell contact (Vaughan and Azad

1990).   Because fleas are able to acquire a sustained *R. felis*-infection from an infected

bloodmeal (the current study), further studies utilizing an *in vivo* model animal model of

arthropod acquisition of *R. felis* from infected bloodmeals will be useful in defining additional

parameters of bloodmeal *R. felis* transmission.

The predominant route of transmission for most insect-transmitted *Rickettsia* (e.g.

R. typhi and *R. prowazekii*) is via fecal transmission (Azad 1990). As the cat flea serves as the

only known biological vector and host of *R. felis*, the potential of horizontal transmission via

fecal transmission has previously been examined. *R. felis* DNA was detected in the feces of fleas

that fed on bovine blood containing *R. felis*-infected flea homogenates, but not human blood

containing *R. felis* from culture six days post-*R. felis* meal (Wedincamp, Jr. and Foil 2002).

Acquisition of *R. felis* infection by flea larvae feeding on feces, flea eggs, and younger instar

larvae positive for *R. felis* gDNA was also examined, but all resulting adults were negative for

R. felis infection (Wedincamp, Jr. and Foil 2002).

In the current study, potential fecal transmission was also examined and *R. felis* gDNA

was detected in feces not only directly after an *R. felis*-infected bloodmeal, but also up to 28-days

post-exposure. The exact number of *R. felis* shed by an individual flea could not be determined

by the assay used as feces was samples from groups of fleas, not individual fleas, which could

contribute to the variable presence of *R. felis* DNA observed over time in any one group. The

high *Rf*17kDa copy-number at 2-dpe likely reflects the large exposure dose of *R. felis* ($\sim 5 \times 10^9$

R. felis/exposure meal) in the infected bloodmeals, followed by the significant decrease observed

at 4-dpe as fleas continued to feed on uninfected blood. *R. felis* has been documented to infect

the midgut epithelium of fleas (Adams et al. 1990); therefore, lysis of a heavily infected cells

would result in *R. felis* being released into the gut and shed with the feces. The continued

presence of *R. felis* gDNA in flea feces at later time points indicates fleas are successfully

infected with *R. felis*, and the increase in *R. felis* 17-kDa copy-number starting at 14-dpe indicates that *R. felis* has successfully infected, replicated, and lysed (or released by some other mechanism) gut cells. However, detection of *R. felis* gDNA in flea feces does not imply viability of *R. felis* in feces. To address the viability of *R. felis* in flea feces, amplification of rickettsial RNA from a portion of feces was attempted. Although *R. felis* RNA was only detected at 21-dpe, the presence of any viable *R. felis* in flea feces may indicate an additional transmission route and further studies on the viability and infectivity of *R. felis* in flea feces are needed. Fecal transmission of other rickettsial species traditionally transmitted by saliva during arthropod feeding, have also been studied. Under experimental conditions *R. rickettsii* and *R. conorii*, both tick-transmitted rickettsial species, have been identified in louse feces by IFA and PCR amplification of the rickettsial *ompA* gene (Houhamdi and Raoult 2006). As with *R. rickettsii* and *R. conorii* being detected in lice feces, the viability and infectiousness of *R. felis* in flea feces is unknown and additional studies will be needed to determine if any of these rickettsial species, in this medium, are infectious to either arthropods or vertebrates.

Arthropods are the primary host of all rickettsial species and many rickettsial species are inherited and maintained via transovarial and transstadial transmission within arthropods (Sakurai et al. 2005). Vertical transmission of *R. felis* has been described in detail and is the primary transmission strategy in the LSU cat flea colony (Wedincamp, Jr. and Foil 2002). Although *R. felis* acquisition and sustained infection in fleas was demonstrated in the current study, all F1 progeny examined for vertically transmitted *R. felis*-infection were negative. As vertical transmission has been described to be the predominant maintenance strategy of *R. felis*, the lack of transmission to F1 progeny in this study is interesting. Several factors may influence successful vertical transmission of *R. felis* to progeny of horizontally infected parents including: meeting a minimum threshold infection density; duration or number of exposures to an *R. felis*-

infected bloodmeal; dissemination to reproductive tissues; and, competing resident microbiota.

In a previous study where *R. felis* infection density in fleas was examined the ratio of rickettsial 17-kDa and cat flea 18S rDNA gene copy numbers were greater (mean log*Rf*17kDa/log*Cf*18S = 0.75) than the levels of infection density observed in *R. felis*-infected fleas in this study (log*Rf*17kDa/log*Cf*18S = 0.50) (Reif et al. 2008). In the current study, *R. felis* infection density increased over the course of time, whereas in the previous study, *R. felis* infection density remained constant over time. The observed difference between the two studies is likely due to the fact that fleas in the earlier study were vertically infected with *R. felis* versus fleas in this study that horizontally acquired *R. felis* via an infected bloodmeal. The absence of *R. felis* infection in progeny in this study may be due to fleas not reaching a threshold level of infection necessary for vertical transmission. Future studies examining the relationship between infection density and successful vertical transmission are needed.

The absence of *R. felis* in F1 progeny via vertical transmission in this study may be the result of incomplete dissemination of *R. felis* in the flea. As previously reported, *R. felis* is a disseminated infection in naturally infected cat fleas and has been identified in the midgut epithelium, ovaries, salivary glands, etc. (Adams et al. 1990, Macaluso et al. 2008). In the current study, the persistent detection of *R. felis* gDNA in flea feces and an absence of infected progeny may suggest that *R. felis* infection is concentrated in flea gut tissues and is not disseminating to other tissues in the flea such as the reproductive tissues. Future studies examining the dissemination of *R. felis* in fleas horizontally infected by feeding on an infected bloodmeal are needed.

One reason for restriction of *R. felis* to specific tissues may be the presence of other flea microbiota. In the EL Laboratory cat flea colony several other bacterial species have been identified including *Wolbachia* spp. and *Staphylococcus* spp. (Pornwiroon et al. 2007).

Wolbachia are also vertically maintained bacteria and may compete with a newly introduced *R. felis* infection in tissues such as the ovaries, preventing *R. felis* from also being vertically transmitted. Competition for vertical transmission has been previously reported between rickettsial species and also between *Rickettsia* and other bacterial species. Among rickettsial species an interference phenomenon has been described where establishment of one rickettsial species inhibits the transovarial transmission of a horizontally acquired second rickettsial species (Burgdorfer 1988, Macaluso et al. 2002). Additionally, *Rickettsia* species may be able to overcome competition pressures by other microbiota, such as in the pea aphid, where *Rickettsia* was observed to significantly suppress the population of the essential *Buchnera* symbiont (Sakurai et al. 2005).

In conclusion this study definitively demonstrates the ability of cat fleas to acquire *R. felis* infection with subsequent development of a persistent infection, likely for their lifespan, after a single *R. felis*-infected bloodmeal. Acquisition of *R. felis* by this route results in infection densities similar to fleas vertically infected with *R. felis*. Further studies are needed to examine the dissemination of horizontally acquired *R. felis* in cat fleas and any interactions with other flea microbiota that could influence vertical transmission to progeny. Also, additional studies examining the viability and infectiousness of *R. felis* in flea feces are needed as *R. felis* gDNA can be detected in flea feces almost one month after infection. This model confirms that fleas are indeed able to acquire *R. felis* infection from an *R. felis*-infected bloodmeal and should serve as a platform to develop *in vivo* models of *R. felis* infection and horizontal transmission between arthropods and vertebrates. Knowledge of *R. felis* transmission strategies is essential for developing a better understanding of *R. felis* ecology.

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CHAPTER 4
DEVELOPING A MURINE MODEL OF *RICKETTSIA FELIS* INFECTION

4.1. Introduction

The agent of flea-borne rickettsiosis, *Rickettsia felis* is currently classified as an emerging pathogen (Parola et al. 2005). The first human case of *R. felis* infection occurred in Texas in 1994 (Schriefer et al. 1994); since then human cases of flea-borne rickettsiosis have been reported in 12 countries around the world including in: Spain (Perez-Arellano et al. 2005, Bernabeu-Wittel et al. 2006, Nogueras et al. 2006, Oteo et al. 2006), Germany (Richter et al. 2002), France (Raoult et al. 2001), Brazil (Raoult et al. 2001, Galvao et al. 2006), Mexico (Zavala-Velazquez et al. 2000, Galvao et al. 2006, Zavala-Velazquez et al. 2006), Thailand (Parola et al. 2003), Taiwan , South Korea (Choi et al. 2005), Laos (Phongmany et al. 2006), Tunisia (Znazen et al. 2006), and Egypt (Parker et al. 2007). In addition to flea-borne rickettsiosis, the disease associated with *R. felis* infection has also been referred to as: cat flea rickettsiosis (Eremeeva et al. 2008), cat flea typhus (Azad et al. 1997), flea-borne spotted-fever (Parola et al. 2005).

In humans, clinical presentation of flea-borne rickettsiosis is not well defined. Commonly reported symptoms/signs are typical of other rickettsial infections and include fever, rash, headache, myalgia and eschar at the bite site with potential progression to neurological and visceral involvement. The non-specificity of recognized symptoms and lack of a specific case definition have likely resulted in misdiagnosis or lack of diagnosis of human cases. Traditionally, diagnosis of rickettsial diseases have been accomplished using serological tests; however, the high cross-reactivity between rickettsial species often makes an exact diagnosis not possible. Recently, more sensitive molecular assays have been developed that are able to specifically identify *R. felis* infection (Henry et al. 2007).

Frequently, in areas where human cases of flea-borne rickettsiosis have been reported, surveys of *R. felis*-infection in local arthropods and peridomestic animals have also been conducted. In every country where a human case of flea-borne rickettsiosis has been reported except Tunisia and Egypt, *R. felis* has been successfully detected in local arthropods. The arthropod species *R. felis* has been most commonly detected from is the flea, specifically the cat flea (*Ctenocephalides felis*). The cat flea is currently the only described biological vector of *R. felis* (Wedincamp, Jr. and Foil 2002). Surveys of local peridomestic animals have also been conducted and *R. felis* has been detected by both molecular and serologic means in opossums (Boostrom et al. 2002) and dogs (Richter et al. 2002).

Despite numerous reports of *R. felis* infections in fleas and mammals, the transmission mechanisms between invertebrate and vertebrate hosts is undefined. Although *R. felis* has been identified molecularly in both arthropods and vertebrates, the direct transmission from an infected arthropod to susceptible vertebrate with subsequent vertebrate infection has not been described. Similar to other pathogenic *Rickettsia* species, flea acquisition of *R. felis* likely requires a persistent infection within the vertebrate host. Before studies can examine the transmission mechanisms between arthropod and vertebrate hosts, a basic understanding of *R. felis* infection in both an arthropod host and vertebrate host, independent of each other is needed. Therefore, development of a murine model of *R. felis* infection was attempted.

Animal models of infection are useful tools in studying disease etiology. Previous vertebrate models of rickettsial infection have provided invaluable insight to understanding infection and transmission parameters, and the associated disease pathology (Perez-Gallardo and Fox 1946, Feng et al. 1993, Walker et al. 1994). Currently there is no vertebrate model of *R. felis* infection. Despite numerous reports of *R. felis* infections in fleas and mammals, the transmission mechanisms between invertebrate and vertebrate hosts is undefined. Characterizing

the infectivity of *R. felis* in vertebrate hosts is critical to deciphering the transmission cycle of *R. felis* in nature. Prior to flea/host transmission studies, a suitable animal of *R. felis* infection must be identified. The hypothesis of this study is that inoculation of a vertebrate host with *R. felis* will result in a disseminated infection and subsequent disease.

Towards understanding *R. felis* infection parameters, including pathogenesis and transmission dynamics the specific objectives of this study are to determine an appropriate mouse strain for studying *R. felis* infection; develop a quantitative real-time PCR (qPCR) assay to detect and quantify *R. felis* infection in a mouse model; examine multiple tissues to determine any *R. felis* tissue trophism; and, compare resulting infection from two different *R. felis* doses.

4.2. Materials and Methods

4.2.1. *Rickettsia* and Preparation of Inoculums

Rickettsia felis (LSU), present in naturally infected cat flea colony maintained at LSU for the past 25 years, was subsequently isolated and cultured in a tick-derived cell line (ISE6) (Pornwiroon et al. 2006). For mouse inoculations, *R. felis* (LSU), passage 4, was partially purified from ISE6 cells and inoculation doses were quantified as previously described (Sunyakumthorn et al. 2008). Briefly, *R. felis* was released from host cells via passage through as 27 ½ g needle ten times and then filtered through a 2 μM syringe filter. Rickettsial viability and enumeration for inoculation doses were accomplished using a *Bac*Light Live/Dead staining kit (Molecular Probes, Eugene, OR), and rickettsiae were counted in a Petroff-Hausser bacteria counting chamber using a Leica microscope as previously described (Sunyakumthorn et al. 2008).

4.2.2. Mice

Five-to-six week-old male C3H/HeN mice (Harlan Sprague-Dawley, Indianapolis, IN) were housed in groups of five and were allowed access to food and water *ad libidum*. Mice were

intravenously inoculated in the lateral tail vein and sacrificed at pre-determined time-points by initial anesthetization with isofluorine followed by cardiac centesis and cervical dislocation. Blood and various tissues were collected and assessed for presence of *R. felis* gDNA as described below. All animal work was conducted in accordance with protocols approved by the LSU Institutional Animal Care and Use Committee, and an approved protocol (#07031) is on file in the office of the Division of Laboratory Animal Medicine at LSU-SVM.

4.2.3. Experimental Design

Sixty five-to-six week old male mice were randomly divided into three groups and intravenously inoculated with *R. felis* in 100 μl of culture medium or culture medium alone in the lateral tail vain. The three groups were identified as: (1) high-dose group (n=24); (2) low-dose group (n=24); (3) control group (n=12). For every collection time point, six mice for both the high- and low-dose groups and four mice for the control group were sacrificed and assessed for *R. felis* infection (Table 4.1). Mice in the high-dose group were inoculated with $1x10^6$ *R. felis* and sacrificed at 1-, 3-, 6-, 14-days post-inoculation (dpi). Mice in the low-dose group were inoculated with $1x10^3$ *R. felis* and sacrificed at 3-, 8-, 14- and 19-dpi. Mice in the control group were inoculated with ISE6 cell lysate and sacrificed at 6-, 14-, and 19-dpi. At each collection point, spleen, liver, kidney, heart, lung, testicle, skin, cerebrum and cerebellum were collected and divided into three portions for subsequent DNA extraction, RNA extraction, or histological preparation. Samples for DNA and RNA extraction were immediately snap-frozen in liquid nitrogen upon collection; and, samples for histology were stored in 10% neutral buffered formalin. During cardiac centesis, blood was collected into tubes with EDTA and reserved for subsequent DNA extraction.

4.2.4. DNA Isolation

Extraction of gDNA from tissue samples was accomplished using QIAGEN DNeasy

Table 4.1. Experimental timeline. Timeline for collection of tissue samples from mice in high-dose, low-dose, and control groups. At each collection point, blood and the following tissues were collected: heart, lung, liver, spleen, kidney, skin, cerebrum, cerebellum, and testicle. Portions of each tissue for DNA and RNA extraction were immediately frozen in liquid nitrogen and stored at -80°C, and portions of tissue for histology processing were preserved in 10% neutral buffered formalin.

Group	day(s) post-inoculation (dpi)																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
High-dose ($1x10^6$)	X		X			X								X					
Low-lose ($1x10^3$)			X					X						X					X
Control (ISE6 lysate)			X		X									X					X

Tissue Kit according to the manufacturer's instructions. Briefly, 25-mg samples of tissue (except for spleen, ~10-mg) were manually homogenized in 200 μl Buffer ATL and 20 μl Proteinase K and incubated for at least 6 hr at 56°C. Skin samples were initially finely ground in a liquid nitrogen bath and then processed as the other samples. Further sample lysis and wash steps were performed as directed with an additional 500 μl wash with Buffer AW2 to help reduce protein contamination. All samples were eluted in 50 μl Buffer AE and stored at -20°C. Extraction of gDNA from blood was accomplished using the QIAGEN DNeasy Tissue Kit (Chatsworth, CA) according to the manufacturer's instructions and eluted in 40 μl Buffer AE.

4.2.5. RNA Isolation and cDNA Synthesis

Extraction of total RNA from ~25 mg mouse tissue samples was accomplished using Qiagen's RNeasy Mini Kit (Chatsworth, CA) according to the manufacturer's instructions for total RNA isolation from tissues. Briefly, tissue disruption and homogenization was performed by combining the tissue samples and two stainless steel 4.77-mm bbs in a 2-ml microcentrifuge tube containing 200 μl of Buffer RLT and shaking in a TissueLyser (Qiagen, Chatsworth, CA), for (2x) 3 min at 30 Hz. Further sample lysis and wash steps were performed as directed and samples were eluted in 30 μl RNase-free water. RNA samples were DNase I treated (Promega, Madison, WI) according to the manufacturer's instructions. From DNase I-treated RNA samples *R. felis* 17-kDa gene-specific (same primers as above) cDNA was synthesized using SuperScript® First-Strand Synthesis System (Invitrogen, Carlsbad, CA). For all samples, no-RT controls were included to verify the absence of DNA contamination. *R. felis* viability was determined by qPCR amplification (as described above) of *R. felis* 17-kDa from prepared cDNA samples.

4.2.6. Detection of Rickettsial gDNA and cDNA by Quantitative Real-time PCR (qPCR)

Individual tissues were assessed for *R. felis* infection by qPCR amplification of a 157-bp

portion of the *R. felis* 17-kDa antigen gene as previously described (Reif et al. 2008) or the

rickettsial outer membrane protein B (*ompB*) (Blair et al. 2004). Briefly, qPCR components and

template that included 2x iTaq SYBR Green Supermix (BioRad, Hercules, CA); 100 nM of each

primer; DNase/RNase-free water; and 5 μl of gDNA/cDNA template (samples), water (negative

control), or serial 10-fold dilutions (1x10^8 to 1 copy) of pCR4-TOPO-*Rf*17kDa were pre-mixed

in 35 μl volumes in 96-well plates and aliquoted in triplicate 10 μl reactions on 384-well plates.

The qPCR was performed with an ABI 7900HT unit (Applied Biosystems, Foster City, CA) at

LSU-SVM using conditions previously described; see Chapter 2. Results were analyzed with

ABI 7900HT sequence detection system (SDS v2.3) software. The specificity of the assay was

verified and the expected single peak for the internal control plasmid and positive gDNA

samples, but not in the water (negative control) samples, was identified in the dissociation curve.

Additionally, representative qPCR products from each trail were verified by gel analysis to

confirm the specificity of the reaction and cloned and sequenced to confirm that mice were

infected with *R. felis*. For *R. felis*-positive liver and spleen samples a second assay was

performed to determine *R. felis* infection density in 100 ng of tissue where both the *R. felis* 17-

kDa (Reif et al. 2008) and the murine adipsin (complement factor D, *cfd*) gene (Cfd1461 5'-

CAGTTTCTTGCTGGCTATTGG-3', Cfd1570 5'-CCACGTAACCACACCTTCG-3') were

amplified and quantified by extrapolating the individual gene Ct values from serial dilutions of

plasmid pCR4-TOPO-*Rf*17kDa+*Mm*Cfd, which contained single-copy portions of both genes as

previously described (Reif et al. 2008). *R. felis*-infection density was quantified as the ratio of

log transformed *Rf*17kDa and *Mm*Cfd copy-numbers (*Rf*17kDa/ *Mm*Cfd) per tissue sample.

<u>4.2.7. Histology</u>

Portions of mouse spleen, liver, kidney, heart, lung, testicle, skin, cerebrum, and

cerebellum were preserved in 10% neutral buffered formalin, infiltrated with paraffin, sectioned

at 5 µM, stained by hematoxylin and eosin, and examined by light microscopy. Spleen samples

were scored for percentage of white pulp, extramedullary hematopoiesis (EMH) and splenitis.

Also for each spleen sample three periarteriolar lymph sheaths (PALS) were measured and the

means compared between samples. Liver samples were scored for cellular infiltrates,

inflammation, vacuolization, and sinus neutrophilia. Heart samples were assessed for

mitochondrial mineralization, myocardial degeneration, and myocardial necrosis. Mouse lung,

kidney, testicle, brain and skin samples were observed for any signs of pathology. Samples were

blinded and histopathology was scored by a board certified veterinary pathologist.

4.2.8. Statistical Analysis

Results from blinded histology scores were ranked and analyzed as metric data (SAS

statistical package, Version 9.1.3, GLM procedure, Cary, NC). High-dose and control group

spleen, liver, and heart samples were examined for potential differences between groups and

over the study period; when overall significance was found, Tukey's honestly significant

difference (HSD) post hoc test was used to examine pairwise differences of means of main

effects. Pairwise *t*-tests of least square means were performed to determine any interaction

effects between group and experimental day. For all comparisons, a *P*-value of < 0.05 was

considered significantly different.

4.3. Results

4.3.1. Course of Disease

No mice inoculated with either $1x10^3$, $1x10^6$ *R. felis* or control mice died or developed

overt disease. Typical signs of rickettsial infection including ruffled fur, lethargy, and hunched

posture were not observed in any group.

4.3.2. Detection of *R. felis* DNA in Mouse Blood and Tissues

For every mouse in the high-dose and control group, gDNA was extracted from portions

of spleen, liver, kidney, heart, lung, testicle, skin, cerebrum, and cerebellum. For low-dose

group mice gDNA was extracted from only liver, spleen, and heart samples. *R. felis* infection of

these tissues was determined by qPCR amplification of the rickettsial 17-kDa antigen gene from

100 ng of total gDNA. All spleen and liver samples 1- and 3-dpi were positive for *R. felis* gDNA

(Table 4.2). On 6- and 14-dpi, the prevalence of *R. felis* in the spleen and liver had decreased to

50% or less. In the high-dose group, *R. felis* gDNA was also detected in heart, lung, brain

(cerebrum and cerebellum), kidney, testicle, and skin samples, although not in as high a

frequency as in the liver and spleen (Table 4.2). In the low-dose group, *R. felis* was only

detected in the spleen of one mouse 8-dpi and in the heart of 1 mouse also at 8-dpi. Because

R. felis gDNA was only detected in two tissue samples in the low-dose group, gDNA was only

extracted from spleen, liver, and heart samples. Additionally, due to the infrequent detection of

R. felis in these tissue, additional gDNA extraction and examination of the corresponding lung,

brain, kidney, testicle, and skin samples was not completed. *R. fel*is gDNA was never detected

in gDNA extracted from mouse blood in any group. All control group mouse tissues were

always negative for *R. felis*.

4.3.3. Determination of *R. felis* Quantity in High-Dose Group Mice Liver and Spleen Samples

Because *R. felis* gDNA was most frequently detected in liver and spleen samples of high-

dose group mice an additional qPCR was performed to determine the quantity of *R. felis* present

in these tissues. All tissue gDNA samples were standardized to 100 ng. Estimation of *R. felis*

quantity was determined using qPCR to quantify the *R. felis* 17-kDa copy-number against a

standard curve (1 to 1×10^8 copies). To serve as a comparison point the murine single-copy

adipsin (*cfd*) gene (Min and Spiegelman 1986) quantity was also determined against the standard

curve. In the liver, the range of *R. felis* infection was determined to be one *R. felis* for every 100-

1100 host cells, with a mean infection of one *R. felis* per 500 host cells. In the spleen, the range

Table 4.2. Detection of *R. felis* gDNA in mouse tissues. Samples of spleen, liver, heart, lung, brain, kidney, testicle, and skin were collected from all mice. Extracted gDNA from tissue samples was examined by qPCR for the presence of *R. felis* 17-kDa gene.   A) Percentage of high-dose group mice positive for *R. felis* gDNA in tissue samples at 1-, 3-, 6-, and 14-dpi (n=6 per collection point). B) Percentage of low-dose group mice positive for *R. felis* gDNA in tissue samples at 3-, 8-, 14-, and 19-dpi (n=6 per collection point). Because *R. felis* was rarely detected in low-dose group mice liver, spleen, and heart samples examination of the remaining tissues for *R. felis* gDNA was not done. Mice in the control group were negative for *R. felis* gDNA at all collection points.

A) High-dose group mice

Tissue	% of mice positive for *R. felis*			
	1-dpi	3-dpi	6-dpi	14-dpi
Spleen	100	67	33	33
Liver	100	67	50	33
Heart	33	0	17	0
Lung	33	0	17	33
Brain	17	0	0	17
Kidney	17	17	0	0
Testicle	17	0	17	17
Skin	0	0	17	0

B) Low-dose group mice

Tissue	% of mice positive for *R. felis*			
	3-dpi	8-dpi	14-dpi	19-dpi
Spleen	0	17	0	0
Liver	0	0	0	0
Heart	0	17	n.d.	n.d.
Lung	n.d.	n.d.	n.d.	n.d.
Brain	n.d.	n.d.	n.d.	n.d.
Kidney	n.d.	n.d.	n.d.	n.d.
Testicle	n.d.	n.d.	n.d.	n.d.
Skin	n.d.	n.d.	n.d.	n.d.

n.d. = not determined

of *R. felis*-infection was determined to be one *R. felis* for every 200-9000 host cells, with a mean infection of one *R. felis* per 5500 host cells.

4.3.4. Detection of *R. felis* RNA in Mouse Tissues

Total RNA was extracted from portions of liver and spleen from high-dose and control group mice. cDNA was synthesized from RNA samples using 17-kDa gene specific primers and examined by qPCR for presence of *R. felis* 17-kDa. *R. felis* 17-kDa cDNA was not detectable in either high-dose or control group liver and spleen samples.

4.3.5. Histopathology

As *R. felis* could only be detected in tissues from the high-dose group, comparisons of histopathology were only conducted between the control and high-dose group. For spleen samples, the following characters were scored: percent white pulp, EMH, and spleenitis (Table 4.3a). Also the mean PALS diameter was calculated and compared. No significant differences were observed between any of the above parameters between the control and high-dose groups. Interestingly, the spleens for both groups were quite reactive, with a significant degree of EMH and a high percentage of white pulp. Also, mild-moderate spleenitis was noted for both groups. In one high-dose group, spleen samples collected 3-dpi, suspicious bacteria were present inside macrophages and surrounded by a high percentage of blast cells. For liver samples, cellular infiltration, inflammation, vacuolization, and sinus neutrophilia were scored (Table 3B). No significant differences in any of these parameters were observed between control and high-dose groups. In both groups, occasional focal accumulations of macrophages and increased vacuolization were observed. For heart samples, myocardial mineralization, myocardial necrosis, and myocardial degeneration were assessed and compared between control and high-dose groups (Table 4.3C). No significant differences in any of the examined parameters were observed between groups. In one mouse in the high-dose group, the heart sample contained

Table 4.3. Histology results for high-dose and control group spleen, liver, and heart samples. (A) Histology results for spleen samples included comparing mean PALS (±SEM, measured at 20X) and scores for percent white pulp, EMH and splenitis between the high-dose and control groups. (B) Histology results for liver samples included comparing scores for cellular infiltration, inflammation, vacuolization, and sinus neutrophila between high-dose and control groups. (C) Histology results for heart samples included comparing the percentage of high-dose and control mice positive for mitochondrial mineralization (including location), myocardial degeneration, and myocardial necrosis.

A) Spleen

Group	dpi	PAL diameter (20X) (±SEM)	% White pulp	EMH	Splenitis
Control	6	5.8 (±0.82)	2	2	moderate diffuse
	14	8.5 (±2.38)	2.5	2	mild diffuse
	19	7.3 (±0.00)	2	2	none-moderate diffuse
High-dose	1	8.4 (±0.38)	2.3	1.5	mild diffuse/multifocal
	3	6.6 (±0.46)	2.2	1.6	mild diffuse
	6	7.0 (±0.38)	2.3	2	mild diffuse
	14	7.1 (±0.63)	3	2.6	none-mild diffuse

Percent white pulp scores: 1=0-25%; 2=25-50%; 3=50-75%; 4=75-100%
EMH score: 1=mild; 2=moderate; 3=marked; 4=severe

B) Liver

Group	dpi	Cellular infiltration	Inflammation	Vacuolization	Sinus neutrophilia
Control	6	1	0	0	0
	14	1	0	0.5	0
	19	0	0	2	0
High-dose	1	0.33	0	0	0
	3	0.5	0	1	0
	6	0	0	0	0
	14	0.5	0	1	0

Cellular infiltration and vacuolization score score: 0=background level; 1=mild; 2=moderate; 3=marked; 4=severe

C) Heart

Group	dpi	Mitochondrial mineralization	Myocardial degeneration	Myocardial necrosis
Control	6	0%	0%	0%
	14	100% (IVS, LVF)	0%	0%
	19	50% (LVF)	0%	0%
High-dose	1	50% (LVF)	0%	0%
	3	17% (IVS)	0%	0%
	6	50% (LVF, LVS)	0%	0%
	14	83% (LVF, LVS, IVS)	17%	17%

Mitochondrial mineralization: % of mice with mitochondrial mineralization and location
(IVS=interventricular septum; LVS=left ventricular septum; LVF=right ventricular free-
Myocardial degeneration and necrosis: % of mice positive

areas with swollen degenerated myocytes and multi-focal myocardial degeneration and necrosis. In another high-dose group mouse, an enlarged right ventricle was noted in the heart sample. In both control and high-dose groups, mitochondrial mineralization in heart samples was observed especially in 14-dpi samples.

4.4. Discussion

Vertebrate models of rickettsial infection have been essential in defining rickettsial pathogenesis and elucidating transmission mechanisms such as with *R. conorii* (Walker et al. 1994) and *R. typhi* (Vaughan and Azad 1990). Characterizing the *R. felis* infectivity in a vertebrate hosts is a critical step in deciphering the transmission cycle of *R. felis* in nature. One challenge in developing a vertebrate model of *R. felis* infection is choice of vertebrate species for a model. The vertebrate model should, when infected with *R. felis*, elicit the same disease response as in humans. The lack of identification of a natural vertebrate reservoir host makes choice of a vertebrate model more difficult. Also, the lack of a human or vertebrate *R. felis* isolate is another challenge in trying to develop a vertebrate model of *R. felis* infection because no information is known about the degree of vertebrate pathogenesis from strains isolated from an arthropod host. Development of a vertebrate model will be a critical tool not only in defining *R. felis* pathogenesis, but also in deciphering *R. felis* transmission strategies between arthropod and vertebrate hosts. This study represents the initial steps toward developing a vertebrate model of *R. felis*-infection.

Common clinical symptoms of both SFG and TG rickettsial diseases include fever, headache and myalgia. Although a specific clinical picture is not yet available for the TRG *Rickettsia*, the non-specific symptoms listed above are also typical. In addition to the wide geographic range of human cases of flea-borne rickettsiosis, the range of reported clinical symptoms/signs is also great. Likely cases of flea-borne spotted fever are underreported or are

misdiagnosed as another rickettsial infection such as murine typhus or Mediterranean spotted fever as several rickettsial species have overlapping geographic distributions (Schriefer et al. 1994, Raoult et al. 2001). Development of a vertebrate *R. felis*-infection model will help refine the clinical picture of flea-borne rickettsiosis and help expedite accurate diagnosis.

Several successful murine models of rickettsial infection have been previously developed including for *R. conorii*, *R. australis* and *R. typhi*. In *R. conorii* infected C3H/HeN mice, rickettsial disease was characterized by a disseminated endothelial infection with death observed in high-dose inoculated mice as the result of vascular injury-based meningoencephalitis and interstitial pneumonia 5 to 6 dpi (Walker et al. 1994). In *R. australis* infected Balb/c mice, rickettsial disease was characterized by progressively severe vasculitis, interstitial pneumonia and multifocal hepatic necrosis (Feng et al. 1993). Finally, in *R. typhi* infected C3H/HeN mice, rickettsial disease was characterized by a disseminated endothelial infection with vascular-based lesions in the brain, lungs, heart, and kidneys (Walker et al. 2000).

In this study no overt disease or death resulted from inoculation of mice with either dose of *R. felis*. Mice appeared healthy with no indications of illness such as ruffled fur, lethargy, or hunched posture; all common signs of rickettsial infection in mice (Walker et al. 1994). Despite the absence of clinical sign/symptoms of disease *R. felis* gDNA was detected in several mouse tissues. Most frequently, *R. felis* gDNA was detected in the liver and spleen; however, *R. felis* gDNA was also detected in the heart, lung, kidney, testicle, brain, and skin of some *R. felis*-inoculated mice. Detection of *R. felis* gDNA in the skin is especially important from a transmission study standpoint as the skin is the location where cat fleas, the major arthropod vector of *R. felis*, feed. Identification of *R. felis* gDNA in the testicle is also interesting as some rickettsial species including *R. rickettsii* and *R. conorii* induce a severe scrotal reaction (deBrito et al. 1973, Walker et al. 1994). *R. felis* gDNA was not detected in any blood samples, indicating

that a period of rickettsemia did not occur or was too brief to be detected on the intervals

assessed. The presence of rickettsial transcripts in mouse tissues was also assessed; however, no

rickettsial 17-kDa transcripts were amplified from either the examined spleen or liver samples.

Lack of rickettsial transcript detection may be due to low (undetectable) rickettsial infection,

masked infection as the result of an overwhelming amount of mouse RNA/DNA, non-viable

R. felis not viable. Further studies utilizing additional doses, strains of *R. felis*, and strains of

mice are needed to further elucidate *R. felis* tissue localization and pathogenesis.

Histopathologic examination of tissue samples did not demonstrate any pathological

changes that could be specifically associated with rickettsial infection. Reactive spleens were

observed in both the control and high-dose groups, likely as a result of the inoculation medium

or an underlying sub-acute infection as spleens from mice in control group were still reactive 14-

dpi. The liver is a frequent location of rickettsial infection, but no anomalous lesions were

observed, except for a few areas of cellular infiltration. The absence of more histological lesions

specific to rickettsial infections in this study indicates that a productive *R. felis* infection did not

occur. Although no vertebrate *R. felis*-infection models currently exist, expected pathology had

included infection and lesions in the vascular endothelium, as a disseminated vascular disease is

the hallmark of all rickettsial species (Walker et al. 1994).

The lack of overt disease and pathology observed in the current study may be due to

insufficient infectious dose, pathogenicity of the rickettsial strain used, susceptibility of mouse

strain used, or development of a non-symptomatic infection (no rickettsemia, no apparent

disease). In the current study, mice were inoculated with one of two different doses of *R. felis*.

The inoculums chosen for this study were based off of inoculums from previously developed

models of *R. conorii* and *R. australis* infection (Feng et al. 1993, Walker et al. 1994, Walker et

al. 2000). Lack of overt *R. felis* infection in mice from both the high-dose and low-dose groups

may have resulted from an insufficient inoculation dose of *R. felis*.   In the future, studies examining additional inoculums of *R. felis* (LSU) on the development of *R. felis* infection, along with establishing an LD_{50}, are needed.

When developing animal models of human diseases, human isolates of the infectious agent or isolates that result in similar disease manifestations in the animal model are commonly used. Differences in strain pathogenicity have been previously described for *Rickettsia*. For example infection with *R. rickettsii* Sheila Smith or Norgard strains are highly virulent where as infection with *R. rickettsii* Morgan, Simpson, or HLP strains results in no or mild disease (Anacker et al. 1986, Anacker et al. 1984).   In the current study, the *R. felis* (LSU) strain, originally isolated from a naturally infected colony of fleas maintained at Louisiana State University, was utilized. The pathogencity of *R. felis* (LSU) is unknown as it has continuously been cycled through the LSU cat flea colony with restricted access to bloodmeal sources. The LSU cat flea colony is maintained on cats, and although the cats used for colony maintenance seroconvert, no overt disease resembling rickettsial infection has been seen. Differences in *R. felis* pathogenicity and transmissibility likely vary between strains/isolates; however, due to the limited strains of *R. felis* available, strain comparisons have not been conducted.

The susceptibility of mouse strains to rickettsial infection also varies and may have impacted the results of the current study. In the current study, C3H/HeN mice which have previously been used to model other rickettsial infections such as *R. conorii* and *R. typhi* (Walker et al. 1994, Walker et al. 2000) were chosen to model *R. felis* infection. Additional susceptible mouse strains that have been employed in developing vertebrate models of rickettsial infection are Balb/c (*R. australis*) (Feng et al. 1993) and C3H/HeJ (*R. conorii*) (Jordan et al. 2008), while C57BL/6 mice were shown to normally be resistant to *R. typhi* (Billings et al. 2001). Additional vertebrate species, including guinea pigs (Anacker et al. 1984) and rats (Shirai et al. 1967), have

also been used to model rickettsial infection. Consideration of non-mouse species in identifying a suitable vertebrate species to model *R. felis* infection in may be necessary. One example of a non-mouse vertebrate species that been repeatedly examined for *R. felis* infection due to its common association with cats fleas are cats. In a survey of cats from veterinary clinics with *R. felis*-infected cat fleas, *R. felis* DNA could not be amplified from cat blood samples (Hawley et al. 2007). However, in another study, all cats experimentally infested with *R. felis*-infected fleas seroconverted by four months and most were positive for *R. felis* by PCR amplification of DNA isolated from blood samples (Wedincamp, Jr. and Foil 2000). Examination of additional mouse strains or alternate vertebrate models, such as guinea pigs or opossums, is needed to determine the most appropriate vertebrate model of *R. felis* infection.

None of the mice in the current study were discernibly ill. Although in this case, the lack of disease is most likely the result of a non-productive infection other studies of rickettsial vertebrate infections have demonstrated that asymptomatic but transmissible infections are possible. For example, rabbits injected with either *R. conorii* or *R. rickettsii* remained asymptomatic throughout the experiment, although they seroconverted and were able to transmit both rickettsial species to feeding arthropods (Houhamdi and Raoult 2006). Vertebrate species that are able to acquire asymptomatic but transmissible rickettsial infections would make ideal reservoir hosts and additional surveys of likely candidates such as cats, dogs and opossums (all common cat flea hosts) are needed. Until the role(s) of vertebrate reservoirs in the transmission of *R. felis* are defined, an understanding of basic *R. felis* ecology is not possible.

In the current study, the clinical signs and symptoms of human flea-borne spotted fever, as reported in the literature, were not reproduced in the present mouse model of *R. felis* infection. Although overt disease was not observed, *R. felis* was still identified in a variety of mouse tissues, similar to the disseminated infection characteristic of other rickettsial species, including

the spleen, liver, heart, lung, and skin. Before the ecology of *R. felis* can be truly appreciated,

basic questions concerning the transmission and pathology of *R. felis* in a vertebrate model must

be answered. Future studies examining alternate mouse strains, alternate animal models,

additional strains of *R. felis* need to be conducted to produce a suitable vertebrate model of

R. felis infection. Once a suitable model is established, additional studies examining

transmission of *R. felis* from an infected animal to a susceptible arthropod vector can be

conducted as well as studies concerning the vertebrate immune response to *R. felis* and the

impact of the immune response can impact disease and transmission.

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CHAPTER 5
DISCUSSION OF RESULTS AND FUTURE DIRECTIONS

5.1. Discussion of Results and Future Directions

Initially described almost 20 years ago (Adams et al. 1990), several basic questions concerning the ecology and epidemiology of *Rickettsia felis* remain unanswered. Identified in numerous arthropods species in 28 countries around the world, the only currently defined biological vector and host of *R. felis* is the cat flea, *Ctenocephalides felis* (Wedincamp, Jr. and Foil 2002). Within cat fleas, *R. felis* is widely disseminated (Adams et al. 1990), however, *R. felis* infection kinetics and biology within in the flea are undescribed. Outside of persistent vertical transmission observed in infected cat flea colonies (Wedincamp, Jr. and Foil 2002) additional mechanisms of transmission are unclear. The role of vertebrates as reservoirs of *R. felis* and their impact on the maintenance of *R. felis* in nature are also unknown. Likely vertebrate reservoirs candidates for *R. felis* include common hosts of cat fleas such as cats, dogs, and opossums.

Listed as an emerging pathogen (Parola et al. 2005), and etiologic agent of flea-borne rickettsiosis, several cases of human *R. felis* infection have been documented around the world. Due to cross-reactivity of commonly employed serologic tests, *R. felis* infection has been misdiagnosed as other rickettsial infections (e.g. murine typhus or Mediteranean spotted-fever) (Schriefer et al. 1994, Raoult et al. 2001). As patients with flea-borne rickettsiosis present with a wide range of symptoms, no clear clinical definition of this disease has been described. Currently, no vertebrate *R. felis*-infection models are available to study *R. felis* pathogenesis. Despite being detected molecularly and serologically in a multitude of arthropods and vertebrates, direct transmission between arthropods and vertebrates has not been observed. It has recently been suggested that all vertebrate infections are incidental, and that arthropods are not

Despite stable vertical transmission in laboratory settings, the occurrence of *R. felis* in numerous species of fleas and ticks in nature suggests that horizontal transmission occurs. Deciphering the role of horizontal transmission in the maintenance of *R. felis* and defining the determinants of *R. felis*-infection in vertebrate and invertebrate hosts are essential. The broad hypothesis of this dissertation research is that horizontal transmission is a necessary maintenance strategy of *R. felis* in nature that is influenced by: persistence in the vector; the ability of naïve vectors to become infected; and, dissemination and replication in the vertebrate host. To address this hypothesis the following were examined: *R. felis*-infection dynamics in the flea vector; the ability of fleas to horizontally acquire *R. felis*-infection; and, *R. felis* infection in a vertebrate model. Before *R. felis* control and prevention measures can be developed and implemented, basic questions concerning the ecology of *R. felis* need to be answered. The studies conducted in this document address some of these basic questions concerning *R. felis* biology and ecology by developing novel assays and model systems of *R. felis* infection.

Recognizing that *R. felis* is maintained in flea cohorts predominantly via vertical transmission, *R. felis* replication kinetics in fleas during metabolically active periods was examined. Because cat fleas are anautogenous, the intimate relationship between *R. felis* and *C. felis* was hypothesized to allow for coordination of rickettsial replication and the metabolically active periods associated with flea bloodmeal acquisition and oogenesis. By developing a qPCR assay *R. felis* infection load (counting *R. felis* 17-kDa copy-numbers/flea) and density (ratio of *R. felis* 17-kDa to *C. felis* 18S rDNA copy-numbers) was examined in individual fleas. In this study, three independent trials were conducted over the course of one year and assessed for: prevalence of *R. felis* infection in the colony; mean *R. felis* infection load; and mean *R. felis* infection density.

Colony *R. felis*-infection prevalence was observed to decline over subsequent trials, with

a 61% decrease (range 35-96%) observed between the first and last trial. Interestingly, as colony *R. felis*-infection prevalence decreased, mean *R. felis*-infection load significantly increased. *C. felis* 18S rDNA copy-number was also observed to increase in subsequent trials. To verify that the observed increase in *R. felis*-infection load was not an artifact of more available flea cells to infect, examination of *R. felis* infection as a function of *R. felis*-infection density was essential. As *R. felis*-infection density was also observed to significantly increase over subsequent trials, we were able to confidently conclude that fleas in the last trial were significantly more infected than fleas in the first two trials.

Flea bloodmeal acquisition and oogenesis are both metabolically active periods that could serve as signals for vertically maintained bacteria to replicate and be transmitted to host progeny. When *R. felis* infection was assessed during these metabolically active periods no significant variation in *R. felis*-infection load was observed across trials. Based on the results of this study a model of *R. felis* infection for the LSU cat flea colony was constructed that depicts an inverse relationship between colony *R. felis*-infection prevalence and individual flea *R. felis*-infection load. From this model arguments could be made that *R. felis* infection is regulated at the population level and that when *R. felis*-infection prevalence in the population decreases, infection loads in individual fleas are increased, perhaps influencing flea fitness to facilitate rickettsial transmission to the next generation of fleas or to a susceptible vertebrate host.

The results of this study will serve as a platform for several additional experiments. In the future, studies are needed to assess the kinetics of *R. felis* infection in flea immature and inactive lifecycle stages. As fleas are able to survive for up to 155 days as adults in their pupal cases (Rust and Dryden 1997), examination of *R. felis* kinetics during periods of inactivity would help elucidate *R. felis* maintenance strategies in nature and possibly rickettsial virulence determinants. Also in the current study *R. felis* infection load and infection density were

examined at the 'whole' flea level. Although significant differences were not observed in either *R. felis*-infection load or density at the whole flea level, significant differences at the tissue level (e.g. reproductive tissues, salivary glands) may occur.   As more densely infected fleas were observed in the last trial, when colony *R. felis*-infection prevalence was lowest, studies examining the ability of fleas with higher *R. felis*-infection densities to more efficiently transmit *R. felis* are needed. In this study, because gender was not always equally represented, detailed information concerning *R. felis*-infection gender differences in the flea were not always possible and needs to be examined further. Future assessment of *R. felis*-infection in the LSU cat flea colony, or in comparisons to other *R. felis*-infected flea colonies, would be interesting to examine whether the trends in colony *R. felis*-infection prevalence and individual flea *R. felis*-infection loads/density observed in this study are supported.

The great variation observed in flea colony *R. felis*-infection prevalence is also interesting as surveys of fleas in nature tend to have a lower prevalence of *R. felis*-infection versus the high *R. felis*-infection prevalence commonly observed in institutional and commercial flea colonies. Whether *R. felis*-infection prevalence fluctuates nature to the degree that was observed in this study is unknown. Examination of factors that could influence *R. felis*-infection prevalence and infection load in fleas such as competing microbiota or flea host bloodmeal choice may illuminate additional variables that influence *R. felis* transmission and maintenance in nature. As the only currently defined biological vector, examination of *R. felis* infection kinetics has only been conducted in cat fleas; however, assessment of *R. felis* kinetics in other flea or arthropod species could elucidate additional arthropod vectors.   Lastly, the influence of *R. felis*-infection on flea fitness also needs to be examined. Vertically maintained bacteria, such as *Wolbachia* spp., commonly manipulate their arthropod host to facilitate their own transmission (Werren 1997). The ability of *R. felis* to influence flea fitness; manipulate the flea to further its own

transmission; or, whether fleas feel selection pressures to either retain or remove *R. felis*-infection from the general population are unknown and need to be examined.

As previously mentioned, vertical transmission (both transovarial and transstadial) has been well-described for *R. felis*; however, *R. felis* horizontal transmission strategies were unclear. Evidence supporting a role for horizontal transmission of *R. felis* included identification of *R. felis* infection in vertebrates and humans and detection of *R. felis* in a wide range of arthropod species. Despite evidence for horizontal *R. felis* transmission in nature, lack of known definitive horizontal transmission mechanism have led to speculation that although accidental transmission to vertebrates and other arthropods sometimes occur, the ability of fleas to acquire *R. felis* from an infected bloodmeal is unlikely (Weinert et al. 2009). Studies demonstrating whether vertebrate-arthropod *R. felis* transmission cycles occur are necessary before *R. felis* maintenance strategies based on horizontal transmission mechanisms are ruled out.

In the second set of experiments, the ability of fleas can acquire *R. felis* from an infectious bloodmeal addressed. Also assessed was if *R. felis* infection acquired by this route was transient or persistent. Subsequently, flea progeny were examined to determine whether fleas infected horizontally via an infected bloodmeal were able to vertically transmit *R. felis*. Lastly, the presence and viability of *R. felis* in flea feces, a transmission strategy employed by other insect-transmitted pathogenic rickettsial species, was examined. To answer these questions an artificial flea feeding system was used to expose fleas to *R. felis*-infected bloodmeals. Several preparations of *R. felis*-infected bloodmeals were compared and *R. felis* infection in both individual fleas and flea feces was quantified using the previously developed qPCR assay.

Using this model of *R. felis* exposure, previously uninfected fleas were successfully demonstrated to acquire *R. felis* infection after feeding for only one day on an infectious

bloodmeal. All *R. felis*-infectious bloodmeal preparations (cell-free and in host cell) were capable of resulting in infection, but fleas that fed on *R. felis*-infected intact host cells lived the longest and had the highest average incidence of infection. The acquired *R. felis*-infections were persistent and could be detected up to 35 days post-exposure (dpe), or until the end of their lives. The mean incidence of infection also increased over time from about ~50% at 7-dpe to ~90% by 28-dpe. The *R. felis*-infection density of fleas in this study (mean log*Rf*17kDa/log*Cf*18S ratio = 0.5) were lower than that observed in the naturally infected fleas in the previous study (mean log*Rf*17kDa/log*Cf*18S ratio = 0.75). This difference in infection density could help explain why we did not observe vertical transmission of *R. felis* to progeny in the horizontally infected fleas as a threshold level of infection may be necessary for transmission to occur. Future studies examining the relationship between infection density and successful vertical transmission are needed.

Interestingly, *R. felis* gDNA was detected in flea feces for up to 28-dpe. *R. felis* gDNA was successfully recovered independent of infectious bloodmeal preparation. Examination of *R. felis* 17-kDa copy-number in fecal samples standardized by weight revealed an expected significant decrease between 2- and 4-dpe after fleas had finished digesting/excreting the infectious bloodmeals and were switched to feed on uninfected blood. At all collection points after 4-dpe a general trend of increasing *R. felis* 17-kda copy-number in flea feces was observed and all subsequent collection points after 4-dpe were significantly higher than the *R. felis* 17-kDa copy-number observed at 4-dpe. *R. felis* viability in feces was also examined and rickettsial transcripts in flea feces at 16-dpe were detected. Assessment of vertical *R. felis* transmission to offspring of horizontally infected fleas was also investigated and, curiously, after examination of approximately 400 F1 progeny from the first trial we were unable to detect *R. felis* infection in any progeny. Although the mean *R. felis*-infection density was lower in this study compared to

the first, some individual fleas that were horizontally infected reached similar *R. felis*-infection density levels as fleas vertically infected. Despite individual horizontally infected fleas reaching vertically infected flea *R. felis*-infection densities, no vertical transmission to progeny was observed, indicating that there may be other influencing factors.

Although this study is the first to definitively demonstrate flea acquisition and persistent *R. felis* infection after feeding on an infectious bloodmeal, several questions still need to be addressed. Examination of tissue dissemination patterns of horizontally acquired *R. felis* are needed to determine if *R. felis* infection is being restricted to the flea gut or whether infection is disseminating to other tissues (e.g. reproductive tissue or salivary glands). If *R. felis* is not being disseminated then examination of factors possibly effecting or restricting dissemination need to be explored along with any impact on subsequent transmission. Competition for resources and vertical transmission have previously been described among rickettsial species (Burgdorfer 1988, Macaluso et al. 2002) and between other bacterial symbionts (Sakurai et al. 2005). As the fleas utilized in this study had other non-rickettsial symboints (Pornwiroon et al. 2007), their impact on *R. felis* infection, dissemination and transmission might explain the inability of *R. felis* infection to be transmitted to flea progeny and could prove an interesting avenue of research. Additionally, an increasing incidence of *R. felis*-infection was observed over the experimental period in this study. Future studies examining mechanisms that could influence *R. felis*-infection prevalence such as death of uninfected fleas (fitness effect) and co-feeding/direct contact with infected fleas are needed.

Detection of *R. felis* gDNA and transcripts in flea feces also offers an interesting area of future research. As insect-transmitted rickettsial pathogens (e.g. *R. typhi*, *R. prowazekii*) are transmitted in insect feces, the previously unrecognized potential of *R. felis* fecal transmission needs to be examined. Further investigation of rickettsial viability, persistence, and

infectiousness are needed to determine whether fecal transmission is a viable *R. felis*

maintenance strategy. If *R. felis* is determined to be both viable and infectious in flea feces,

acquisition of *R. felis* infection by larval fleas ingesting infected adult flea feces will need to be

examined.

In terms of transmission via oral acquisition, the infectious dose of *R. felis* for flea

infection needs to be determined. Along with identifying the infectious dose, the following

variables need to be considered when determining *R. felis* transmission efficiency: quantity of

R. felis circulating in host; period of time host blood is infectious; the mean number of *R. felis*

ingested per bloodmeal; the mean number of flea feeding events per day; and, the average

lifespan of the flea on host. Detailed studies on the high transmission efficiency of *Rickettsia*

typhi, another flea-transmitted rickettsial pathogen, have revealed that fleas are only able to

acquire *R. typhi* from a rat host during periods of rickettsemia and require ingestion of only a few

organisms to acquire infection and subsequently transmit *R. typhi* to a new host (Farhang-Azad

et al. 1983, Vaughan and Azad 1990).

In the last series of preliminary experiments, steps were taken to develop a vertebrate

R. felis-infection model. Previous vertebrate models of rickettsial infection have provided

valuable insight towards understanding rickettsial pathogenesis and infection/transmission

parameters (Feng et al. 1993, Walker et al. 1994, Walker et al. 2000). With questions

surrounding the occurrence of a definitive *R. felis* vertebrate reservoir and the wide spectrum of

clinical symptoms reported in human cases of flea-borne rickettsiosis, development of a

vertebrate *R. felis* infection model will serve as an invaluable tool to study *R. felis* pathogenesis

and transmission. C3H/HeN mice were intravenously injected with one of two different *R. felis*

inoculums with the objective of developing a vertebrate model of *R. felis* infection. *R. felis*

infection in mice was assessed over 19 days. Mouse health was monitored daily and at specific

collection points a variety of mouse tissues were assessed for *R. felis* presence using qPCR. Tissues were also prepared for histologic examination of *R. felis* pathology.

In this study, no overt disease or death was observed in mice inoculated with either dose of *R. felis*. *R. felis* gDNA was detected in mouse heart, lung, kidney, testicle, brain and skin samples, although at a lower frequency compared to liver and spleen samples. When tissue samples were examined for presence of rickettsial transcripts, no rickettsial products were able to be amplifed. Histological examination of liver and spleen samples from high-dose and control group mice did not reveal any pathology that could be specifically attributed to *R. felis* infection. Spleen reactivity was observed in both exposed and control groups and likely the result of the inoculation medium or an underlying sub-acute infection. As this study represents the initial steps in developing a vertebrate *R. felis*-infection model, several directions are indicated for future studies. Because no overt clinical disease/pathology or death was observed in either experimental group, examination of different inoculation dosages and determination of the LD_{50} are needed. Lack of rickettsial transcript detection in mouse tissues is likely the result of a low infection rate, or a rapid resolution of infection by the mouse. Additional mouse strains and/or vertebrate species should also be investigated before determining the most appropriate vertebrate model of *R. felis* infection. Variables, such as differences in *R. felis* strain pathogenesis and vertebrate host susceptibility, also need to be addressed. Most vertebrate models of rickettsial infection utilize human isolates; however, no human or vertebrate *R. felis* isolates are currently available. Future work defining a clinical disease and associated pathology and host immune response will require a vertebrate model that mimics *R. felis* disease as observed in nature. Studies on *R. felis* transmission between arthropods and vertebrates and their implications on *R. felis* maintenance in nature are also needed before the ecology of *R. felis* can be fully appreciated.

Like all vector-borne diseases, the parameters of infection are multifocal. The research described in this dissertation sought to understand the ecology of *R. felis* transmission by examining the pathogen, vector, and the vertebrate host. Within a flea cohort *R. felis*-infection prevalence is dynamic and appears to be inversely correlated with individual flea *R. felis* infection load/density. This study definitely demonstrates that fleas are able to acquire a persistent *R. felis* infection after feeding on an infectious bloodmeal. Finally, studies towards developing a vertebrate model of *R. felis* infection were initiated. Before appropriate *R. felis* control and prevention measures developed, questions concerning the basic ecology of *R. felis* first need to be answered. The results of these studies will serve as a platform for future work as several areas of research still remain concerning the biology and ecology of *R. felis*.

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APPENDIX
COMMONLY USED ABBREVIATIONS

ANOVA – Analysis of variance

cDNA – Complementary DNA

*Cf*18S – Portion of *C. felis* 18S rRNA gene

cfd – Murine complement factor D

Ct – Critical threshold

DFA – Direct immunofluorescent assay

DNA – Deoxyribonucleic acid

dpe – Days post-exposure

dpi – Days post-inoculation

EL – Elward II Laboratories

ELB – Original name of *Rickettsia felis*

ELISA – Enzyme-linked immunosorbent assay

EMH – Extramedullary hematopoiesis

gDNA – Genomic DNA

gltA – Rickettsial citrate synthase gene

h – Hour(s)

HotSHOT – Hot sodium hydroxide and tris

HSD – Honestly significant difference

IFA – Indirect immunofluorescent assay

ISE6 – *Ixodes scapularis* cell line

LD50 – Lethal dose 50 (dose which causes 50% mortality)

LSU – Louisiana State University

m – Minute

m – Minute(s)

MIF – Microimmunofluorescent assay

ompA – Rickettsial outer membrane protein A

ompB – Rickettsial outer membrane protein B

PALS – Periarteriolar lymph sheath

PCR – Polymerase chain reaction

qPCR – Quantitative Real-time PCR

rDNA – Ribosomal DNA

*Rf*17kDa – Portion of *R. felis* 17-kDa antigen gene

RFLP – Restriction fragment-length polymorphism

RNA – Ribonucleic acid

rRNA – Ribosomal RNA

RT-PCR – Reverse transcription PCR

s – Second(s)

SD – Standard deviation

SEM – Standard error of the mean

SFG – Spotted-fever group

TEM – Transmission electron microscopy

TG – Typhus group

TRG – Transitional group

APPENDIX
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Sincerely,

 Kathryn Reif

PhD Student

Department of Pathobiological Sciences

School of Veterinary Medicine

Louisiana State University

Baton Rouge, Louisiana 70803

PH: (225) 578-7419

Email: kreif1@lsu.edu

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Sent: Friday, February 27, 2009 11:23 AM

To: barbara lau

VITA

Kathryn Elizabeth Reif is the daughter of Mr. Luke John Reif and Mrs. Susan Elizabeth Reif. She was born in Garfield Heights, Ohio, in 1982, and is the older sister of Nicholas Alexander and Sarah Marie Reif. For high school Kathryn attended Beaumont School, Cleveland Heights, Ohio, where she met Ms. Stephanie Levi, a guest speaker from Case Western Reserve University and her first research mentor. After high school, Kathryn attended Ohio Wesleyan Reserve University, Delaware, Ohio, where she graduated with a Bachelor of Arts degree in zoology and a minor in psychology in 2004. At Ohio Wesleyan Kathryn further became interested in research with Dr. Ramon Carreno as her mentor. At Ohio Wesleyan she published her first peer-reviewed journal article in the Journal of Parasitology. After college, Kathryn earned a Master of Public Health degree in tropical medicine at Tulane University's School of Public Health. At Tulane Kathryn worked as a research assistant for Dr. Dawn Wesson, surveying local mosquito populations for West Nile virus.   Resolute that she wanted to pursue a research career in vector biology, Kathryn began her doctorate degree in the Department of Pathobiological Sciences at the School of Veterinary Medicine and Louisiana State University under the guidance of Dr. Kevin Macaluso. In Dr. Macaluso's laboratory, Kathryn studied arthropod and vertebrate determinants for horizontal transmission of *Rickettsia felis*. Several chapters of her dissertation research have been published. Kathryn will graduate with the degree of Doctor of Philosophy in veterinary sciences with a concentration in pathobiological sciences in May, 2009. With her doctorate in-hand, after graduation Kathryn will pursue a post-doctoral fellowship in vector biology with the future goal of establishing her own independent research program.