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RICKETTSIA FELIS, TRANSMISSION MECHANISMS OF AN EMERGING FLEA-BORNE RICKETTSIOSIS

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
Biomedical and Veterinary Medical Sciences
Through the Department of Pathobiological Sciences

by
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ABSTRACT

Rickettsia felis is an emerging insect-borne rickettsial pathogen and the causative agent of flea-borne spotted fever. First described as a human pathogen from the United States in 1991, *R. felis* is now identified throughout the world and considered a common cause of fever in Africa. The cosmopolitan distribution of this pathogen is credited to the equally widespread occurrence of cat fleas (*Ctenocephalides felis*), the primary vector and reservoir of *R. felis*. Additionally, *R. felis* has been identified in other hematophagous arthropods (including numerous species of fleas, ticks, mosquitoes, and mites). Most transmission cycles of pathogenic *Rickettsia* include transovarial and transstadial passages in their arthropod hosts as well as transmission to new vectors through the infectious blood of vertebrate amplifying hosts. The continuous molecular detection of *R. felis* from other blood-feeding vectors supports the notion of infectious transmission cycles; however, naturally infected mammalian blood or tissues have never been shown to be a source of *R. felis* infection from vertebrate to arthropod host. Here we demonstrate that horizontal transmission of *R. felis* occurs independent of a rickettsemic vertebrate host. The combination of intraspecific and interspecific cofeeding transmission of *R. felis* on a vertebrate host, sustained transmission of *R. felis* between cofeeding cat fleas in an artificial host system, and support by modeling demonstrated cofeeding as an important mechanism of pathogen maintenance and transmission within flea populations. Additionally, our results indicate that not only are *R. felis*-exposed cat fleas infectious following a brief incubation period, but utilization of a mechanical mechanism may also explain the rapid rate of spread that typifies *R. felis* flea-borne transmission within experimental and computational models. Elucidation of the *R. felis* transmission cycle is necessary to further our understanding of this emerging rickettsiosis.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Insect-borne rickettsiae are among the most influential zoonotic pathogens in human populations throughout the world, with both historic (*e.g.* louse-borne epidemic typhus during Napoleon's retreat from Moscow) [1] and current (*e.g.* reemergence of flea-borne endemic typhus in southern California and Texas) [2, 3] outbreaks. Recently, a third insect-borne rickettsial pathogen, *Rickettsia felis*, has progressed from a sporadic disease in the United States to a common cause of fever in Africa [4]. First described in 1990 from colonized cat fleas (*Ctenocephalides felis*) [5], this intracellular Gram-negative bacterium was associated with human disease by 1991 [6]. Many years passed before the species itself was formally validated by molecular criteria in 2001 and isolation of the reference strain (Marseille-URRWXCal2) from cat fleas was completed shortly thereafter in 2002 [7, 8]. The definitive description of *R. felis* as the causative agent of flea-borne spotted fever has dramatically increased the appearance of this pathogen in the literature, with roughly 315 peer-reviewed articles currently and more than 90% of which were published after 2002. The ease of molecular tools, specifically polymerase chain reaction (PCR), to detect pathogens from around the globe has confirmed *R. felis* infections from every continent except Antarctica [4, 6, 9]. Within the last decade, several advances have been made towards the understanding of basic *R. felis* biology (*e.g.* genomics and pathogenicity); yet, some deficiencies (*e.g.* transmission mechanisms, epidemiology, and species diversity) remain and continue to hinder investigative advances for this universal emerging pathogen.

1.2. Transmission biology of *Rickettsia felis*

Following the initial detection of *R. felis* from an isolated cat flea colony, several other commercial and institutional organizations confirmed the presence of *R. felis* in additional laboratory-reared cat flea colonies (reviewed in [10]). Sustained *R. felis* infections within cat flea populations were first postulated to occur through stable vertical transmission based on the detection of rickettsiae in flea reproductive tissues [11]. Later reports using PCR analyses confirmed vertical transmission of *R. felis* in colonized cat fleas in both freshly deposited flea eggs (transovarial transmission) and newly emerged, unfed adult fleas (transstadial transmission) [11, 12]. Subsequently, the cat flea was considered not only the primary vector of *R. felis*, but also the reservoir host due to the maintenance of infection solely within the vector population [12]. Although vertical transmission has been demonstrated, prevalence of *R. felis* among cat flea colonies exhibits tremendous variability. For example, prevalence of *R. felis*-infection in adult cat fleas from a single colony ranged from 35 – 96% over the course of one year [13], while comparison of F_1 infection rates from distinct *R. felis*-infected cat flea colonies may range from 0 – 100 % based on unknown mechanisms [10]. An inverse correlation was observed between colony *R. felis*-infection prevalence and *R. felis*-infection load in individual cat-fed fleas, suggesting that vertical transmission of *R. felis* is a maintenance strategy for persistence within cat flea populations [13]; however, vertical transmission efficiency of *R. felis* in cat fleas fed on bovine blood, as opposed to cat-fed colonies, was shown to severely diminish after 12 consecutive generations [14]. The inefficient transfer of *R. felis* from adult to progeny fleas was potentially linked to the vertebrate blood source, but cat fleas lack true host specificity and *R. felis*-infected arthropods have been recovered from numerous vertebrate species (*e.g.* cats, dogs, rodents,

opossums, hedgehogs, horses, sheep, goats, gerbils and monkeys) [4, 10, 15]. Given that vertical transmission of *R. felis* is not 100% efficient, it is probable that horizontal amplification is required for maintenance of this pathogen within vector populations.

Further studies with cat flea colonies lacking a constitutive *R. felis*-infection demonstrated favorable host-pathogen associations for horizontal transmission. The initial report showed that uninfected cat fleas were able to acquire *R. felis* by feeding on a simulated infectious bloodmeal, and this newly acquired infection persists the remainder of the vectors' lifespan [16]. Following *R. felis* acquisition in previously uninfected cat fleas, the infection then disseminates from the gut to the hemocoel and other tissues before reaching the salivary glands [17]. Subsequent transmission of *R. felis* to vertebrate hosts is based on serum samples positive to rickettsial antigen and to a lesser extent PCR-positive tissue samples, including blood, resulting from exposure to infected cat fleas (reviewed in [10]). Ultimately, horizontal transmission of *R. felis* was demonstrated through a shared bloodmeal between *R. felis*-infected and uninfected cat fleas in an artificial host system [18]. Contrary to other vector-borne pathogens, there appears to be no correlation between rickettsial distribution in flea tissues and distinct transmission routes, *i.e.* horizontal transmission events occur well before the spread of *R. felis* to flea salivary glands (authors' unpublished data).

The majority of our current understanding of the life cycle of *R. felis* in nature is derived from *R. felis*/*C. felis* laboratory models. The dilemma in this transmission cycle is the subsequent acquisition of viable *R. felis* by cat fleas from vertebrate hosts to complete the "flea to mammal to flea" succession comparable to other insect-borne rickettsial pathogens.

Transmission of *R. felis* from cat fleas to vertebrate hosts is presumed to occur through infectious flea bite and potentially infected flea feces, which are also comprised of rickettsiae [16]. Among the mammalian species found to be seropositive or PCR-positive for *R. felis* in nature include cats, dogs, opossums, raccoons, rodents, and humans [10, 19-22]. A definitive mammalian host with a systemic *R. felis* infection has not been identified, and may vary by geographic location (*e.g.* lack of marsupials in Africa, Asia, and Europe) and distribution of arthropod vectors (*e.g.* sites that have few, if any, cat fleas) [10, 23]. A recent study generated *R. felis*-infected BALB/c mice via an artificial route, and subsequently produced infectious *Anopheles gambiae* mosquitoes that caused transient rickettsemia in naïve mice [24]; nevertheless, naturally infected mammalian blood or tissues have never been shown as a source of *R. felis* infection from vertebrate to arthropod hosts.

The transmission biology of flea-borne spotted fever is complicated further by the progressive accumulation of field surveys reporting molecular detection of this infectious agent from other vectors, *i.e.* more than 40 additional species of fleas, ticks, mites, and mosquitoes (Table 1.1) [4]. Given the infrequency of a systemic vertebrate infection, the presence of *R. felis* in these additional arthropod species is unclear. Successful transmission of pathogens between actively blood-feeding arthropods in the absence of a disseminated vertebrate infection has been demonstrated (reviewed in [25]). This transmission event, referred to as co-feeding, is reliant on the temporal and spatial dynamics of infected and uninfected arthropods as they blood feed. The infected arthropod is both the vector and the reservoir for the pathogen, while the vertebrate acts as a conduit for infection of naïve arthropods. The potential for co-feeding transmission of *R. felis* between cat fleas was demonstrated with the use of a shared bloodmeal in an artificial host system [18]. Recently,

Table 1.1. Geographic distribution of *R. felis* in wild-caught arthropods since 2009 review [10].

Country	Vector	Prevalence of Infection	Reference
Albania	*Ctenocephalides felis*	3% (10/371)	[26]
Algeria	*Archeopsylla erinacei*	96% (316/331)	[27]
	Xenopsylla cheopis, Leptopsylla segnis	15% (10/69)	[28]
Australia	fleas	ND	[29]
	C. felis	ND	[30]
	Liposcelis bostrychophila	ND	[31]
Brazil	*C. felis*	38% (268/701)	[32]
	Amblyomma humerale	14% (1/7)	[33]
	ticks and fleas	ND	[34]
	C. felis	ND	[35]
Chile	*Rhipicephalus sanguineus*	ND	[36]
China	*Eulaelaps stabularis*	ND	[37]
	C. felis	95% (57/60)	[38]
	R. sanguineus	10% (15/146)	
	Linognathus setosus	16% (6/37)	
	Anopheles sinensis, Culex pipiens	6% (25/428)	
Colombia	*C. felis, Ctenocephalides canis, Pulex irritans*	ND	[39]
Costa Rica	*C. felis*	ND	[40]
	C. felis	ND	[41]
Côte d'Ivoire	*Anopheles gambiae*	1% (1/77)	[42]

Table 1.1 continued

Cyprus	*X. cheopis*	1% (4/400)	[43]
Czech Republic	fleas	18% (6/33)	[44]
Democratic Republic of Congo (Kinshasa)	*C. felis*	95% (37/39)	[45]
	C. canis	42% (10/24)	
	C. felis	57% (13/23)	[46]
Democratic Republic of Congo (Ituri)	*C. felis*	23% (15/64)	[46]
	Leptopsylla aethiopica	9% (1/11)	
	Echidnophaga gallinacea	5% (1/21)	
Ethiopia	fleas	21% (63/303)	[47]
	C. felis	100% (3/3)	[48]
	P. irritans	43% (23/53)	
	fleas	ND	[49]
France	*A. erinacei*	99% (128/129)	[50]
	A. erinacei	11% (2/19)	[51]
Gabon	*Aedes albopictus*	3% (3/96)	[52]
Guatemala	*C. felis*	ND	[40]
Hungary	*C. felis*	ND	[53]
Indonesia	*X. cheopis*	ND	[54]
Italy	*C. felis*	26% (34/132)	[55]
	fleas	ND	[56]
	C. felis	12% (38/320)	[57]
	C. felis	31% (9/29)	[58]
Ivory Coast	*C. canis*	50% (1/2)	[59]

Table 1.1 continued

Kenya	*X. cheopis, C. felis, C. canis, P. irritans, E. gallinacea*	ND	[60]
Korea	*Ctenophthalmus congeneroides, Stenoponia sidimi, Rhadinopsylla insolita*	ND	[61]
Laos	*C. canis, C. felis, Ctenocephalides orientis*	59% (13/22)	[62]
Lebanon	*C. felis*	16% (17/104)	[63]
	C. felis	44% (8/18)	[64]
Malaysia	*C. felis*	32% (57/177)	[22]
	C. felis	4% (4/95)	[65]
	C. fels	75% (337/450)	[66]
Mexico	*C. felis*	25% (1/4)	[67]
	Polygenis odiosus	33% (1/3)	
Morocco	fleas	20% (112/554)	[68]
New Caledonia	*C. felis*	81% (17/21)	[69]
Netherlands	*C. canis, C. felis*	ND	[70]
Panama	*C. felis*	35% (7/20)	[71]
Peru	*C. felis*	67% (2/3)	[72]
Reunion Island	*X. cheopis, Xenopsylla brasiliensis*	2% (5/205)	[73]

Table 1.1 continued

Senegal	*Aedes luteocephalus*	< 1% (1/203)	[74]
	Anopheles arabiensis	1% (2/154)	
	Anopheles ziemanni	14% (1/7)	
	Anopheles pharoensis	10% (1/10)	
	Anopheles funestus	29% (2/7)	
	Mansonia uniformis	25% (2/8)	
	Cimex hemipterus	3% (1/39)	
Slovakia	*Ctenophthalmus agyrtes*, *Ctenophthalmus solutus*, *Ctenophthalmus uncinatus*, *Nosopsyllus fasciatus*	11% (34/315)	[75]
Spain	*C. felis*	26% (20/118)	[76]
	C. felis	44% (34/78)	[77]
	C. felis	3% (2/76)	[78]
Taiwan	*C. felis*	ND	[79]
	C. felis	21% (90/420)	[80]
	Stivalius aporus, *Acropsylla episema*	1% (2/160)	[81]
Tunisia	*C. felis*	9% (2/22)	[82]
	C. felis	< 1% (1/322)	[83]
Turkey	*Rhipicephalus bursa*	ND	[84]
United Republic of Tanzania	*C. felis*	65% (13/20)	[46]
	C. canis	71% (5/7)	
	Ctenophthalmus calceatus	25% (5/20)	

Table 1.1 continued

USA	*C. felis*	ND	[85]
	C. felis, P. irritans, X. cheopis, E. gallinacea, Diamanus montanus	ND	[86]
	Amblyomma maculatum	ND	[87]
	X. cheopis	ND	[88]
	L. bostrychophila	ND	[89]
	Carios capensis	ND	[90]
	C. felis, P. irritans, X. cheopis, E. gallinacea, Diamanus montanus, L. segnis	ND	[91]
	fleas	ND	[92]
Uruguay	*C. canis, C. felis*	41% (27/66)	[93]
West Indies	*C. felis*	ND	[94]

ND, not determined.

both intra- and interspecific transmission of *R. felis* between co-feeding arthropods on a vertebrate host was demonstrated (Figure 1., C and 1., D) [95]. Analyses revealed that infected cat fleas transmitted *R. felis* to naïve cat fleas and Oriental rat fleas (*Xenopsylla cheopis*) via flea bite on a non-rickettsemic vertebrate host [95]. Also, cat fleas infected by co-feeding were infectious to newly emerged uninfected cat fleas in an artificial system (Figure 1., E) [95]. Furthermore, a stochastic model was utilized to demonstrate that co-feeding is sufficient to explain the enzootic spread of *R. felis* amongst populations of the biological vector [95]. These results implicate cat fleas in the spread of *R. felis* amongst different vectors, and the demonstration of co-feeding transmission of *R. felis* through a vertebrate host represents a novel transmission paradigm for insect-borne *Rickettsia*.

1.3. Epidemiology of *Rickettsia felis*

Flea-borne spotted fever is considered an emergent global threat to human health, with cases likely underestimated due to similarities in clinical signs with other febrile illnesses (*e.g.* fever, rash, headache, and myalgia) and limited access to appropriate laboratory tests (*e.g.* molecular diagnostics) [4, 10, 15]. The first human case of *R. felis* infection was misdiagnosed as flea-borne endemic typhus (*Rickettsia typhi*) because the available serological reagents were unable to distinguish between the two rickettsial species [6]. A retrospective investigation for *R. felis* among endemic typhus patients was initiated because field surveys revealed the presence of this agent within suspected vectors and mammalian hosts of *R. typhi* in the United States [96-98]. Comparable to endemic typhus, serological and molecular analyses have implicated cat fleas and Virginia opossums (*Didelphis virginiana*) as respective vectors and hosts of *R. felis* in suburban regions of California and Texas [21, 96, 98]. The suburban cycle of endemic typhus is unique to the

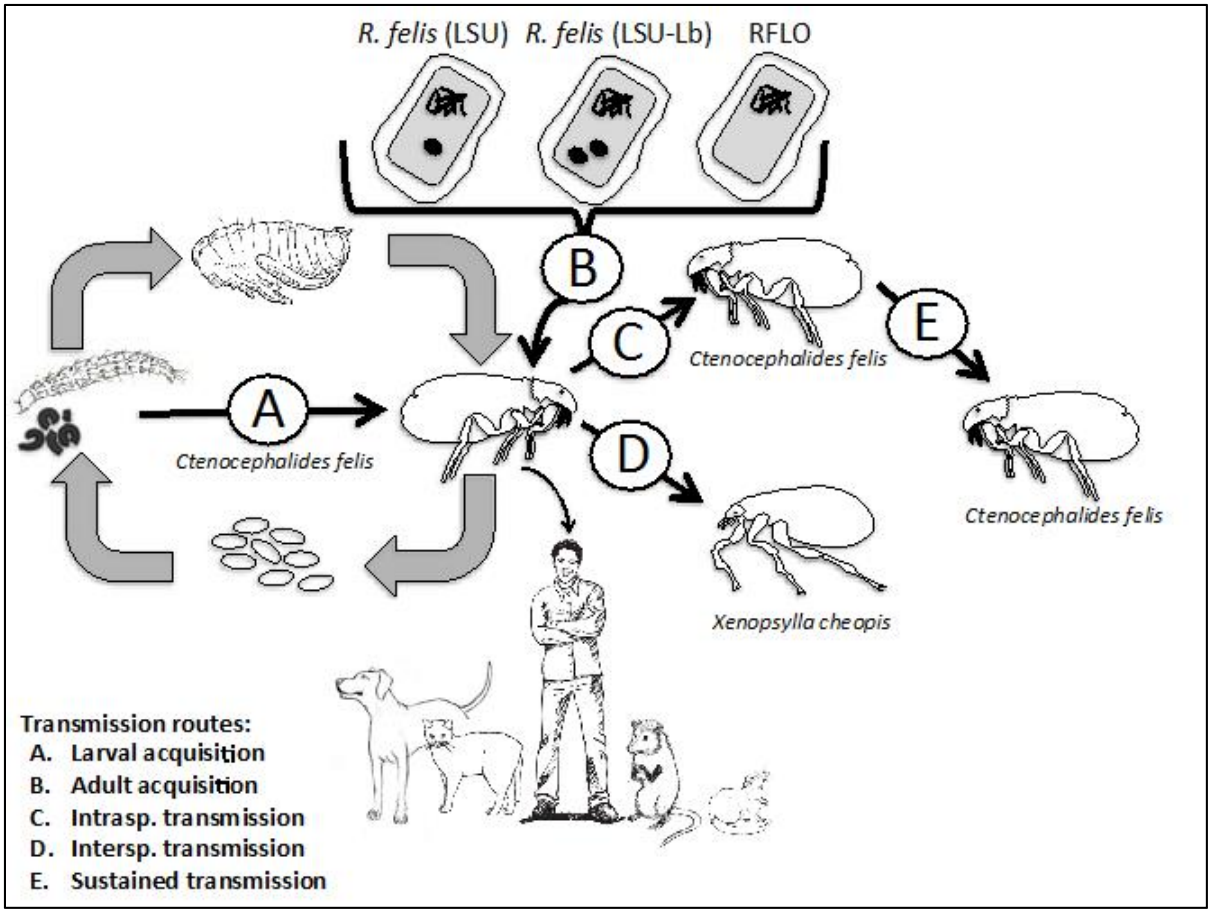

Figure 1.1. The proposed and described transmission routes necessary for persistence and maintenance of *R. felis* infections within the environment. (A) Vertical non-transovarial transmission, *i.e.* larval acquisition by infectious adult feces, of *R. felis* within cat flea colonies requires experimental confirmation. (B) Adult acquisition bioassays with *R. felis* str. LSU and LSU-Lb resulted in infected cat fleas; however, acquisition bioassays with RFLOs have not been attempted. (C) Intraspecific transmission of *R. felis* between co-feeding cat fleas was demonstrated both in an artificial system and on a vertebrate host. (D) Interspecific transmission of *R. felis* between co-feeding cat fleas and rat fleas was observed on a vertebrate host. (E) Sustained transmission of *R. felis* by co-feeding was demonstrated by the continuous spread of infection to newly emerged uninfected cat fleas in an artificial system over the course of four weeks.

United States due to urban expansion into suburban environments and most likely supplementary to the classic association of *R. typhi* with rat fleas and commensal rats (*Rattus* sp.) [99]. Interestingly, a recent survey revealed a higher prevalence of *R. felis* among Oriental rat fleas and Norwegian rats (*Rattus norvegicus*) than *R. typhi* in endemic typhus

areas of Los Angeles [88]. It is unclear whether this urban focus was newly established or represents an expansion of a persistent low-level exposure rate of rat populations to *R. felis*-infected fleas. The vulnerability of human populations to flea-borne rickettsiae is of particular concern in developed countries where aggressive pest management programs may not control for ectoparasites, which can result in the relocation of arthropods to new hosts (*e.g.* humans and their pets) following rodent extermination. Given the indiscriminate feeding habits of cat fleas [15], *R. felis* is essentially a household rickettsiosis in human populations where peri-domestic animals (*e.g.* cats, dogs, opossums) are in close contact.

Much of the latest work concerning the epidemiology of *R. felis* has been conducted almost exclusively in Africa due to the considerable frequency of flea-borne spotted fever in hospitalized febrile patients. In sub-Saharan Africa, *R. felis* is described as a common (3-15%) cause of illness among patients with "fever of unknown origin" in malaria-endemic regions [20, 74, 100]. Remarkably, the incidence of human *R. felis* infections was higher than that of malaria in two of the studied villages of Senegal [100]. This high proportion of *R. felis* infections reported within the last 5 years is in stark contrast to the total number of infections (~100 human cases) documented worldwide [4]. Again, although *R. felis* is classified as an emerging pathogen, it is unclear whether this increased incidence in Africa reflects an overall trend or represents an endemic state previously unknown for this disease. Commonalities (*e.g.* geographic distribution, seasonality, target population, incidence of relapses or re-infections, and asymptomatic infections) were observed between the epidemiology of *R. felis* and *Plasmodium falciparum* infections in Africa, which were initially hypothesized to coincide because of a proposed common vector, *Anopheles* mosquitoes [74]. At the time of the Mediannikov et al. [74] publication, the role of *Anopheles* in the transmission of *R. felis*

was ambiguous; however, the transmission potential of *R. felis* by *Anopheles gambiae* (the primary malaria vector in sub-Saharan Africa) was recently demonstrated in a simulated model [24]. Other arthropods infected with *R. felis* in Africa include numerous species of fleas, mosquitoes, and mites, as well as an individual bed bug [74]. The vertebrate reservoir host responsible for maintenance of *R. felis* in Africa is unknown, but molecular evidence for the presence of *R. felis* in African apes (chimpanzees, gorillas, and bonobos) was derived from PCR-positive stool samples [101]. It was suggested that similar to malaria and other rickettsial species (*e.g.* louse-borne epidemic typhus) the reservoir host of *R. felis* in Africa might be primates, including humans [101]. As such, human fecal samples collected from two Senegalese villages with documented *R. felis* infections were PCR-positive for rickettsial DNA [102]. Conversely, it was demonstrated that for predatory apes (chimpanzees and bonobos) the ingestion of an infected prey species and associated ectoparasites might contribute significantly to the presence of parasite nucleic acids in fecal samples and caution should be used when interpreting these molecular analyses [103].

1.4. Genetic diversity of *Rickettsia felis*

Historically, the genus *Rickettsia* (Rickettsiaceae) was designated as typhus group (TG) or spotted fever group (SFG) rickettsiae; however, *R. felis* displayed phenotypic oddities that confounded its categorization as either TG or SFG, *e.g.* association with insect, hemolytic activity, actin-based motility, transovarial maintenance in the vector hosts, and serological cross-reactivity [104]. Additionally, genetic analyses of *R. felis* revealed a large genome size relative to other rickettsiae, and the presence of plasmids [105]. Combined analyses of genome and biological characteristics suggested that additional groups exist within the genus *Rickettsia*, including a sister clade of the SFG now known as the transitional

group (TRG) and a non-pathogenic clade, thought to be basal to all other groups, called the ancestral group (AG) [104]. *Rickettsia felis* is a member of the TRG rickettsiae, which may explain certain anomalies (*e.g.* lack of a definitive mammalian host) as this bacterium continues to undergo major life history transitions.

Several strains of *R. felis* have been isolated from colonized and wild-caught arthropods [106, 107], including the non-hematophagous, parthenogenic booklouse *Liposcelis bostrychophila* (Insecta: Psocoptera) [89]. In the booklouse host, *R. felis* is an obligate mutualist required for the early development of the oocyte and is maintained 100% transovarially [108, 109]. Given that flea-borne strains are considered facultative parasites of the vector, distinct strains of *R. felis* employ different transmission routes for sustained infection within unique arthropod populations [110]. In an effort to determine whether genetic variability determines *R. felis* host specialization, the sequenced genomes of two strains, *R. felis* (str. LSU-Lb) isolated from a booklice colony and *R. felis* (str. LSU) isolated from a cat flea colony, were compared to the flea-derived *R. felis* reference strain (str. URRWXCal2) [110]. Sequence analyses revealed genomic heterogeneity across the three strains of *R. felis*, suggesting that spatial isolation (str. URRWXCal2 vs. str. LSU) and potential host specialization (flea vs. booklouse) have resulted from genetic divergence [110]. Specifically, the discovery of a second, unique plasmid (pLbaR) in the *R. felis* str. LSU-Lb assembly provides evidence for host-specific strain variation [110]. This discovery coincides with other studies that demonstrated differences in plasmid numbers between *R. felis* strains, with some strains having no plasmids and others having two [111, 112]. Towards this understanding, experimental bioassays were generated to determine acquisition of *R. felis* str. LSU-Lb by a colony of cat fleas, as well as subsequent prevalence and

infection load dynamics (Figure 1., B). Surprisingly, not only did cat fleas become infected with the booklice strain of *R. felis*, there were also negligible differences in prevalence and infection loads between both strains within the same cat flea colony. Additionally, similar to *R. felis* str. LSU, no overt fitness effect on cat fleas infected with *R. felis* str. LSU-Lb was observed, including the production and development of F_1 progeny (authors' unpublished data). Thus, the selective forces operating on *R. felis* genomes from strains associated with different arthropod vectors remain unknown and require further examination.

Within the last decade, numerous reports have identified *Rickettsia felis*-like organisms (RFLOs) in different arthropods, including cat fleas (Table 1.2), throughout the world based on multilocus sequence typing (MLST). A gene sequenced-based criterion was proposed for the identification of *Rickettsia* isolates at the genus, group, and species level [113]. As such, the number of newly identified *Rickettsia*, specifically RFLOs, has dramatically increased since this recent designation. The proposed genetic guidelines rely on similarities (*i.e.* percent homology) in the sequences of the 16S rRNA (*rrs*) (≥99.8%) gene and four protein-coding genes, the *gltA* (≥99.9%), *ompA* (≥98.8%), and *ompB* (≥99.2%) genes and gene D (≥99.3%) to existing *Rickettsia* species [113]. The concern with this approach is that 0.2% divergence in the *rrs* gene is the borderline for separation of 2 *Rickettsia* species, whereas 1% divergence is known to mark the borders of naturally occurring bacterial species [137]. For example, two recently described *Rickettsia* species isolated from cat fleas, *Candidatus* Rickettsia asemboensis and *Candidatus* Rickettsia senegalensis, showed 99.5% and 99.65% similarity to the *rrs* gene in validated species of *R. felis*, respectively [60, 130]. Given the potential for genetic diversity of *R. felis* isolates due to spatial isolation, a more suitable approach to justify the separation of RFLOs into species

Table 1.2. Geographic distribution of RFLO in wild-caught arthropods.

Country	Vector	Prevalence of Infection	Reference
Brazil	*Ctenocephalides felis*	ND	[35]
China	*Eulaelaps stabularis*	ND	[37]
Côte d'Ivoire	*Anopheles gambiae, Anopheles melas*	7% (5/77)	[42]
Costa Rica	*C. felis*	ND	[40]
Croatia	*Haemaphysalis sulcata*	23% (23/101)	[114]
Czech Republic	fleas	3% (1/33)	[44]
Ecuador	*C. felis*	100% (8/8)	[115]
Egypt	*Echidnophaga gallinacea*	100% (12/12)	[116]
	Ornithonyssus bacoti	ND	[117]
France	*Archaeopsylla erinacei*	50% (2/4)	[64]
Gabon	*Ctencephalides canis*	100% (12/12)	[64]
	An. gambiae	1% (1/88)	[42]
	An. melas	9% (6/67)	
Germany	*Archaeopsylla erinacei*	96% (144/150)	[118]
Hungary	*Pulex irritans*	ND	[53]
India	fleas	78% (7/9)	[119]
	C. felis	73% (56/77)	[120]
Iran	*Pediobius rotundatus*	20% (1/5)	[121]
Israel	*Xenopsylla ramesis, Synosternus cleopatrae*	ND	[122]
Japan	*C. felis*	39% (26/67)	[123]
Kenya	*Xenopsylla cheopis, C. felis, C. canis, P. irritans, E. gallinacea*	ND	[60]
	C. canis, C. felis	ND	[124]

Table 1.2 continued

Malaysia	*C. felis*	3% (6/209)	[125]
Peru	*C. felis*	96% (71/74)	[126]
Portugal	*Ornithodoros erraticus*	ND	[127]
Senegal	*Synosternus pallidus*	91% (31/34)	[128]
	Glossina morsitans	100% (78/78)	[129]
	C. felis	17% (5/29)	[130]
Slovakia	*Ctenophthalmus agyrtes, Ctenophthalmus solutus, Ctenophthalmus uncinatus, Nosopsyllus fasciatus*	11% (34/315)	[75]
Spain	*C. canis, C. felis*	28% (25/88)	[131]
Taiwan	*Leptotrombidium* chigger mites, *Ixodes granulatus*, Mesostigmata mites	ND	[132]
Thailand	*C. canis, C. felis*	43% (66/152)	[133]
Thai-Myanmar border	*C. canis, C. felis*	4% (4/54)	[134]
USA	*C. felis*	100% (19/19)	[135]
	C. felis	ND	[136]
	Carios capensis	ND	[90]

ND, not determined.

may be to seek ecological, genomic or phenotypic differences among the major clusters resolved by MLST [137].Recently, the whole-genome of *Candidatus* Rickettsia asemboensis was sequenced [138], and future comparative analyses may reveal genotypic differences responsible for phenotypic characteristics.

1.5. Prospective research for *Rickettsia felis*

The transmission routes required for persistence and maintenance of *R. felis* infections in endemic-disease foci remains unclear (Figures 1., A-E). Excretion of viable rickettsiae in the feces of infected arthropods is crucial in transmission cycles for both louse-borne epidemic typhus (*Rickettsia prowazekii*) and flea-borne endemic typhus (*R. typhi*) [99, 139]. The direct inoculation of fecal bacteria by scratching at the bite site constitutes as a persistent source of infection from arthropod to vertebrate hosts. Although *R. felis*-infected cat fleas generate feces with detectable levels of rickettsial transcript [16], the transfer of bacteria from freshly deposited adult feces to susceptible vertebrates has not been demonstrated. Another flea-borne pathogen, *Bartonella henselae*, achieves successful transmission from adult fleas to their progeny via vertical non-transovarial transmission [140]. Vertical transmission of *Bartonella* species was demonstrated, but a previous study showed the absence of transovarial transmission of *B. henselae* within flea colonies [141]; however, when flea larvae were exposed to *Bartonella*-positive adult flea feces then the larvae acquired an infection that was maintained through adulthood [140]. Thus, vertical non-transovarial transmission of *R. felis* should be tested within cat flea colonies as an additional route of pathogen maintenance in vector populations (Figure 1., A).

The lack of a description of a definitive vertebrate host impedes epidemiological studies of *R. felis* throughout the world. Doubts have been raised about whether *R. felis*

transmission from mammal to arthropod occurs given the efficiency of pathogen transfer between co-feeding fleas without a systemic vertebrate infection [95]; however, field surveys frequently identify mammalian hosts (*e.g.* cats, dogs, opossums, rodents) as either seropositive or PCR-positive for *R. felis* infections in endemic disease foci. Transmission of *R. felis* within cat flea colonies has proved variable and adaptable, with decreased colony prevalence signaling to increase infection burdens in individual fleas [13]. Thus, only occasional amplification from vertebrate hosts may be needed to enhance or maintain *R. felis* in nature. The latest reports from urban environments have emphasized the potential of domestic cats and dogs as mammalian reservoirs of *R. felis* infections [30, 32, 55, 142-145], while studies from uninhabited localities suggest the importance of rodents and opossums [22, 146]. Accordingly, it appears that a peri-domestic cycle exists for *R. felis* where components of this enzootic cycle are present, *e.g.* free-ranging cats and dogs, commensal rodents and opossums, and associated flea species. Future studies should address Koch's postulates to identify *R. felis* as the causative agent of vertebrate infection, specifically isolation of *R. felis* for culture from these proposed reservoir hosts.

Recently, *R. felis* infections in febrile and afebrile patients were diagnosed by PCR detection in human blood samples [74, 147]; thus, it was proposed that perhaps humans could be the natural reservoir for *R. felis*, as they are for another insect-borne rickettsial species (*R. prowazekii*). The transmission cycle for *R. prowazekii* is louse to human to louse, with lice ingesting bacteria by blood-feeding on infected humans and subsequently transferring the bacterium to humans by excretion of infectious feces at the bite site [139]. A delayed complication of *R. prowazekii* is Brill-Zinsser disease, or recrudescent typhus, in which mild symptoms reappear after a latent period [139]. Humans with recrudescent typhus are still

capable of infecting lice and spreading the disease [139]. Similarly, *R. felis* DNA was detected in the blood of a patient at multiple time points over a 1.5-month interval. While this initial observation suggests episodic rickettsial infection (relapse or reinfection) in humans, these samples were taken from a child in the absence of antimicrobial therapy [100]. The occurrence of relapses or reinfections of *R. felis* should be investigated further with adult patients administered antibiotic treatment. Additional studies reported that not all patients diagnosed as PCR-positive for *R. felis* infection generated anti-rickettsial antibodies, which researchers proposed supports the notion of a recurrent infection [74, 148]; however, supplementary data may marginalize diagnoses of *R. felis* infection based on PCR-positive blood samples. For example, *R. felis* DNA was detected in skin swabs from healthy individuals in a Senegal village where roughly 7% of the villagers possess an *R. felis* infection [149, 150]. This study highlights the potential for blood samples from afebrile patients to become polluted by skin surface contaminants prior to molecular analyses [149]. Furthermore, the discovery of *R. felis* in blood samples from asymptomatic persons challenges existing paradigms about pathogenic rickettsiae. Such as, the magnitude of rickettsial growth required for PCR detection in the bloodstream of patients is typically fatal, yet these afebrile individuals had no adverse symptoms [151]. Rickettsioses in febrile and afebrile persons should be confirmed by culture, but as stated previously *R. felis* has not been isolated from a vertebrate host, even in severe human cases. Thus, a human isolate must be obtained before conclusions are drawn on the role of people in *R. felis* epidemiology.

The genetic diversity within the *R. felis* genotype appears to be vast, with different isolates shown to consist of unique individual qualities. Whether RFLOs warrant species designation is unclear, but there are disparities among this genogroup that may lead to a

microbial-dependent influence on *R. felis* prevalence. For example, interspecific competition of rickettsiae in ticks is well documented, with a primary infection responsible for the interference or blocking of a secondary infection [152-154]. Thus, the high prevalence of RFLOs in areas where *R. felis* infections appear low or absent may be due to an interference event followed by perpetuation of the primary infection within a closed arthropod population. The transmission biology as well as the pathogenicity of RFLOs is unknown, but these organisms are detected in arthropods known to bite humans. Future work with RFLOs should identify, if any, phenotypic characteristics associated with genotypic diversity and focus on acquisition, dissemination, and transmission of these organisms by their respective arthropod hosts (Figure 1., B).

1.6. Conclusions

Every year there are new reports of arthropod, animal, and human cases of *R. felis* from additional countries, and the influx of RFLOs may result in a similar trend. Active surveillance of *R. felis* infections among hospitalized febrile patients will determine when an endemic state has been reached by this emerging pathogen, as well as indicate spread to populations outside of endemic disease foci. Advance genetic analyses of *Rickettsia* species should include criteria for ecological, genomic and phenotypic differences in addition to sequence homology. In order to determine the specific roles of both the vertebrate and arthropod host in the transmission cycle of *R. felis*, it is critical to continue the development and implementation of molecular tools and bioassays necessary for more accurate risk assessment and efficacious control measures.

Most pathogenic species of *Rickettsia* are cycled through transovarial and transstadial passages in their arthropod hosts, and transmitted to new hematophagous vectors through the

infectious blood of vertebrate amplifying hosts [155]. The continuous molecular detection of *R. felis* from other blood-feeding vectors supports the notion of infectious transmission cycles [4]; however, naturally infected mammalian blood or tissues have never been shown to be a source of *R. felis* infection from vertebrate to arthropod host. Therefore, despite the demonstration of horizontal transmission in an artificial host system [18], the principal route of rickettsial pathogens from systemically infected vertebrates to uninfected arthropods may not be applicable to the *R. felis* transmission cycle. The broad hypothesis of this dissertation research is that horizontal transmission of *R. felis* occurs independent of a rickettsemic vertebrate host. The specific objectives of this dissertation were to: (1) examine horizontal transmission of *R. felis* via vector cofeeding on a vertebrate host; (2) determine the extrinsic incubation period of *R. felis* within cat fleas; and, (3) assess the mechanisms utilized for horizontal transmission of *R. felis* by cat fleas.

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CHAPTER 2
COFEEDING INTRA- AND INTERSPECIFIC TRANSMISSION OF AN EMERGING INSECT-BORNE RICKETTSIAL PATHOGEN[1]

2.1. Introduction

Insect-borne rickettsial diseases have dramatically shaped human history (*e.g.* louse-borne epidemic typhus was responsible for the deaths of more French soldiers than warfare during Napoleon's retreat from Moscow) [1]. Presently, infections are encountered in populations living in unsanitary, crowded conditions [1, 2] as urban expansion into suburban environments worldwide has generated ideal ecosystems for infectious disease outbreaks caused by these prevalent pathogens (*e.g.* reemergence of flea-borne endemic typhus in southern California and Texas) [3, 4]. Observed with considerable frequency, a third insect-borne rickettsial pathogen, *Rickettsia felis*, was identified as the causative agent of the emerging flea-borne spotted fever in hospitalized patients with acute febrile illness [5-15]. Since the first human case reported from Texas in 1994, *R. felis* has been detected from every continent except Antarctica [5, 12, 16]. The widespread range of *R. felis* corresponds to the cosmopolitan distribution of the primary hematophagous vector for this pathogen, the cat flea (*Ctenocephalides felis*) [17]. Cat fleas are arguably one of the most common flea species worldwide and lack true host specificity [18]; therefore, *R. felis* is essentially a household rickettsiosis in human populations where peri-domestic animals (*e.g.* cats, dogs, opossums) are in close contact.

Insect-borne rickettsial pathogens follow the most common horizontal transmission cycle of vector-borne pathogens which includes three sequential components: (i) an infectious (donor) arthropod introduces an inoculum of the pathogen to a vertebrate host

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during blood meal acquisition; (ii) a susceptible vertebrate host develops a systemic infection with circulating pathogen in its bloodstream; and (iii) a naïve (recipient) arthropod imbibes the pathogen from subsequent blood feeding on the now infectious vertebrate host [19]. It is the generalist blood-feeding behavior of most arthropod vectors that increases the potential for emerging diseases by providing a novel infection route between animals and humans [20]. Maintenance of vector-borne pathogens through this type of horizontal transmission is dependent upon competent vertebrates to provide an infectious bloodmeal to recipient arthropods; however, persistently infected animals that serve as reservoirs of pathogens for arthropod vectors are inconsistently available in nature [21]. Unless vertical transmission events are 100% efficient then additional horizontal amplification is required for the maintenance of pathogens within host populations [22]; thus, vertical transmission of certain vector-borne pathogens eliminates the need for a vertebrate host by passing the infection from adult arthropods to their offspring.

Sustained *R. felis* infections within cat flea populations were first postulated to occur through stable vertical transmission [23]; however, this transmission route is shown to be highly variable with F_1 infection rates ranging from 0 – 100% within commercial and institutional flea colonies [17]. Thus, vertical transmission alone does not sufficiently explain maintenance of *R. felis* within flea populations. Although not confirmed on a vertebrate host, the potential for horizontal transmission of *R. felis* between cat fleas has been demonstrated with the use of a shared bloodmeal in an artificial host system [24]. The transmission of *R. felis* between infected (donor) and naïve (recipient) fleas during feeding events suggests the potential for a rapid expansion of infection through horizontal transmission, but the sustained transmission of *R. felis* from recipient to other naïve cat fleas has not been assessed.

Complicating the epidemiology of flea-borne spotted fever are progressively accumulating field surveys reporting molecular detection of this infectious agent from other human-biting vectors (more than 40 other species of fleas, ticks, mites, and mosquitoes) [12]. Vectorial capacity for *R. felis* has not been assessed in these additional arthropod species and a vertebrate reservoir has not been identified for *R. felis*, in spite of numerous field studies and laboratory attempts to delineate a host based on animals naturally infested with *R. felis*-infected cat fleas (*e.g.* cats, dogs, opossums, rats) [25-32]. Although most peri-domestic animals implicated in the transmission of *R. felis* are seropositive to rickettsial antigen, certain individuals may show no correlation between seroprevalence and *R. felis*-infected cat fleas [25, 31]. Moreover, *R. felis* has been identified by molecular detection from the blood, skin, and internal organs of suspected reservoir hosts [26, 33-37], but viable bacteria have never been isolated from these tissues. A recent study generated *R. felis*-infected mice (inbred mouse strain BALB/c) via an artificial inoculation route, and subsequently produced infectious *Anopheles gambiae* mosquitoes that caused transient rickettsemia in naïve mice [38]; however, naturally infected mammalian blood or tissues have never been shown to be a source of *R. felis* infection from vertebrate to arthropod host [39]. Additionally, much debate surrounds the likelihood of freely circulating rickettsiae in the blood of vertebrates from non-fatal cases [40]. Therefore, despite the demonstration of horizontal transmission in an artificial host system [24], the principal route from systemically infected vertebrates to uninfected arthropods may not be applicable to the *R. felis* transmission cycle.

Successful horizontal transmission of pathogens between actively blood-feeding arthropods in the absence of a disseminated vertebrate infection has been demonstrated (reviewed in [22]). This transmission event, referred to as cofeeding, is reliant on the

temporal and spatial dynamics of infected and uninfected arthropods as they blood feed. The infected arthropod is both the vector and the reservoir for the pathogen, while the vertebrate acts as a conduit for infection of naïve arthropods. For example, guinea pigs are non-competent hosts for Thogoto virus (family Orthomyxoviridae) transmitted by African ticks (*Rhipicephalus appendiculatus*); yet, as long as the infected and uninfected ticks feed simultaneously, albeit physically separated, then transmission of this tick-borne virus between ticks occurs independent of a viremic host [41]. Similar results are observed for tick-borne encephalitis virus (family Flaviviridae), including cofeeding transmission with the use of both traditional (*Ixodes ricinus*) and non-traditional (*Rhipicephalus appendiculatus*) vector species [42, 43]. Cofeeding transmission is not limited to tick-borne viruses and is a confirmed route for transmission of *Rickettsia conorii israelensis* between *Rhipicephalus sanguineus* ticks [44]. Also, as opposed to the long-term cofeeding transmission behavior of ticks, experimental results revealed transfer of West Nile virus (family Flaviviridae) between intermittent cofeeding mosquito species (*Culex* and *Aedes* spp.) [45]. Although cofeeding transmission was demonstrated, these pathogens are also maintained by the classic transmission paradigm of an infectious vertebrate host, which has not been demonstrated for *R. felis*. Despite the absence of *R. felis*-infectious bloodmeals in vertebrate reservoir hosts, no studies have examined cofeeding transmission as an alternative mechanism to explain the presence of this pathogen amongst widely distinct arthropods. Thus, we hypothesized that if cofeeding transmission with *R. felis*-infected cat fleas accounts for the incidence of *R. felis* in additional blood-feeding arthropods, then transfer of the pathogen is independent of a rickettsemic vertebrate host.

In the present study, we utilized two flea species, *C. felis* and *Xenopsylla cheopis* (Oriental rat flea), to study the transmission of *R. felis* between cofeeding arthropods on a vertebrate host. *Xenopsylla cheopis* is the biological vector of *Rickettsia typhi*, but *R. felis* is routinely detected in wild-caught individuals and is even considered more prevalent than *R. typhi* in some *X. cheopis* populations [34]. A murine model was developed to conduct rickettsial cofeeding transmission bioassays between *R. felis*-infected donor cat fleas and uninfected recipient cat fleas (intraspecific transmission) and rat fleas (interspecific transmission), respectively. Specifically, we examined (i) cofeeding transmission between donor and recipient cat fleas in the same feeding capsule (cofed bioassays) in which donor cat fleas were exposed to either a low dose (5×10^9 rickettsiae/mL) or high dose (5×10^{10} rickettsiae/mL) infectious bloodmeal prior to association with recipient fleas, (ii) cofeeding transmission between donor and recipient cat fleas in separate feeding capsules (cross-fed bioassays) positioned 20 mm apart using both sets of donor cat fleas exposed to low and high dosages prior to placement in capsules, and (iii) cofeeding transmission between donor cat fleas and recipient rat fleas in the same feeding capsule using low and high dose exposed donor cat fleas. Additionally, successive horizontal transmission bioassays were conducted in an artificial host system with recipient cat fleas generated from cofeeding with donor fleas then placed with additional naïve cat fleas in order to assess the persistence of *R. felis* within the vector population through cofeeding transmission. Furthermore, we utilized a stochastic model to demonstrate that cofeeding transmission is sufficient to explain the enzootic spread of *R. felis* between cat fleas. Our results implicate cat fleas in the spread of *R. felis* amongst different vectors, and the demonstration of cofeeding transmission of *R. felis* through a

vertebrate host represents a novel transmission paradigm for insect-borne *Rickettsia* and furthers our understanding of this emerging rickettsiosis.

2.2. Materials and Methods

2.2.1. Species and strains of bacteria, fleas, and mice

The *R. felis* strain used was originally obtained from the Louisiana State University cat flea colony (*R. felis*; LSU; passage 3) and maintained in an *Ixodes scapularis* embryonic cell line (ISE6), provided by T. Kurtti (University of Minnesota), in modified L15B growth medium [46]. Rickettsial infections within culture were monitored using the Diff-Quik staining procedure [46], and the number of rickettsiae was enumerated by the *Bac*Light viability stain kit [47]. Newly emerged, *Rickettsia*-uninfected cat fleas were purchased from Elward II (Soquel, CA, USA), and given 2 mL of heat inactivated (HI) defibrinated bovine blood (HemoStat Laboratories) within an artificial dog unit [48]. Prior to exposure of their first bloodmeal, a portion of these experimental cat fleas were tested to verify the absence of *R. felis* infection with the use of quantitative real-time polymerase chain reaction (qPCR) analyses [49]. The remaining cat fleas were allowed to feed on the bovine blood for 24 hrs without disturbance prior to use in bioassays. Rat fleas were generously provided by B. Joseph Hinnebusch (Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT) and used in bioassays immediately following their arrival to LSU. Five week old, male, mice strain C3H/HeJ were purchased from Jackson Laboratory as a murine model organism.

2.2.2. Ethics Statement

This study was carried out in accordance with the following: Animal Welfare Act (9 CFR Ch. 1 Subpart C 2.31 (c) (1 – 8)), Guide for the care and use of Agricultural Animals in

Agricultural Research and Training (Chap. 1), and the Public Health Service Policy on Human Care and Use of Laboratory Animals (Section IV.B. (1 – 8)). All animal research performed under the approval of the LSU Institutional Animal Care and Use Committee (IACUC) (Protocol Number: 13-034).

2.2.3. Cat flea bloodmeal treatments in the artificial dog unit

Following the 24 hr period of pre-feeding on HI bovine blood, cat fleas were divided into three groups, starved for 5-6 hrs, and given one of three bloodmeal treatments: *Rickettsia felis*-infected bloodmeal, Rhodamine B-labeled bloodmeal, or control bloodmeal. Intact *R. felis*-infected cells were used following bacterial count, and diluted to inoculation doses containing $5x10^9$ rickettsiae (low dose) or $5x10^{10}$ rickettsiae (high dose). *Rickettsia felis*-infected cells were pelleted by centrifugation at 13 000 x *g* for 10 min and resuspended in 600 µL of HI bovine blood. Cat fleas were allowed to feed on the *R. felis*-infected bloodmeal for 24 hrs, after which fleas fed on an uninfected bloodmeal for an additional 48 hrs. In order to differentiate between cat fleas exposed or unexposed to a *R. felis*-infected bloodmeal, the biomarker Rhodamine B (RB) was used as previously described [24]. For a control bloodmeal, 2 mL of unaltered (*i.e.*, without rickettsiae or RB) HI bovine blood was used as a treatment to generate control cat fleas for the duration of the experiment.

2.2.4. Rickettsial horizontal transmission bioassays on C3H/HeJ mice

Four bioassays were established (acquisition, cofed, cross-fed, and control) with cat fleas exposed to the *R. felis*-infected bloodmeal (donor cat fleas), labeled with RB (recipient cat fleas), or unaltered (control cat fleas) to examine rickettsial transmission (Figure 2.1, A).

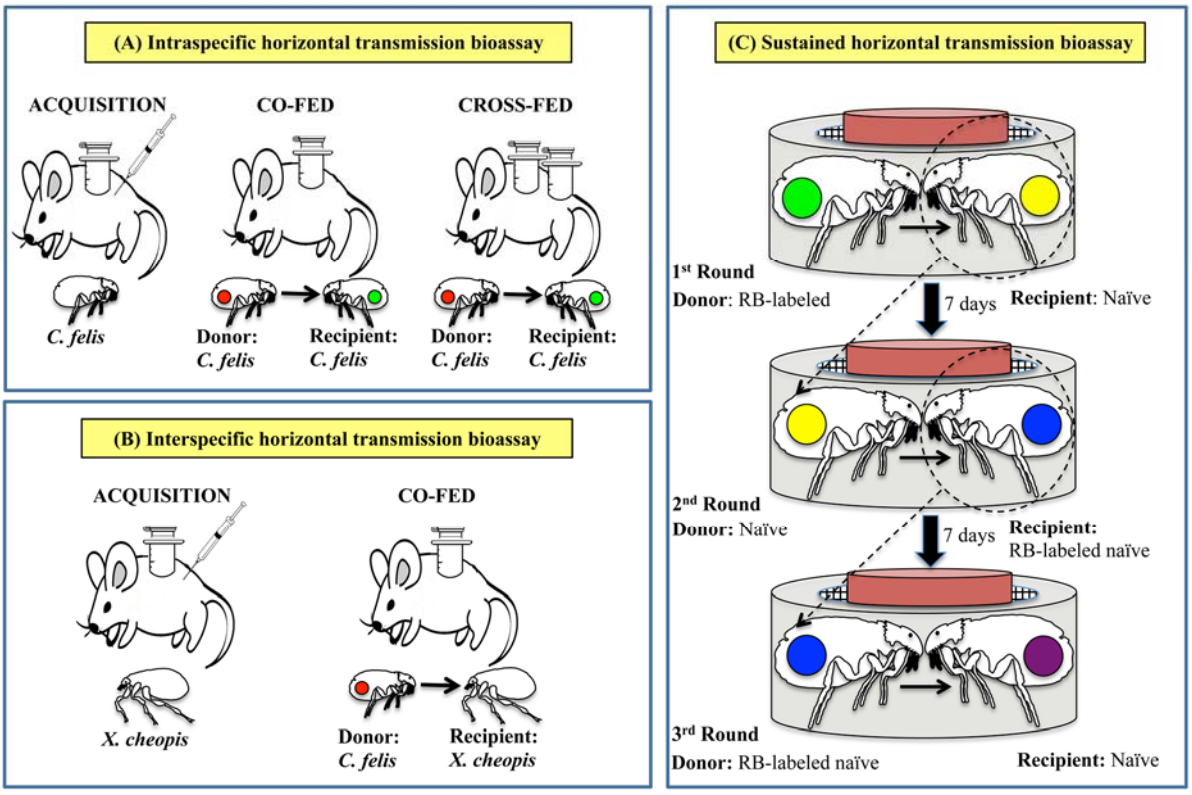

Figure 2.1. Rickettsial horizontal transmission bioassays. (A) Cat fleas (*Ctenocephalides felis*) were infected by ingestion of *Rickettsia felis* in an intradermal (ID) bleb or by cofeeding naïve cat fleas (green circle) with *R. felis*-infected cat fleas (red circle) for 24 hrs. Cofed bioassays consisted of donor and recipient cat fleas in the same feeding capsule, while cross-fed bioassays involved placement of donor and recipient cat fleas in different feeding capsules on the same mouse. (B) Rat fleas (*Xenopsylla cheopis*) were infected by ingestion of *R. felis* in an ID bleb or by feeding naïve rat fleas with *R. felis*-infected cat fleas (red circle). Cofed bioassays consisted of donor cat fleas (*C. felis*) and recipient rat fleas (*X. cheopis*) in the same feeding capsule. (C) Successive horizontal transmission bioassays were conducted in an artificial host system with recipient and naïve cat fleas. Following a week of cofeeding with *R. felis*-infected donor cat fleas (not pictured), the recipient cat fleas (green circle) were grouped with naïve cat fleas (yellow circle) for 7 days (1st round). The recipient cat fleas were then removed and replaced by naïve cat fleas (blue circle) labeled with Rhodamine B for 7 days (2nd round). Finally, the naïve cat fleas were removed and replaced by additional naïve cat fleas (purple circle) for the final 7 days (3rd round).

For each bioassay, fleas were placed in a feeding capsule created from a modified 1.7 mL microcentrifuge tube and adhered to the flank of the mouse with a 1:4 mixture of beeswax and rosin [50]. To determine if cat fleas could acquire *R. felis* from a vertebrate host, C3H/HeJ mice received an intradermal (ID) inoculation with $5x10^9$ rickettsiae in 100 µL of

SPG buffer (referred to as a bleb) and 10 cat fleas were placed into a feeding capsule adhered over the bleb. The cofed bioassays consisted of 10 donor cat fleas and 10 recipient cat fleas in the same feeding capsule. The cross-fed bioassays involved placement of 10 donor cat fleas in one feeding capsule and 10 recipient cat fleas in a different feeding capsule on the same mouse. Low and high dose infectious bloodmeals were fed to two distinct groups of donor cat fleas and each group was utilized in independent cofed and cross-fed bioassays. The control bioassays used 10 control cat fleas in the same feeding capsule. Sexual transmission of *R. felis* between cofeeding cat fleas *in vitro* has been reported [24], therefore all intraspecific bioassays were conducted with only female cat fleas.

To examine interspecific rickettsial transmission between cat fleas and rat fleas on a vertebrate host, three of the four previously described bioassays (acquisition, cofed, and control) were used (Figure 2.1, B). Identical to intraspecific bioassays, blebs were constructed to determine acquisition of *R. felis* infection by rat fleas from the C3H/HeJ mice with use of the same methods described above. The cofed bioassays consisted of 10 donor cat fleas exposed to the high dose infectious bloodmeal and 10 recipient rat fleas in the same feeding capsule. Likewise, the control bioassay used 10 unaltered rat fleas in the same feeding capsule. All aforementioned intra- and interspecific bioassays were conducted in three separate trials for a 24 hr period. After this 24 hr period, the mice were humanely euthanized with carbon dioxide followed by cervical dislocation. Skin at the site of capsule placement and away from the site was collected aseptically and placed in 10% formalin for histopathological evaluation. Additionally, skin between capsules was collected from crossfed animals, placed into RNAlater (Ambion), and stored at -80ºC for RNA extraction.

2.2.5. Sustained rickettsial horizontal transmission bioassay

In order to demonstrate sustained transmission of an *R. felis* infection within the vector population, successive horizontal transmission bioassays (three rounds total) were conducted in an artificial host system (Figure 2.1, C). Following exposure to a high dose *R. felis*-infected bloodmeal, donor cat fleas were housed with recipient cat fleas as previously described [24] for 7 days. Recipient cat fleas were then grouped with naïve cat fleas for 7 days (1st round) afterwards the recipient cat fleas were removed and replaced by naïve cat fleas labeled with RB (2nd round). The original naïve cat fleas from the first round are the donor cat fleas in the second round of transmission bioassays. Finally, the naïve cat fleas were removed and replaced by additional naïve cat fleas for the final 7 days (3rd round). Given that the infection prevalence of recipient cat fleas in an artificial host is approximately 10.0%, the initial horizontal transmission bioassay included 200 donor cat fleas and 200 recipient cat fleas in an attempt to ensure a successful transmission event as well as securing enough fleas to complete the 4-week experiment. After each succeeding transmission bioassay, there was a decrease in the number of donor cat fleas therefore an equal number of recipient cat fleas was used to create the new cage each week. The first round used 200 donor and recipient cat fleas, second round used 165 donor and recipient cat fleas, and the third round used 85 donor and recipient cat fleas.

2.2.6. Detection of *Rickettsia* in fleas and mice

After the above experimentation, the collected fleas were washed with 10% bleach for 5 minutes, 70% ethanol for 5 minutes, and sterile distilled water for 5 minutes (three times). Fleas were then placed in microcentrifuge tubes and homogenized with a combination of liquid nitrogen and sterile plastic pestles. Genomic DNA (gDNA) was extracted using

Qiagen DNeasy Tissue Kit according to the manufacturer's instructions and eluted in 25 µL PCR-grade H_2O. A negative environmental control (DNA extraction reagents without biological sample) was utilized for each DNA extraction process, as well as a negative control for the qPCR (ultrapure sterile water in the place of template). All gDNA preparations were stored at -20°C. Quantitative PCR analyses used the plasmid pCR4-TOPO-Rf17kda+Cf18SrDNA as a standard template to create serial 10-fold dilutions ($1x10^9$ to 10 copies) as described previously [49]. The qPCR was performed with a LightCycler 480 Real-Time PCR system (Roche), and results were presented as quantified rickettsial copy numbers per individual flea lysate. Additionally, once mice were sacrificed, whole blood was collected via cardiocentesis into EDTA tubes and gDNA was extracted for qPCR following the same methodology as above in an attempt to delineate a disseminated *R. felis* vertebrate infection.

In order to examine the potential viability of *R. felis* transmitted between cofeeding cat fleas (*i.e.* transmission of transcriptionally active organisms and not deceased organismal DNA), rickettsial RNA was isolated from skin samples between capsules of mice in cross-fed bioassays to synthesize complementary DNA (cDNA). Following bioassays, tissues were collected near feeding capsule sites and placed in RNAlater for storage at -80°C. Extraction of RNA from skin samples was accomplished using Qiagen's RNeasy Mini Kit according to the manufacturer's instructions for total RNA isolation from tissues. Briefly, tissue disruption and homogenization were performed by combining the tissue samples with two stainless steel beads in a microcentrifuge tube containing Buffer RLT, followed by shaking in a TissueLyser (Qiagen) [51]. Further sample lysis and wash steps were performed according to the manufacturer's instructions, and samples were eluted in 30 µL RNase-free water. RNA

samples were DNase I treated (Promega) according to the manufacturer's instructions. The DNase I-treated RNA samples synthesized *R. felis* 17-kDa gene-specific cDNA using the random hexamers approach in the SuperScript® First-Strand Synthesis System (Invitrogen). In order to confirm the absence of DNA contamination, no-RT controls were included for all samples. Viability of *R. felis* was determined by qPCR amplification (as described above) of *R. felis* 17-kDa from prepared cDNA [52].

2.2.7. Histopathology and immunohistochemistry

After formalin fixation, skin samples were paraffin-embedded and sections were cut for both hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) with a polyclonal anti-*Rickettsia* antibody (diluted 1/1000) as previously described [51]. Skin sections were blindly examined by a board-certified veterinary anatomical pathologist, and dermatitis was categorized as absent (non-significant lesions), mild (rare to infrequent small foci of inflammatory cells (1-4 cells) in the superficial dermis, overall <20% of all cells), moderate (several medium foci of inflammatory cells (5-10 cells) extending from the superficial to deep dermis, overall 20-50% of all cells) or severe (frequent large multifocal to coalescing foci of inflammatory cells (>10 cells) extending from the superficial to deep dermis and into subcutaneous fat (panniculitis), overall >50% of all cells).

2.2.8. Statistical analyses and model of cofeeding transmission

A Fisher's exact test was performed to examine independence between the proportion of *R. felis* infections in donor cat fleas versus recipient cat fleas in the cofed and cross-fed bioassays, independence between the proportion of *R. felis* infections in recipient cat fleas versus low and high infectious dosages in the cofed and cross-fed bioassays, as well as independence between *R. felis* infections in recipient cat fleas versus recipient rat fleas in the

high dose cofed bioassays. Additional comparisons within bioassays were made by a Mann-Whitney test between total rickettsial infection loads. Also, a Kruskal-Wallis test was used to compare rickettsial infection loads between rounds of sustained transmission bioassays, followed by a Dunn's multiple comparison test when significance was observed. All statistical analyses were performed using GraphPad Prism Version 6 (GraphPad Software, Inc. 2013), and differences were considered significant at $p \leq 0.05$.

A stochastic, event-driven model was constructed to determine whether cofeeding transmission amongst an isolated cat flea population is capable of supporting pathogen persistence in the absence of rickettsemic vertebrate hosts. Given the absence of vertical transmission in our previous studies [24, 52], this parameter is not incorporated in the cofeeding transmission model for sustainability. Model parameter values were defined by reviews of the literature and data generated in the current study (Table 2.1). The transition rates for the stochastic simulation model are stated in Table 2.2. The framework for these

Table 2.1. Parameter values and definitions derived from experimental data or published literature for *Ctenocephalides felis*.

Parameter (value)	Definition	Reference
a (once daily)	The daily biting rate of fleas with vertebrates	[53]
b (variable)	The probability of infection of a 'recipient' flea by a 'donor' flea	From data (Table 1)
f (4.5% every 7 days)	The daily flea transfer rate from one vertebrate host to another	[54]
B (1000 fleas every 28 days)	The recruitment rate of new fleas	Set to maintain constant density of flea population
μ^{-1} (28 days)	The average lifespan of a flea	Personal observation utilizing the artificial membrane system

Table 2.2. Transition rates for the stochastic simulation model.

Event	Change in State	Transition Rate
Transmission from donor to recipient flea	$S_f \rightarrow I_f$	$b*a*(C_v/N_v)*S_f$
Susceptible flea death	$S_f \rightarrow S_{f.\mu}$	$\mu*S_f$
Infected flea death	$I_f \rightarrow I_{f.\mu}$	$\mu*I_f$
Contamination of a vertebrate through infestation with at least one infectious flea	$U_v \rightarrow C_v$	$a*(I_f/N_f)*U_v$
De-contamination of a vertebrate through loss of all infectious fleas	$C_v \rightarrow U_v$	$f*(I_f/N_f)*C_v$

compartments was based on the following conditional states: fleas are either 'susceptible' to *R. felis* infection (S_f) or, after *R. felis* infection, 'infectious' to other fleas (I_f); and vertebrate hosts are either 'uncontaminated' in the absence of infectious fleas [55] or 'contaminated' in the presence of at least one infectious flea (C_v), independent of vertebrate systemic infection (Figure 2.2). Additionally, vertebrate species are assumed to be in a closed population (N_v = 100 total vertebrates), and flea density is assumed to be constant ($S_f + I_f = N_f$) by defining the recruitment rate (B) as approximately equal to the average mortality rate of the flea population (μ^{-1}) (Table 2.1). Stochastic realizations of the model were simulated using the tau-leap approximation to Gillespie's algorithm [56]. The model simulations ran for 280 days (equivalent to approximately 10 flea generations), and a time-step of $^1/_8$ days was chosen for maximized computational efficiency and accuracy [57]. All model simulations were performed in R version 3.0.1.

In order to investigate the role of cofeeding transmission in the context of pathogen introduction and persistence, the model was initialized with a single infectious flea and simulated with n = 1000 realizations; probability of pathogen transmission and persistence

was then calculated following the introduction of this single infectious flea. Transmission was defined as secondary infection of previously susceptible fleas in the system. Persistence was defined as the probability that the simulated system achieved equilibrium with the number of infected fleas at a value greater than zero. Additional metrics, such as peak of transmission intensity, were examined by centering all epidemic curves on the peak of transmission and averaging the variables at each centered time point to achieve a single, average epidemic curve. This enabled comparison of transmission dynamics by varying

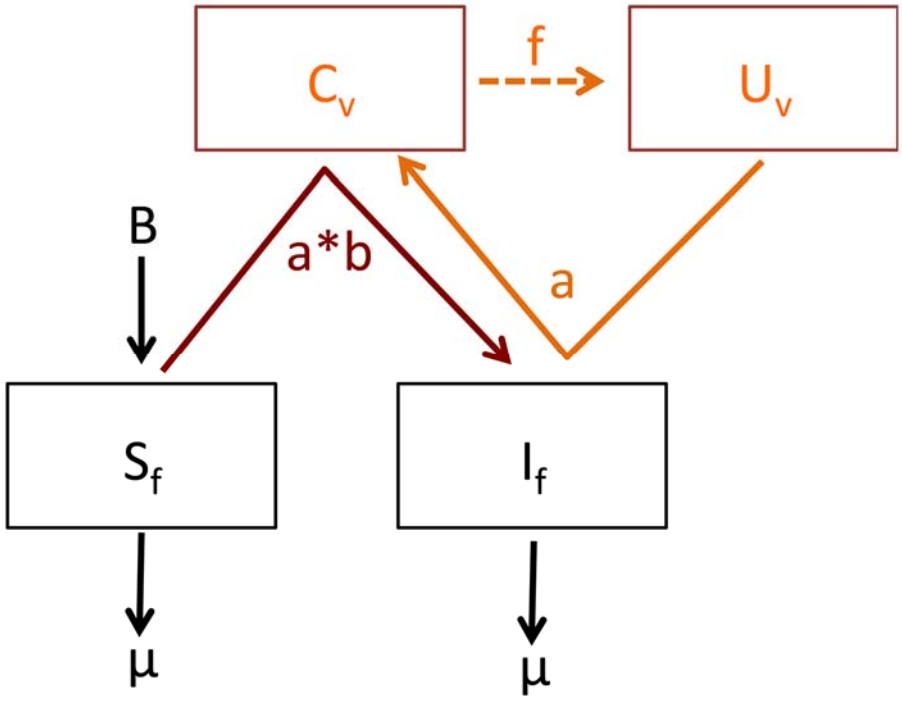

Figure 2.2. Schematic of the compartmental model. Fleas are either 'susceptible' to *Rickettsia felis* infection (S_f) or, after *R. felis* infection, 'infectious' to other arthropods (I_f); and vertebrate hosts are either 'uncontaminated' in the absence of infectious fleas (Huvenne & Smagghe 2010) or 'contaminated' in the presence of at least one infectious flea (C_V). Additionally, flea density is assumed to be constant by defining the recruitment rate (B) as approximately equal to the average mortality rate of the flea population (per μm). The model also incorporates the daily biting rate of fleas (a), the probability of cofeeding transmission (b) and the transfer rate of fleas from one vertebrate host to another (f).

the probability of cofeeding transmission (b) parameterized by the results from the experimental work in the current investigation.

2.3. Results

2.3.1. Horizontal transmission of *R. felis* occurs between cofeeding cat fleas

To determine if cat fleas could acquire *R. felis* infection from a murine host during feeding, an intradermal inoculation (or bleb) of $5x10^9$ rickettsiae in 100 µL of SPG buffer was generated on the dorsal surface of the mouse, and cat fleas were placed in a single feeding capsule adhered directly over the site of inoculation (Figure 2.1, A). These acquisition bioassays generated *R. felis* infections in recipient cat fleas (10.0 – 20.0%) as evidenced by qPCR (Table 2.3), and rickettsial infection loads (determined by quantifying the copy number of *Rf*17kDa per individual flea lysate) ranged from $5.8 \times 10^2 – 1.5 \times 10^3$ rickettsiae/flea. Following confirmation of *R. felis* acquisition, cofed (donor and recipient cat fleas in a single feeding capsule) and cross-fed (donor and recipient cat fleas in separate feeding capsules) bioassays (Figure 2.1, A) were conducted in which donor cat fleas were exposed to either a low dose (5×10^9 rickettsiae/mL) or high dose (5×10^{10} rickettsiae/mL) infectious bloodmeal using an artificial host system prior to on host-experiments. Uninfected recipient cat fleas became positive for *R. felis* after cofeeding transmission with *R. felis*-infected donor cat fleas in both the cofed and cross-fed bioassays. The low dose cofed bioassays yielded an infection prevalence of 16.7% in donor cat fleas and produced *R. felis* infections in 10.0% of the recipient cat fleas in all three trials (Table 2.3). The high dose cofed bioassays generated *R. felis* infections in 100.0% of the donor cat fleas and yielded an infection prevalence of 16.7% in recipient cat fleas (Table 2.3). The low and high dose cross-fed bioassays resulted in an infection prevalence of 30.0% and 100.0% in donor cat fleas,

Table 2.3. Horizontal transmission of *Rickettsia felis* between cofeeding fleas on a vertebrate host.

Group	***C. felis***		***X. cheopis***	
	Prevalence (%)	**Mean infection load (±SEM)**	**Prevalence (%)**	**Mean infection load (±SEM)**
Acquisition				
Recipient fleas	4/30 (13.3)	1.0×10^3 ($\pm 2.0 \times 10^2$)	5/30 (16.7)	2.6×10^4 ($\pm 1.8 \times 10^4$)
Co-fed: Low dose				
Donor fleas	5/30 (16.7)	2.4×10^5 Φ ($\pm 2.4 \times 10^5$)	11/30 (36.7)	Not assessed
Recipient fleas	3/30 (10.0) ψ	1.6×10^3 ($\pm 1.4 \times 10^3$)	0/30 (0.0)	NA
Co-fed: High dose				
Donor fleas	30/30 (100.0)*	2.7×10^6* Φ ($\pm 4.4 \times 10^5$)	30/30 (100.0)	5.9×10^6 ($\pm 1.3 \times 10^6$)
Recipient fleas	6/30 (20.0) ψ	1.6×10^3 ($\pm 5.7 \times 10^2$)	8/30 (26.7)	1.4×10^3 ($\pm 5.0 \times 10^2$)
Cross-fed: Low dose				
Donor fleas	9/30 (30.0)*	1.5×10^4 ($\pm 8.0 \times 10^3$)	Not assessed	Not assessed
Recipient fleas	1/30 (3.3) ψ	7.5×10^2 (NA)	Not assessed	Not assessed
Cross-fed: High dose				
Donor fleas	30/30 (100.0)*	9.7×10^5 ($\pm 1.8 \times 10^5$)	Not assessed	Not assessed
Recipient fleas	1/30 (3.3) ψ	2.0×10^2 (NA)	Not assessed	Not assessed
Control				
Control fleas	0/30 (0.0)	NA	0/30 (0.0)	NA

Female cat fleas were given one of two infectious doses of *R. felis* during acquisition feeding (donor fleas) and subsequently co-fed on mice. Acquisition of novel infection by recipient fleas (*C. felis* or *X. cheopis*) was assessed by qPCR. Rickettsial infection loads were determined by quantifying the copy number of *Rf*17kDa per individual flea lysate.
* A significant difference was observed in the prevalence and/or infection load between donor and recipient fleas within the same bioassay group.
ψ A significant difference was detected in the prevalence between recipient fleas of co-fed (low and high dose combined) and cross-fed bioassays (low and high dose combined).
Φ A significant difference was identified in the infection load between donor fleas of low and high dose co-fed bioassays; NA = Not applicable.

respectively, and both dose experiments resulted in 10.0% acquisition of infection by recipient cat fleas for one of three trials. No significant difference between the number of *R. felis*-infected donor and recipient cat fleas in low dose cofed bioassays were present, while significant differences were observed between the number of *R. felis*-infected donor and recipient cat fleas in high dose cofed bioassays as well as low dose and high dose cross-fed bioassays. Additionally, a significant difference was detected between the number of *R. felis*-infected recipient cat fleas between cofed and cross-fed bioassays. No significant difference was observed between mean rickettsial load of donor and recipient cat fleas in low dose bioassays (Table 2.3); whereas, mean *R. felis* infection load was significantly different between donor and recipient cat fleas in high dose bioassays (Table 2.3). A significant difference in mean rickettsial load was demonstrated between donor cat fleas in low and high dose bioassays (Table 2.3); however, no significant difference was observed between mean rickettsial infection loads in recipient cat fleas of low versus high dose bioassays (Table 2.3). All control recipient cat fleas in the control bioassays remained uninfected for the duration of the experiment, and mice blood samples were negative for *R. felis* infection in all bioassays. Thus, similar to horizontal transmission observed in an artificial host system [24], *R. felis* is consistently transferred between cofeeding cat fleas on a vertebrate host. Furthermore, the on-host results suggest that proficient transmission depends on the distance between cofeeding donor and recipient fleas, rather than the number of infectious donor fleas.

2.3.2. Interspecific transmission of *R. felis* occurs between cofeeding fleas

Field studies have reported molecular identification of *R. felis* in other arthropod species feeding on the same host as *R. felis*-infected cat fleas [17]; of particular interest for this study is the detection of *R. felis* in rat fleas. In order to demonstrate the capacity of rat

fleas to acquire *R. felis* infection from a murine host, an acquisition bioassay was conducted with identical methodology as described above for cat fleas (Figure 2.1, B). Positive *R. felis* infections in recipient rat fleas (0.0 – 40.0%) were confirmed by qPCR (Table 2.3), and rickettsial infection load ranged from $4.0 \times 10^2 – 9.8 \times 10^4$ rickettsiae/flea in acquisition bioassays. Following confirmation of *R. felis* acquisition by rat fleas, cofed bioassays (donor cat fleas and recipient rat fleas in the same feeding capsule) (Figure 2.1, B) were conducted in which donor cat fleas were exposed to either the low dose or high dose infectious bloodmeal in an artificial host system prior to on host-experiments. Recipient rat fleas became positive for *R. felis* only after cofeeding transmission with donor cat fleas administered the high dose *R. felis*-infected bloodmeal (Table 2.3). The high dose cofed bioassays generated an *R. felis* infection in 100.0% of the donor cat fleas and yielded an infection prevalence of 26.7% in recipient rat fleas (Table 2.3). No significant difference was observed between the number of *R. felis*-infected recipient cat fleas and recipient rat fleas (Table 2.3), nor was a significant difference detected between mean rickettsial infection loads in recipient cat fleas and recipient rat fleas in high dose cofed bioassays (Table 2.3). All control recipient rat fleas in the control bioassays were negative for *R. felis* infection, and mice blood samples were negative for *R. felis* infection in all bioassays. Given the prevalence of *R. felis* infections documented from a variety of arthropods, results from this study suggest that other arthropods sufficiently acquire the pathogen by cofeeding transmission in close proximity to *R. felis*-infected cat fleas.

2.3.3. Transcriptionally active *R. felis* was detected in mouse skin between cofeeding fleas

Acquisition bioassays demonstrated the ability of cat fleas to acquire rickettsiae while feeding on a vertebrate host, however the viability of *R. felis* introduced by donor cat fleas and subsequently consumed by recipient cat fleas was unclear; therefore, RNA from mouse

skin of cross-fed bioassays between the two feeding capsules (*i.e.* suggesting dispersal of rickettsial organisms between feeding sites) was isolated. The viability of *R. felis* in mouse skin samples from cross-fed bioassays was confirmed by amplification of *R. felis* 17-kDa from cDNA synthesized from mouse skin total RNA extracts. All no-RT samples were negative for the presence of *R. felis* gene products. Moreover, H&E staining followed by histopathological evaluation revealed moderate neutrophilic dermatitis for the same tissue samples. Though utilization of the anti-*Rickettsia* antibody on acquisition bioassay samples demonstrated intralesional rickettsial antigen expression in skin samples, IHC for *Rickettsia* in cross-fed bioassays were negative; however, the amount of *R. felis* present between the two bioassays is likely disproportionate. During acquisition bioassays a bleb (~ 5 x 10^7 rickettsiae) was inoculated directly into the dermis, whereas in cross-fed bioassays the arthropod vector injects *R. felis* (of unknown quantity) at the feeding site, followed by diffusion between capsules to the skin site assessed. The presence of *R. felis* RNA in the skin between the two capsules supports the likelihood of cofeeding transmission between cat fleas.

2.3.4. Cofeeding transmission of *R. felis* is sustainable amongst cat flea populations

In order to assess persistence of an *R. felis* infection within the vector population, successive horizontal transmission bioassays (three rounds total) were conducted in an artificial host system to determine if recipient cat fleas were infectious following 7 days of cofeeding transmission with *R. felis*-infected donor cat fleas (Figure 2.1, C). Recipient cat fleas were grouped with naïve cat fleas for 7 days (1st round) then the recipient cat fleas were removed and replaced by naïve cat fleas labeled with Rhodamine B (RB) (2nd round). The original naïve cat fleas from the first round are the donor cat fleas in the second round of

transmission bioassays, etc. The three consecutive cofed bioassays generated an *R. felis* infection prevalence of 3.6% in first round recipient, 7.1% in second round recipient, and 4.7% in third round recipient cat fleas. Additionally, the average (± SEM) rickettsial load significantly decreased in recipient cat fleas from the first round of transmission bioassays (3.1×10^4/flea lysate ± 9.0×10^3) compared to the last round (6.0×10^1/flea lysate ±1.1×10^1). Although rickettsial loads decreased following successive horizontal transmission bioassays, sustained transmission of *R. felis* was demonstrated.

2.3.5. Cofeeding transmission is sufficient to cause secondary transmission events after introduction of an infected flea(s), and can lead to persistence of the pathogen

A stochastic compartmental model was constructed to determine whether cofeeding transmission was capable of supporting *R. felis* persistence amongst blood-feeding arthropods in the absence of rickettsemic vertebrate hosts. The likelihood of transmission was not affected by the probability of cofeeding transmission (b) from donor fleas to recipient fleas. When (b) was 10.0%, 20.0%, or 26.7%, the probability of transmission was 0.735 with 95% CI (0.731, 0.739), 0.747 with 95% CI (0.743, 0.751), and 0.767 with 95% CI (0.763, 0.771), respectively. In Figures 2.3 and 2.4, the initial peak followed by a drop in prevalence represents model transmission events where a single infected flea is introduced to a closed population. The number of susceptible fleas is 100% at the beginning of the simulations, which creates a spike in the number of "newly", infected fleas per time point. As the system approaches equilibrium, the susceptibility profile of the population is altered because the number of susceptible fleas is not 100% and the initial peak observed is no longer achievable. Interestingly, if transmission was achieved initially, there appeared to be no barriers to progression of the system towards equilibrium, *i.e.* persistent number of infected fleas at a value greater than zero (Figure 2.3). While the probability of transmission

and persistence was not affected by the probability of cofeeding transmission (b), there were differences in the transmission dynamics. For b = 10.0%, the time-to-peak was on average 3 weeks, while it was only 2 weeks for b = 20.0% and b = 26.7% (Figure 2.4). Additionally, the time to equilibrium was also affected by the value of b. For b = 10.0%, the time to equilibrium was 6 weeks from peak (or 10 weeks from the onset of transmission after initial introduction event); for b = 20.0% and b = 26.7% the time to equilibrium was 4 weeks (or 7 weeks from transmission onset) (Figure 2.4). The percent of fleas infected at equilibrium differed by ≤ 4% (approximately: 18.4% for b = 10.0%, 21.1% for b = 20.0%, and 22.0% for b = 26.7%) and thus is not a telling metric of the effects of differences in cofeeding transmission probabilities. Therefore, the combination of intraspecific and interspecific cofeeding transmission of *R. felis* on a vertebrate host, sustained transmission of *R. felis* between cofeeding cat fleas in an artificial system, and support by modeling demonstrates cofeeding as an important mechanism of pathogen maintenance and transmission within flea populations.

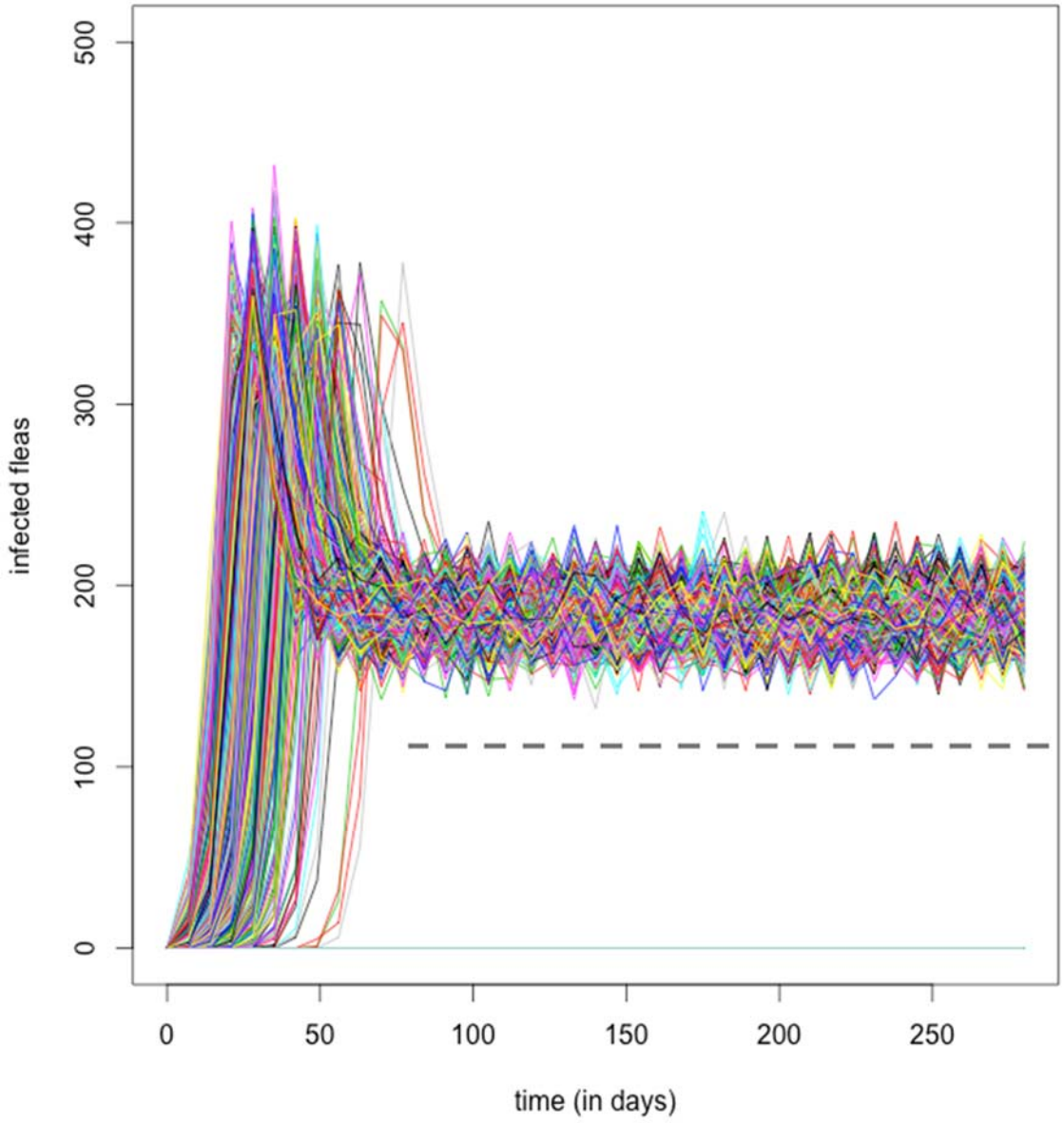

Figure 2.3. Simulations (n = 1000) of the cofeeding model with the probability of cofeeding transmission at 10%. The simulations that have reached equilibrium (above grey dashed line) exhibit relatively constant numbers of infected fleas.

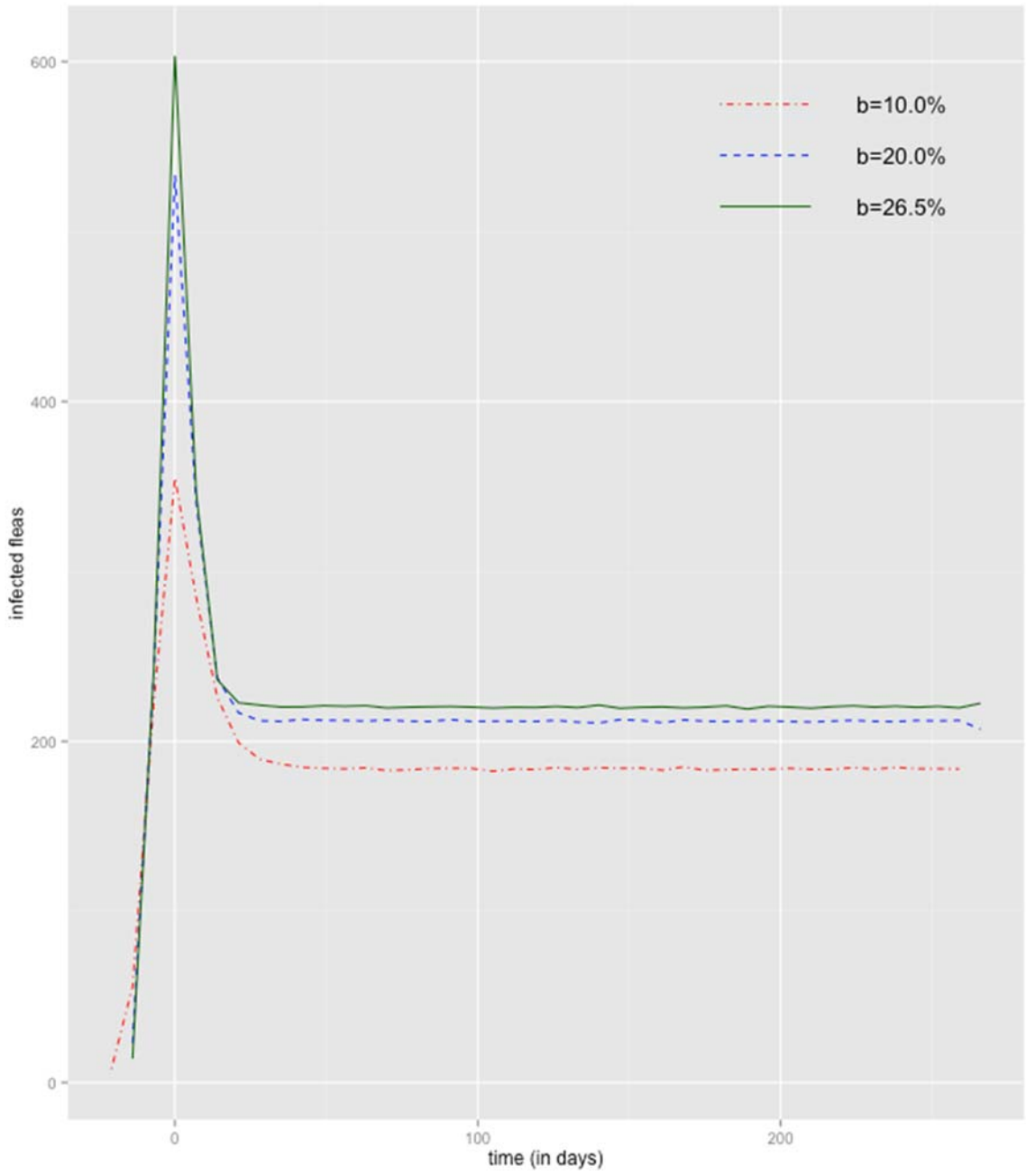

Figure 2.4. Transmission curves of the three scenarios simulated. Peak of transmission intensity was examined by centering all epidemic curves and varying the probability of cofeeding transmission (b) at each centered time point to achieve a single, average epidemic curve.

2.4. Discussion

Rickettsial transmission by arthropods can be vertical or horizontal; furthermore, transmission route and bacterial virulence are interdependent. Vertical transmission favors the evolution of benign associations, whereas frequent horizontal transmission between vectors favors virulent *Rickettsia* species [58, 59]. Unique to *R. felis*, both transmission paradigms have been identified within cat flea populations and may coexist with no adverse cost to flea fitness [23, 24, 60]. In addition to being a cosmopolitan flea-borne pathogen, *R. felis* is also a vertically maintained endosymbiont of non-hematophagous booklice (psocids) [61]. In the booklouse host, *R. felis* is an obligate mutualist required for the early development of the oocyte and is maintained 100% transovarially [61, 62]. Unknown factors account for the variable prevalence of *R. felis* observed with vertical transmission amongst colonized populations of cat fleas [24]. For *R. felis* to be maintained within and between arthropod populations, horizontal transmission must be utilized; however, a competent rickettsemic vertebrate host that can serve as a reservoir for *R. felis* is deemed either scarce or absent [17]. Our results demonstrate efficient exchange of *R. felis* between infected donor cat fleas and uninfected recipient cat fleas (intraspecific transmission) and rat fleas (interspecific transmission), respectively, through cofeeding transmission on an uninfected vertebrate host.

In contrast to *R. felis*, horizontal transmission of other insect-borne rickettsial pathogens, such as *R. typhi* and *Rickettsia prowazekii* (the agent of louse-borne epidemic typhus), occurs primarily through infected insect feces [63, 64]. Additionally, both horizontal transmission via flea bite and vertical transmission via transovarial and transstadial mechanisms are reported for *R. typhi*, although at a lower rate compared to fecal transmission [63]. Similarities exist between transmission routes utilized by rickettsial pathogens,

therefore the ability of fleas to transmit *R. typhi* both horizontally and vertically suggest comparable mechanisms are possible for *R. felis* transmission. We previously demonstrated horizontal transmission between cofeeding *R. felis*-infected donor and recipient cat fleas with the use of a shared bloodmeal in an artificial feeding system [24]. After a 24-hour period, all trials yielded a 6.7% prevalence of *R. felis*-infected recipient cat fleas in spite of a significantly higher prevalence in *R. felis*-infected donor cat fleas [24]. Using a comparable population of donor cat fleas on a live host produced positive *R. felis* infections in 10.0% of the recipient cat fleas in all trials. The potential for enhanced transmission of *R. felis* between cofeeding arthropods through the vertebrate host's skin requires further study. Interestingly, although the high dose infectious bloodmeal generated 100.0% *R. felis*-infected donor cat fleas, utilization of low and high dose infectious bloodmeals showed no significant difference between the number of *R. felis*-infected recipient cat fleas in cofed and cross-fed bioassays, respectively. Thus, the transmission rate of *R. felis* to recipient cat fleas does not increase with the number of infectious donor cat fleas used during transmission bioassays.

A necessary condition for transmission of pathogens between cofeeding arthropods is that infected and uninfected vectors feed rather simultaneously in space and time [22]. Cofeeding transmission in space is characteristic for most ectoparasite species because host-grooming behavior often results in spatial aggregations on certain parts of the body [22]. The highest percentage of cat fleas found on stray cats is on the smallest surface of the head and neck area, approximately 46.0% of feeding cat fleas are within a few centimeters of others [65]. Under all experimental conditions of the current study, infection of recipient cat fleas was consistently higher when grouped in the same container as the donor cat fleas (cofed bioassays), compared with when they were grouped separately (cross-fed bioassays). This

result is similar to cofeeding transmission of tick-borne encephalitis virus on field mice in which most virus transmission occurred (72.0%) when donor and recipient ticks were allowed to feed in close proximity, and transmission diminished (38.0%) when donor and recipient ticks were separated on non-immune animals [66]. Thus, combination of the high success rate of *R. felis* transmission between donor and recipient fleas in our cofed bioassays and basic flea biology suggests the likelihood of cofeeding transmission on vertebrate hosts in nature.

The transmission of *R. felis* between cofeeding cat fleas on a vertebrate host has broad implications towards infection of, and potential transmission by, other hematophagous arthropods. The current study is the first experimental demonstration of interspecific transmission of *R. felis*, and highlights the potential for cofeeding transmission to explain the presence of *R. felis* in a variety of blood-feeding vectors. Although use of low and high dose infectious bloodmeals showed no significant difference between the number of *R. felis*-infected recipient cat fleas, the high dose infectious bloodmeal was necessary for the transfer of *R. felis* between donor cat fleas and recipient rat fleas. Failure of rat fleas to acquire an *R. felis* infection with the lower infectious dose may indicate that acquisition is dose dependent; yet, there was no significant difference between *R. felis* acquisition or infection loads in recipient cat and rat fleas utilizing the higher infectious dose. Interspecific cofeeding transmission of vector-borne viruses has been demonstrated for both tick-borne encephalitis virus [43], as well as mosquito-transmitted West Nile virus [45] which is more applicable for this study given the similar short-term feeding behavior of mosquitoes and fleas. Subsequently, viral infections resulted in potentially competent non-traditional vectors based on dissemination of West Nile virus infection in *Aedes albopictus*. While we demonstrated

that rat fleas could acquire *R. felis* during cofeeding transmission events, the role of rat fleas as vectors for this pathogen remains undefined.

The selection of a vertebrate host to examine horizontal transmission parameters of *R. felis* proved challenging because a definitive mammalian host has not been identified in the transmission cycle for this pathogen and, given the expansive geographical range of *R. felis*, may vary depending on location [17]. Serological-based studies have implicated several peri-domestic animals (*e.g.* cats, dogs, opossums, rats) based on seropositive individuals independent from laboratory experiments [25-31]; yet, these retrospective diagnoses only provide signs of the presence of *R. felis* in the environment as opposed to identification of a reservoir vertebrate host. The mouse strain C3H/HeJ has been utilized in previous studies to examine transmission of *Rickettsia* that produce mild infections, such as *R. conorii* and *R. parkeri* [51, 67]. In the current study, all blood samples collected via cardiac puncture were qPCR negative for *R. felis* infection, indicating that experimental mice did not harbor a systemic infection. Although rickettsemia was not detected during our short-term study, other murine models for rickettsial species have observed disseminated infections at one-day post inoculation [38, 68]. The current study utilized the arthropod vector to introduce *R. felis* to the vertebrate host, quantification of the biologically relevant inoculation dose may provide valuable insight into the actual transmission mechanisms employed in nature. Furthermore, acquisition bioassays did not result in systemic vertebrate infection with ID inoculations, but cat fleas that acquired *R. felis* infection through these blebs had rickettsial loads similar to constitutively *R. felis*-infected cat fleas fed on cat hosts [49]. Therefore, this study demonstrates the prospective use of C3H/HeJ as a murine model to further examine the *R. felis* transmission cycle with cat fleas.

Horizontal transmission of *R. felis* by infected donor cat fleas to uninfected recipient cat fleas was demonstrated in an artificial feeding system, but it was apparent that the recipient cat fleas had a lower *R. felis* density when compared to *R. felis*-infected donor cat fleas [24]. While perpetuation of *R. felis* transmission by recipient cat fleas was likely, as cat fleas are a biological vector for *R. felis*, the maintenance of *R. felis* by horizontal transmission amongst this arthropod population required further investigation. Our results demonstrated horizontal transmission of *R. felis* occurred over a 4-week period by interchanging infected and uninfected cofeeding cat fleas in an artificial system. Although *R. felis* prevalence in recipient populations was variable between time points and rickettsial load decreased after each succeeding transmission bioassay, similar results were demonstrated in a vertically maintained, *R. felis*-infected cat flea population. Reif et al. (2008) showed that *R. felis*-infection prevalence and individual *R. felis*-infection load in cat flea colonies are inversely correlated, *i.e.* the populations with the highest prevalence of *R. felis* infection had the lowest mean individual *R. felis*-infection load. Similar findings in the current study showed first round recipient cat fleas had lower prevalence compared to the last round, but the highest average *R. felis*-infection load. In support of our assumption that both vertical and horizontal transmission are needed for the persistence of *R. felis* within cat flea populations, this flexibility in *R. felis* prevalence and infection density may represent a maintenance strategy required for sustained transmission.

Given the low occurrence of disseminated *R. felis* infections in the blood of vertebrate hosts and high occurrence of *R. felis*-infected arthropods in field surveys [17], we sought to determine whether cofeeding transmission was capable of supporting pathogen persistence in the absence of competent vertebrate hosts. In the current model system, sustainable

transmission is achieved with rates as low as 1%, although the number of cat fleas infected at equilibrium is proportionally lower. Cursory exploration of the other parameters utilized demonstrated the limits of cofeeding transmission given this phenomenon. For instance, biting rate notably affects the probability of sustained transmission, given that biting rates account for two events: first, the flea must contract the pathogen and second, the flea must transmit the pathogen. Similarly, cat fleas are considered immediately infectious upon *R. felis* exposure due to cofeeding transmission in relation to a lengthy 28-day lifespan (there are no adverse effects on flea fitness observed in *R. felis*-infected cat fleas), which generates a relatively high proportion of infectious to naïve cat fleas compared to other systems [69]. Exploration of other noteworthy parameters (*e.g.* vertical transmission) may reveal that cofeeding is not solely responsible for sustainable transmission; however, the model demonstrates that cofeeding is not the limiting factor of *R. felis* transmission success. As such, simulation modeling indicated that cofeeding transmission is sufficient to cause secondary transmission events after introduction of an infected flea and can lead to persistence of the pathogen. There are limitations to the model, for example, the vertebrate population is assumed to be closed, *i.e.* a constant number of vertebrates in the system; also, flea density is assumed to be constant, *i.e.* the average recruitment rate is approximately equal to the average mortality rate of the flea population. Contamination of a vertebrate for subsequent cofeeding transmission was assumed to be independent of distance between fleas, *i.e.* all susceptible fleas on a particular contaminated vertebrate have an equal probability of acquiring an infection through cofeeding transmission. Even though distance between cofeeding arthropods has been shown to affect successful transmission from donor to recipient individuals [45, 66], this assumption is made for numerous mosquito-borne disease

models in that homogenous mixing of mosquitoes results in an equal chance of contact [57]. In addition, alternative forms of flea mortality, such as vertebrate grooming habits [54], were not assessed, nor was seasonality of biting rate. Although these assumptions were required, support by modeling for the enzootic spread of *R. felis* through cofeeding transmission implies that this route of transmission is fundamental, not merely supplemental, for the maintenance and spread of *R. felis* infections.

In summary, this study provides novel evidence to support the hypothesis that maintenance of *R. felis* within the vector population is facilitated by horizontal transmission between cofeeding arthropods on a vertebrate host. This represents a unique transmission mechanism for insect-borne rickettsial pathogens. Also, a murine model that may approximate horizontal transmission in wild cat flea populations and offer insight into the transmission cycle intersecting with human hosts has been developed. The maintenance of *R. felis* in populations of fleas is enhanced by horizontal transmission in combination with vertical transmission. Additional studies are needed to elucidate the potential transmission of *R. felis* by rat fleas and differences observed in *R. felis* acquisition between the two flea species.

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CHAPTER 3
TRANSMISSION MECHANISMS OF AN EMERGING INSECT-BORNE RICKETTSIAL PATHOGEN

3.1. Introduction

Rickettsia felis is the causative agent of an emerging vector-borne rickettsiosis transmitted by cat fleas, *Ctenocephalides felis*, and is recognized as a common (3-15%) cause of fever among febrile patients in sub-Saharan Africa [1-5]. In addition to the high proportion of *R. felis* infections in humans from a malaria-endemic region, this pathogen has been detected in other vertebrate hosts (including cats, dogs, opossums, raccoons, rodents, and monkeys) and is present on every continent except Antarctica [6-11]. Moreover, *R. felis* has been identified in other hematophagous arthropods (including numerous species of fleas, ticks, mosquitoes, and mites) throughout the world (reviewed in [12]); nonetheless, the cosmopolitan cat flea is implicated as the primary biological vector based on field and laboratory studies [13-21]. Although maintenance of *R. felis* in nature is poorly understood, both experimental and computational transmission models indicate that this bacterium circulates in enzootic cycles through infectious cofeeding (*i.e.* pathogen transmission occurs between actively blood-feeding arthropods in the absence of a disseminated vertebrate infection) by cat fleas on vertebrate hosts [22]. As such, there is a low occurrence of *R. felis* infections in the blood of vertebrate hosts and high occurrence of *R. felis*-infected arthropods in field surveys [12, 23]. Additionally, experimental demonstration of interspecific transmission of *R. felis* on a vertebrate host between cat fleas and Oriental rat fleas (*Xenopsylla cheopis*) highlights the potential for cofeeding transmission to explain the presence of *R. felis* in a variety of blood-feeding vectors [22]. Currently, the role of the

vertebrate host in the transmission biology of *R. felis*, beyond providing a substrate for pathogen transfer between cofeeding arthropods, is unclear and requires further investigation.

Transmission of flea-borne bacterial pathogens is multifaceted and often each species has several transmission routes to ensure maintenance within the environment [24]. For example, agents of cat scratch disease (*Bartonella henselae*) and murine typhus (*Rickettsia typhi*) utilize horizontal transmission via contaminated flea feces deposited on the host as the primary source of infection to vertebrates [25-27]. Additional horizontal transmission occurs for these pathogens via regurgitation of bacteria from the flea's midgut into the bite site, but requires a lengthy incubation period and occurs to a lesser extent compared to fecal transmission [24, 26]. Contrary to other flea-borne bacterial agents but similar to tick-borne rickettsial pathogens, horizontal transmission of *R. felis* can occur by infectious saliva at the bite site. Support for this saliva transmission mechanism includes identification of *R. felis* in the salivary glands of infected cat fleas [28, 29], and amplification of rickettsial DNA in the blood, as well as seroconversion, of vertebrate hosts exposed to feeding cat fleas with *R. felis* infection [14, 18]. Further evidence for transmission through infectious saliva is the transfer of bacteria between cat fleas cofeeding on a shared bloodmeal, which has been demonstrated in an artificial host system and on a vertebrate host [21, 22]. Based on the hydrodynamic force in the food canal of cat fleas (*i.e.* backwards, away from the bite site, whereas saliva flows forward into the bite site) and the rapid turnover of cat flea midgut contents (*i.e.* clearance of excessive bacteria), regurgitation of blood containing bacteria from cat fleas seems to be an unlikely scenario for transmission [30]; however, no direct evidence for or against this mechanism has been demonstrated.

The journey of an infectious agent within a vector from ingestion to subsequent transmission to a new host (*i.e.* extrinsic incubation period or EIP) relies on a series of complex vector-pathogen interactions [31]. As such, although most hematophagous arthropods feed on a wide variety of vertebrate hosts, not all arthropods that ingest an infectious bloodmeal will be a competent vector or exhibit the vectorial capacity for proficient pathogen transmission [31]. In order for a vector to be competent for a pathogen, the organism must overcome arthropod midgut infection and escape barriers, as well as salivary gland infection and escape barriers if transmission occurs via infectious saliva [32]. The migration of a pathogen from an arthropods' midgut to salivary glands is considered a relatively lengthy event (ranging from days to weeks depending on the vector and/or pathogen), and often corresponds to the time needed for replication and/or cyclic development of the infectious agent [33]. Recently, the infection kinetics of bloodmeal-acquired *R. felis* in cat fleas was observed by immunofluorescence assays (IFA) at weekly intervals for 28 days [29]. This study revealed that in previously uninfected cat fleas the dissemination of *R. felis* from midgut to salivary glands requires 7 or more days post-exposure (dpe) to an *R. felis*-infected bloodmeal. Based on these data, the probable EIP needed for horizontal transmission of *R. felis* by infectious cat flea saliva is approximately 7 days. However, cofeeding transmission bioassays demonstrated that cat fleas exposed to an *R. felis*-infected bloodmeal are infectious to naïve fleas after 24 hours (hrs) (in both an artificial host system and on vertebrate hosts) [21, 22]. Thus, the EIP of *R. felis* within the biological vector remains unknown, though knowledge of this threshold is central to determining the earliest time point at which feeding *R. felis*-infected cat fleas may be infectious to a susceptible host, including humans.

Vector-borne pathogen transmission is considered biological if an incubation period is required before passage within the vector or consequent transmission to a new host [33]. In contrast, mechanical transmission does not require multiplication or development of the organism within the vector, and transmission to a new host occurs by incidental contact with the vector, such as carriage by the insects' feet, proboscis, or gastrointestinal tract [31]. Frequently, biological and mechanical transmission of pathogens co-exists in the same geographic area, in the same hosts, and even by the same vectors [34]. Mechanical transmission is typically considered an alternative mechanism to biological transmission, such as when transmission of a pathogen occurs in geographic areas devoid of the biological vector; however, under specific circumstances mechanical transmission may be as efficient as biological transmission [35]. Another mode of transmission has been observed for the flea-borne bacterium of plague (*Yersinia pestis*), termed "early-phase", where transmission occurs before a designated incubation period; but, certain aspects of this transmission event have impeded confirmation as to whether this is a biological or mechanical mechanism [30]. While horizontal transmission of *R. felis* by cat fleas via infectious saliva is considered biological, the specific mechanism utilized before *R. felis* disseminates to the salivary glands is unclear. Given that *R. felis* is routinely detected in other blood-feeding arthropods, demonstration of nonspecific mechanical transfer may incriminate other human-biting vectors in the transmission cycle of this pathogen.

In this study, we aimed first to designate the EIP of *R. felis* within cat fleas, and second to further elucidate the transmission mechanism (*e.g.* biological or mechanical) utilized by *R. felis* among cofeeding fleas prior to a disseminated arthropod infection. Given that pathogen transmission before passage within the vector would indicate that microbial

replication and development in the arthropod are not required, we hypothesized that a mechanical mechanism is responsible for the observed early-phase transmission of *R. felis* between cofeeding fleas. Horizontal transmission bioassays were developed in an artificial host system to assess temporal dynamics of *R. felis* between cofeeding cat fleas, including exposure time to produce infectious donor fleas and association time to transmit infection to recipient fleas. Additional experiments examined the proportion of *R. felis*-exposed cat fleas with contaminated mouthparts, as well as the potential for cat fleas to release *R. felis* from soiled food and/or salivary canals following exposure to an infectious bloodmeal. The potential for mechanical transmission of *R. felis* by cofeeding cat fleas was further examined using fluorescent latex beads to simulate transfer of an inanimate object, which would not require a biological mechanism to achieve transmission. Our results indicate that not only are *R. felis*-exposed cat fleas infectious following a brief incubation period, but utilization of a mechanical mechanism may also explain the rapid rate of spread that typifies *R. felis* flea-borne transmission within experimental and computational models.

3.2. Materials and Methods

3.2.1. Source of fleas and cultivation of *Rickettsia*-infected fleas

Newly emerged, *Rickettsia*-uninfected cat fleas (*C. felis* Bouche) were purchased from Elward II (Soquel, CA, USA), and reared within an artificial host system as described previously [36]. The Louisiana State University (LSU) strain of *R. felis* was maintained in an *Ixodes scapularis* embryonic cell line (ISE6) [37], and *R. felis*-infected bloodmeals were created using an inoculation dose of $5x10^{10}$ rickettsiae per mL following enumeration by the *Bac*Light viability stain kit [22]. In order to differentiate between cat fleas exposed (donor)

or unexposed (recipient) to a *R. felis*-infected bloodmeal, the biomarker Rhodamine B (RB) was used to label recipient fleas prior to experimentations [21].

3.2.2. Experimental design

3.2.2.1. Kinetics of cofeeding transmission bioassays. In order to examine temporal dynamics of rickettsial transmission, donor cat fleas were placed in one of two experimental groups within an artificial host system (Figure 3.1., A & 3.1., B). The first group was exposed to an infectious bloodmeal for 1, 3, 6, or 12 hrs, then divided into feeding capsules containing 30 donor cat fleas and 30 recipient cat fleas for each time point (exposure bioassays, Figure 3.1., A). Each bioassay was conducted in three separate trials and fleas were housed together for a 24-hr period on defibrinated bovine blood (non-heat inactivated). The second group was exposed to an infectious bloodmeal for 24 hrs, and then divided into feeding capsules containing 30 donor cat fleas and 30 recipient cat fleas (association bioassays, Figure 3.1., B). Each bioassay was conducted in three separate trials and fleas were allowed to cofeed together for a 1, 3, 6, or 12-hr period on defibrinated bovine blood (non-heat inactivated). Immediately following each kinetics bioassay, the entire feeding capsule with all fleas was stored in the -20°C freezer for future DNA extractions and quantitative polymerase chain reaction (qPCR) analyses. All bioassays were conducted with only female cat fleas to eliminate sexual transmission of *R. felis* within each experimental group [21].

3.2.2.2. Mechanism of early-phase rickettsial transmission. A two-fold approach was used to differentiate the mechanism (*i.e.* biological or mechanical) responsible for early-phase transmission of *R. felis* by cofeeding cat fleas. The first approach compared the

presence of *R. felis* in the salivary glands versus the mouthparts of cat fleas following short-term exposure events. Although previous work did not detect the presence of *R. felis* in the

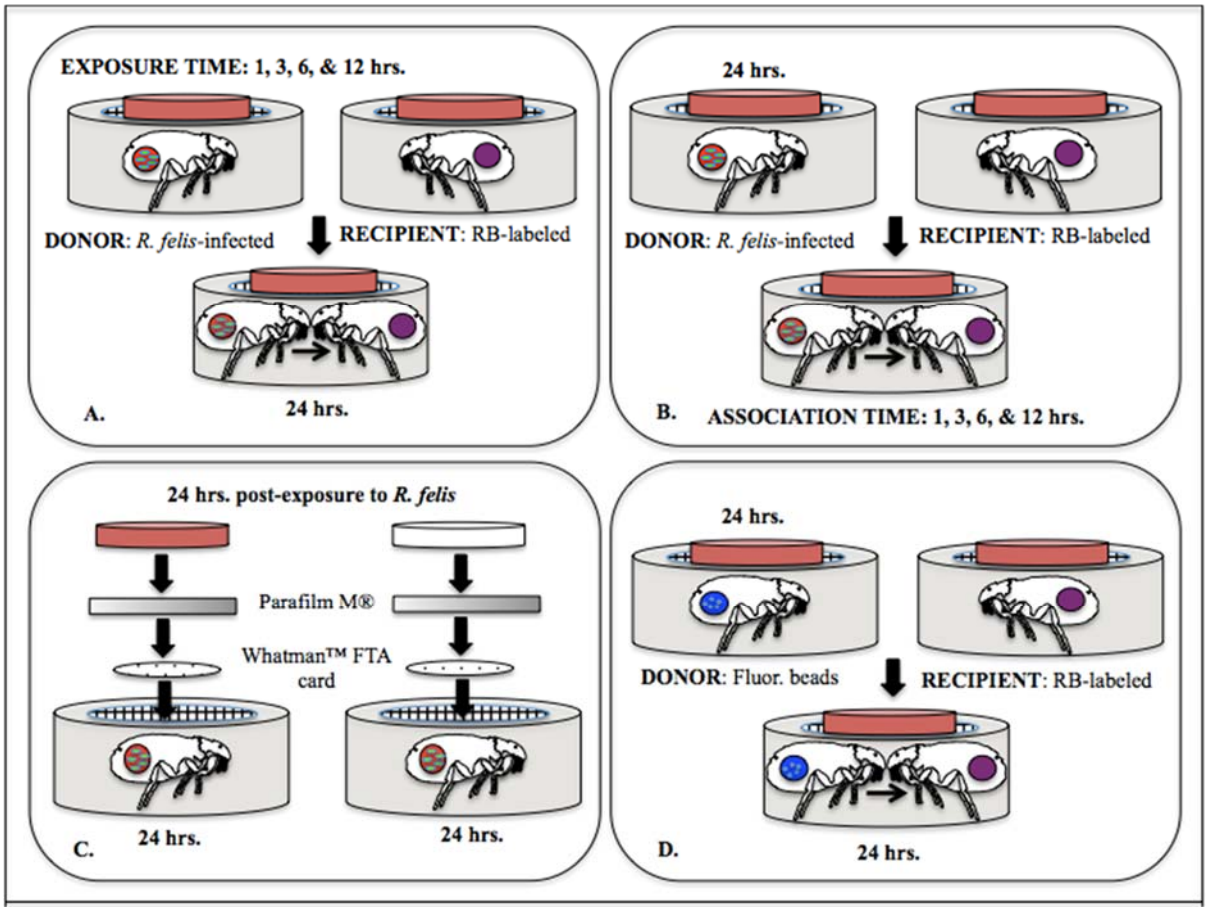

Figure 3.1. Diagram of experimental designs. (A) Cat fleas were exposed to an infectious bloodmeal for 1, 3, 6, or 12 hrs, and then divided into feeding capsules containing naïve cat fleas for 24 hrs (exposure time bioassays). (B) Cat fleas were exposed to an infectious bloodmeal for 24 hrs, and then divided into feeding capsules containing naïve cat fleas for 1, 3, 6, or 12 hrs **(**association time bioassays). (C) Whatman™ FTA cards were placed in flea cages after 24 hpe to an *R. felis*-infected bloodmeal. Cat fleas either had access to blood or the bloodmeal was removed for the duration of the experiment. (D) Cat fleas were exposed to an "infectious" bloodmeal containing fluorescent latex beads for 24 hrs, and then were placed with naïve fleas for 24 hrs.

salivary glands of cat fleas less than 7 dpe to an infectious bloodmeal [29], a portion of fleas (n = 100) from this study were dissected after a 24-hr exposure period to confirm that original observation with a few procedural modifications (detailed below). Salivary glands from these fleas were removed, washed in phosphate-buffered saline (PBS), and then the paired tissues were either fixed with acetone onto slides for IFA (n = 50) or placed in microcentrifuge tubes with Buffer ATL for DNA extractions and qPCR analyses (n = 50). A positive control group was also dissected following the same protocol, but the salivary glands were removed from these fleas 28 dpe to an *R. felis*-infected bloodmeal. In order to determine if cat fleas harbor *R. felis* on their mouthparts in addition to their midgut 24 hrs post-exposure (hpe), a portion of fleas (n = 70) had the upper half of their head (containing the mouthparts) removed for IFA and DNA extractions (Figure 3.2). The remainder corresponding flea bodies were collected in separate tubes for DNA extraction, and flea lysates produced from both the head and body portion were analyzed for *R. felis* by qPCR. An additional group of fleas (n = 50) were exposed to an infectious bloodmeal for 24 hrs, and then permitted to feed on uninfected bovine blood for 24 hrs. Following this 48-hr incubation period, these fleas were dissected for IFA and DNA extractions as described above. Also, Whatman™ FTA cards (filter paper designed to collect and isolate nucleic acid samples for PCR analysis; GE Healthcare™) were placed in donor flea cages after a 24-hr exposure period to examine the release of *R. felis* during flea feeding and/or probing events. The Whatman™ FTA cards were placed outside the flea cages against the upper portion of the screen mesh that provides cat fleas access to blood within the artificial host system (Figure 3.1., C), thus ensuring that only the flea's mouthparts had contact with the cards [38]. Two separate trials were conducted in the presence of the Whatman™ FTA cards, wherein cat

fleas either had access to blood or the bloodmeal was removed for the duration of the experiment (Figure 3.1., C). Cat fleas were surface sterilized (10% bleach for 5 min, 70% ethanol for 5 min, and three rinses with sterile distilled water for 5 min each) prior to the blood-free trials in order to eliminate residual bloodmeal present on the mouthparts between feedings. For analyses, a small disc was punched from the Whatman™ FTA card, then the paper was washed per the manufacturer's instructions (twice with FTA® Purification Reagent and twice with TE^{-1} buffer, 5 min each), and air-dried overnight before use as template for traditional PCR [37].

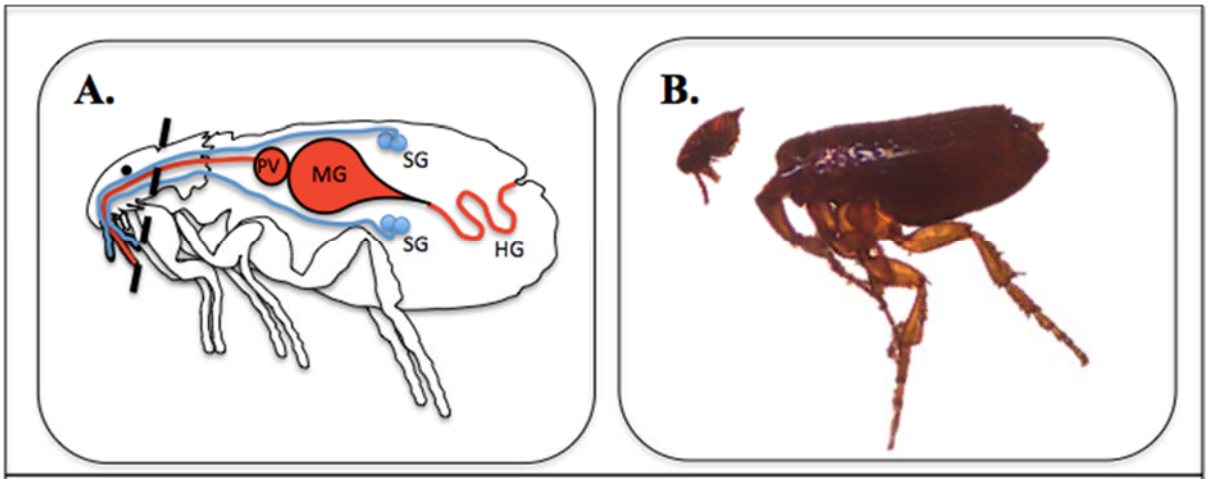

Figure 3.2. Flea dissections. (A) Diagram of flea internal anatomy. The dash line represents where the incision for dissections was made. PV = proventriculus; MG = midgut; HG = hindgut; SG = salivary glands. (B) Photographic image of flea dissections to determine the presence of *R. felis* in flea mouthparts versus midgut at 24 hpe to an infectious bloodmeal.

The second approach duplicated the cofeeding bioassays employed in a previous study [21], but instead utilized fluorescent latex beads in the place of *R. felis* infection (Figure 3.1., D). Product specifications for the specific beads used in this study include: (a) amine-modified polystyrene particles from Sigma-Aldrich© (product number: L2778), (b) 1.0 µm mean particle size, and (c) red fluorescent dye with maximum excitation of 505 nm to

585 nm and maximum emission of 550 nm to 645 nm. Fifty cat fleas were exposed to a mock "infectious" bloodmeal containing $1x10^9$ fluorescent latex beads in 600 uL of heat-inactivated bovine blood for a 24-hr period. These now "donor" fleas were then grouped together with RB-labeled recipient fleas (n = 50) for an additional 24 hrs. Following cofeeding bioassays, both donor and recipient cat fleas were dissected to remove the midgut for visual examination using a confocal fluorescent microscope (Olympus FluoView FV10i). Flea midguts were washed in PBS and placed on slides where they were mounted and counterstained using VECTASHIELD® Hard Set ™ with DAPI (Vector Laboratories Inc.). Additionally, Whatman™ FTA cards were placed in donor flea cages after a 24-hr exposure period to visualize the release of fluorescent beads by probing cat fleas (access to blood was not permitted). Prior to the placement of cards within flea cages as described above, cat fleas were surface sterilized to remove external beads that may have accumulated on the mouthparts. All cards were removed after 24 hrs and examined for beads using a fluorescent dissecting scope (Olympus MVX10).

3.2.3. Detection of *Rickettsia* in fleas

For all experiments, the collected flea samples (*e.g.* whole fleas, individual sections, or salivary glands) were surface sterilized and genomic DNA (gDNA) was extracted using the Qiagen DNeasy Tissue Kit according to the manufacturer's protocol and eluted in 25 µL PCR-grade H_2O. A negative environmental control (DNA extraction reagents without biological sample) was utilized for each DNA extraction process, as well as a negative control for the qPCR (ultrapure sterile water in the place of template). All gDNA preparations were stored at -80°C. Quantitative and traditional PCR conditions for detection of the rickettsial 17-kDa antigen gene and the *C. felis* 18S rDNA gene were performed as

described previously [17, 37]. Quantitative PCR results were presented as either quantified rickettsial copy numbers per individual flea lysate or the ratio of *R. felis* 17-kDa to *C. felis* 18SrDNA gene copy number. Amplified products from traditional PCR of Whatman™ FTA cards were visualized on 1.5% agarose gels, and then cloned into the pCR4-TOPO vector (Invitrogen) according to the manufacturer's protocol for DNA sequencing and analysis. At least three clones of each PCR amplicon were sequenced by the dye terminator method on a 3130 genetic analyzer (Applied Biosystems) at LSU (School of Veterinary Medicine). Sequence analyses were carried out using Vector NTI software (Invitrogen), and nucleotide similarities were compared using the GenBank database.

For the IFA, paired salivary glands were fixed in multi-well slides with ice-cold acetone for 10 min; then they were simultaneously permeabilized and blocked with 0.1% Triton X-100 and 2% bovine serum albumin (BSA) in PBS for 15 minutes. Rickettsiae were labeled with a polyclonal antibody against *Rickettsia* organisms generated in rabbits (I7198 Anti-*Rick*) and created at the National Institutes of Health's Rocky Mountain Laboratories (generously donated by Ted Hackstadt). Anti-*Rickettsia* serum was diluted at 1:1000 in blocking buffer (0.1% Triton X-100/2% BSA solution), and then slides with the diluted primary antibody were incubated in the dark for 1 hour. Additional slides in which no primary antibody was added served as a control for nonspecific binding, and were incubated with PBS for 1 hour in the dark. Goat anti-rabbit AlexaFluor488 conjugate (Invitrogen) served as the secondary antibody, and was diluted at 1:1000 in blocking buffer (0.1% Triton X-100/2% BSA solution) and in incubated in the dark for 1 hour. Coverslips were mounted with VECTASHIELD® Hard Set ™ with DAPI (Vector Laboratories Inc.) for nuclear counterstaining. Immunofluorescence assays on the upper half of the removed flea heads

used an identical protocol, with the exception of the initial preparation of the tissue prior to fixation with acetone. Following flea dissections, each head was placed onto a slide within a circle drawn with a diamond point scriber (2 rows of 5 circles per slide). Multiple coverslips were placed over the entirety of the slide and the heads were then compressed between the coverslips and slide. Coverslips were then removed and discarded, and any large remnants of exoskeleton were detached from the slide with fine forceps to prevent trapping conjugate during the staining procedure [39]. All slides were visualized using a fluorescent confocal microscope (Olympus FluoView FV10i).

3.2.4. Statistical analysis

A Kruskal-Wallis test was used to compare rickettsial infection loads between donor cat fleas within each kinetics bioassay, followed by a Dunn's multiple comparison test when significance was observed. A Mann–Whitney U-test made comparisons within the mechanistic bioassays between total rickettsial infection loads as well as the ratio of *R. felis* to *C. felis* gene copy number between the head and body region of infected cat fleas. All statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software), and differences were considered significant at $P \leq 0.05$.

3.3. Results

3.3.1. Cofeeding transmission of *R. felis* to naïve fleas is dependent upon the exposure time to produce infectious fleas and the association time with infected fleas

In order to determine the length of time needed to produce an infectious cat flea, donor fleas were exposed to an infectious bloodmeal for 1, 3, 6, or 12 hrs then placed with recipient cat fleas for 24 hrs (exposure time bioassay; Figure 3.1., A). In converse, donor cat fleas exposed to an infectious bloodmeal for 24 hrs were housed with recipient cat fleas for 1, 3, 6, or 12 hrs to determine the length of time needed for *R. felis* transmission to occur

between cofeeding fleas (association time bioassay; Figure 3.1., B). After 1 hr and 3 hrs of exposure to an *R. felis*-infected bloodmeal, approximately 53% and 67% of the donor cat fleas were positive as evidenced by qPCR, respectively; however, transmission of *R. felis* to uninfected recipient fleas was not observed at these exposure time points (Table 3.1). Uninfected recipient cat fleas only became positive for *R. felis* after cofeeding with infected donor cat fleas exposed for 6 and 12 hrs to an *R. felis*-infected bloodmeal. The 6-hr exposure time point yielded an infection prevalence of 69% in donor cat fleas and produced *R. felis* infections in 3% of the recipient cat fleas; whereas, a 12-hr exposure period resulted in an *R. felis* infection prevalence of 76% and 7% in donor and recipient cat fleas, respectively (Table 3.1). Comparisons of mean rickettsial load between donor cat fleas from each exposure time point revealed no significant differences, except between the 3-hr and 6-hr exposure periods. Following a 24-hr exposure period, infection prevalence of *R. felis* in donor cat fleas was 74%, 64%, 61%, and 63% in the 1-hr, 3-hr, 6-hr, and 12-hr association bioassays, respectively; nevertheless, transmission of *R. felis* to uninfected recipient fleas was not observed at these association time points (Table 3.1). No significant difference was detected between the mean rickettsial loads of donor cat fleas from each association period. Thus, *R. felis*-infected cat fleas are subsequently infectious to others via cofeeding after a 6-hr incubation period, but *R. felis* transmission to uninfected cat fleas does not occur if cofeeding with infected cat fleas is 12 hours or less.

Table 3.1. Temporal dynamics of rickettsial transmission between cofeeding cat fleas.

	Donor cat fleas		**Recipient cat fleas**	
Exposure (hrs)	**Prevalence (%)**	**Mean infection load (±SEM)**	**Prevalence (%)**	**Mean infection load (±SEM)**
1	48/90 (53)	4.12E+04 (±1.30E04)	0/90 (0)	0.00E+00
3	60/90 (67)	3.33E+04 (±7.39E03)	0/90 (0)	0.00E+00
6	62/90 (69)	4.78E+04 (±9.95E+03)	3/90 (3)	2.60E+03 (±2.16E03)
12	68/90 (76)	3.27E+06 (±2.79E06)	6/90 (7)	6.12E+03 (±3.12E03)
Association (hrs)				
1	67/90 (74)	1.27E+07 (±1.12E07)	0/90 (0)	0.00E+00
3	58/90 (64)	9.23E+03 (±3.12E03)	0/90 (0)	0.00E+00
6	55/90 (61)	2.80E+04 (±1.35E04)	0/90 (0)	0.00E+00
12	57/90 (63)	7.66E+04 (±6.24E04)	0/90 (0)	0.00E+00

Cat fleas were either exposed to an infectious bloodmeal for 1, 3, 6, or 12 hrs, and then divided into feeding capsules containing naïve cat fleas for 24 hrs (exposure bioassay), or exposed to an infectious bloodmeal for 24 hrs, and then divided into feeding capsules containing naïve cat fleas for 1, 3, 6, or 12 hrs **(**association bioassay). Acquisition of novel infection by recipient fleas was assessed by qPCR. Rickettsial infection loads were determined by quantifying the copy number of Rf17-kDa per individual flea lysate.

3.3.2. Cat flea salivary glands are not the anatomical source of *R. felis* for early-phase transmission

In order to differentiate the mechanism (*i.e.* biological or mechanical) responsible for early-phase transmission of *R. felis* by cofeeding cat fleas, the presence of *R. felis* was compared between the salivary glands verses the mouthparts of cat fleas following a 24-hr exposure to an infectious bloodmeal. *Rickettsia felis* was not detected in the salivary glands of cat fleas via IFA following this short-term event (24 hpe), as opposed to the positive control group where rickettsial antigen was identified 28 dpe to an *R. felis*-infected bloodmeal (Figure 3.3). Quantitative PCR analyses confirmed the lack of rickettsiae at 24 hrs

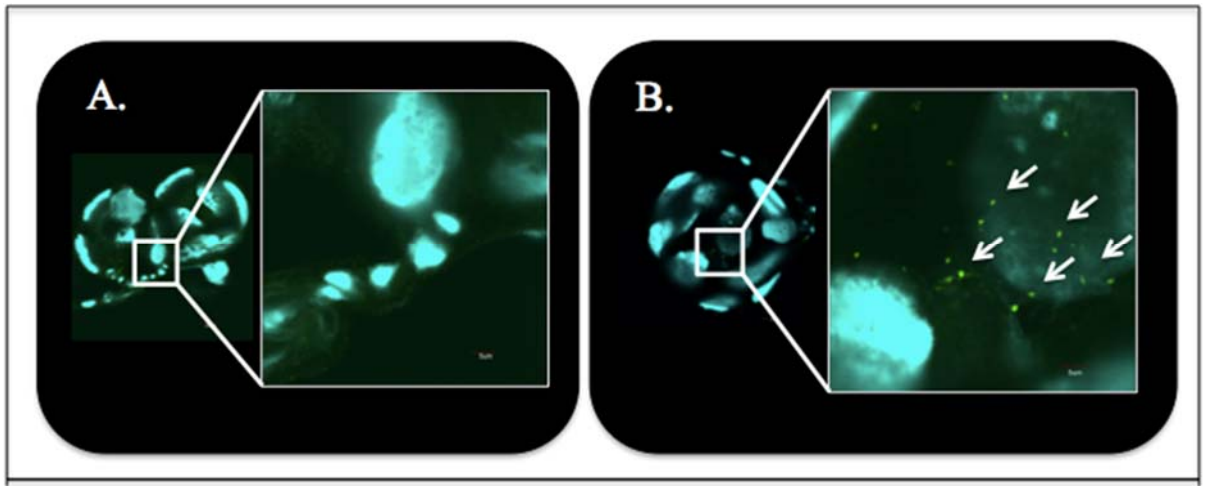

Figure 3.3. Dissemination of *Rickettsia* to flea salivary glands. **(A)** No rickettsial antigen is present at 1 dpe to an *R. felis*-infected bloodmeal. **(B)** Presence of rickettsial antigen (labeled green, indicated by arrows) at 28 dpe to an *R. felis*-infected bloodmeal (positive control).

with no amplification of the *R. felis* gene in the salivary glands assessed from the same time point. Correspondingly, 10% (7/70) of the heads removed from cat fleas were positive for *R. felis* as evidenced by qPCR after 24 hrs exposure to an infectious bloodmeal; however, no definitive organisms were detected via IFA. Additionally, a significant difference was observed between the average (± SEM) rickettsial load detected within the head ($1.5x10^3 \pm 1.3x10^3$) and body ($1.3x10^5 \pm 9.0x10^4$) between corresponding flea lysates, as well as between the ratio of *R. felis* to *C. felis* genes between the head ($7.2x10^{-3} \pm 6.6x10^{-3}$) and body ($2.4x10^{-2} \pm 2.0x10^{-2}$) segments. Moreover, 4% (2/50) of the heads removed from cat fleas 48 hpe confirmed the presence *R. felis* by qPCR analyses, but again no definitive organisms were detected via IFA. The average (± SEM) rickettsial load detected in flea heads at 48 hrs ($7.9x10^1 \pm 1.6x10^1$) was significantly less than flea heads collected at 24 hrs ($1.5x10^3 \pm 1.3x10^3$), thus further decreasing the likelihood for visualization by fluorescent microscopy. Consequently, these results suggested that *R. felis* resides within the mouthparts, not the salivary glands, of cat fleas following a 24-hr exposure to an infectious bloodmeal.

3.3.3. Cat fleas release *R. felis* from contaminated mouthparts during probing events

Feeding behavior of cat fleas includes probing expeditions to locate vertebrate capillaries before initiation of blood consumption. These brief probes may be sufficient to inoculate residual *R. felis* that remains within the mouthparts between intermittent feedings. Whatman™ FTA cards were placed in donor flea cages after a 24-hr exposure period to determine the release of *R. felis* during cat flea feeding and/or probing events in both the presence and absence of blood. The presence of rickettsial DNA from Whatman™ FTA cards was confirmed by traditional PCR amplification and sequencing of portions of the *Rickettsia* genus-specific 17-kDa antigen in both trials (Figure 3.4., A & 3.4., B). Nucleotide

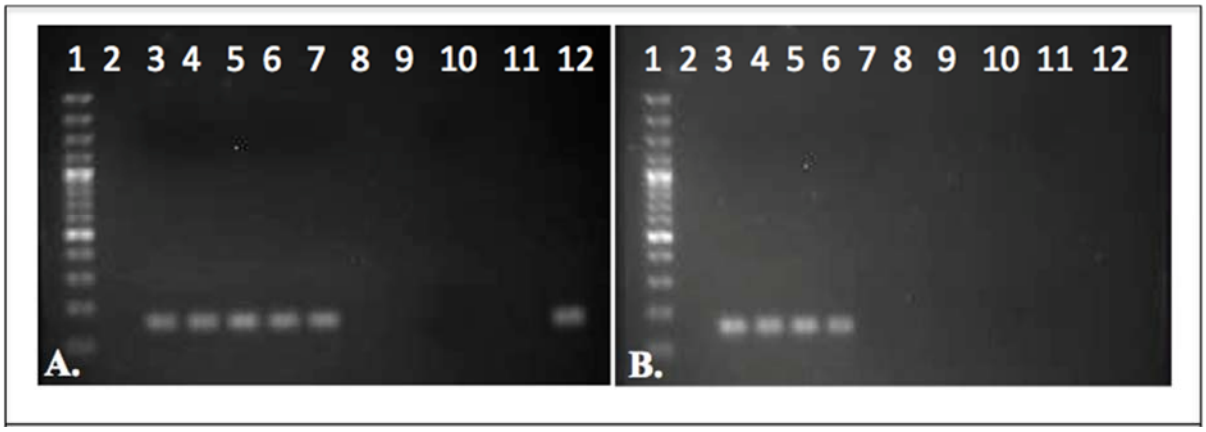

Figure 3.4. PCR detection of rickettsial 17-kDa antigen gene in Whatman™ FTA cards. (A) Lane 1, 100 bp DNA marker; Lane 2, blank; Lanes 3-7, single disc punch from five different cards exposed to *R. felis*-infected cat fleas in the presence of blood. Lanes 8-10, blank; Lane 11, environmental control; Lane 12, positive PCR *R. felis* genomic DNA**.** (B) Lane 1, 100 bp DNA marker; Lanes 2, & 7-11, blank; Lanes 3, 4, 5, single disc punch from three different cards exposed to *R. felis*-infected, surface sterilized cats fleas in the absence of blood; Lane 6, positive PCR *R. felis* genomic DNA; Lane 12, environmental control.

sequences of the 17-kDa antigen (434 bp) genes were identical to those of the sequences reported for *R. felis* in the GenBank database (accession numbers CP000053 and AF195118). Interestingly, although flea mouthparts were unable to penetrate through the cards to feed in trials with access to blood (feeding occurred at the periphery not covered by paper), droplets of blood were deposited along the surface of cards exposed to these fleas (Figure 3.5). In contrast, cat fleas that were surface sterilized prior to placement with Whatman™ FTA cards in the absence of blood did not leave evidence of feeding and/or probing (Figure 3.5), yet *R. felis* was still detected and confirmed by traditional PCR and sequencing of the 17-kDa antigen (Figure 3.4., B). Thus, these data provide initial evidence for the persistence of *R. felis* within residual blood deposited from the food and/or salivary canals while probing, as well as the potential for bacteria to adhere to the inside of these stylets and consequently discharged through probing events.

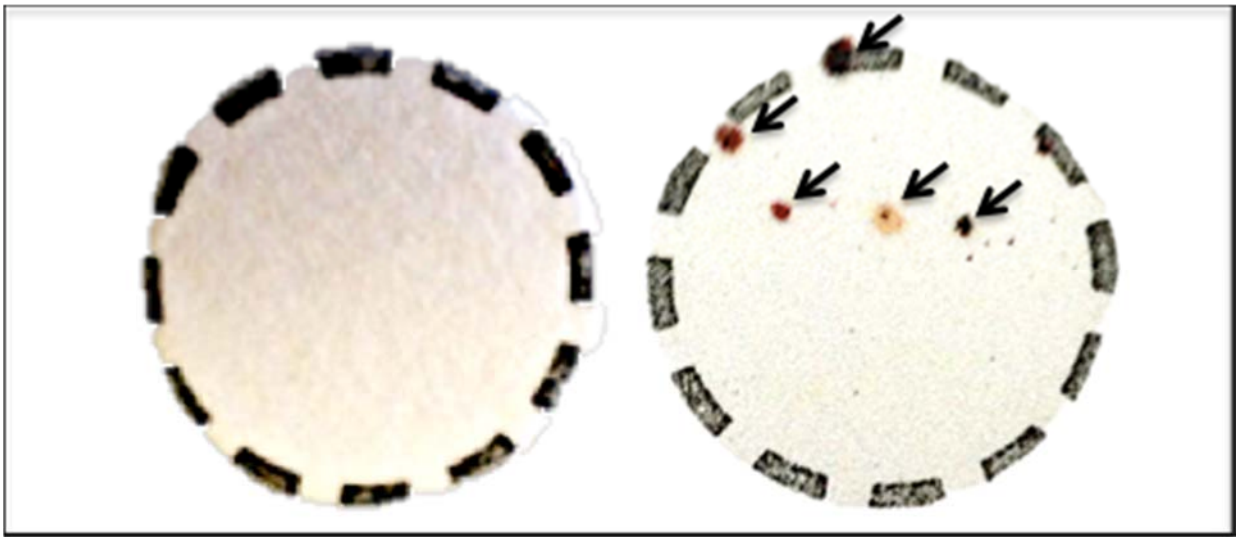

Figure 3.5. FTA cards exposed to cat fleas in the absence (left) and presence (right) of blood. Residual blood droplets (arrows) were deposited when cat fleas had access to blood.

3.3.4. Early-phase transmission of *R. felis* is due to a mechanical mechanism

Given now the evidence for *R. felis* on the mouthparts of cat fleas following a 24-hr exposure to an infectious bloodmeal, the potential for mechanical transmission by cofeeding fleas was further evaluated with the use of size-matched fluorescent latex beads as opposed to a live pathogen. Cofeeding bioassays were conducted with donor cat fleas exposed to these fluorescent beads in a bloodmeal, and then donor and recipient fleas were allowed to feed together on fresh bovine blood for 24 hrs prior to midgut dissections to assess transmission. Following a 24-hr exposure to this "infectious" bloodmeal, donor cat fleas possessed large quantities of fluorescent beads within their midgut (Figure 3.6., A). Intriguingly, recipient cat fleas were found to harbor fluorescent beads within their midgut after cofeeding with these donor fleas for 24 hrs (Figure 3.6., B). Additionally, donor cat fleas deposited these beads onto FTA cards following surface sterilization prior to placement within flea cages with no access to blood (Figure 3.7). Therefore, based on these data, the mechanism responsible for

early-phase transmission of *R. felis* between cofeeding cat fleas is determined mechanical by this criterion.

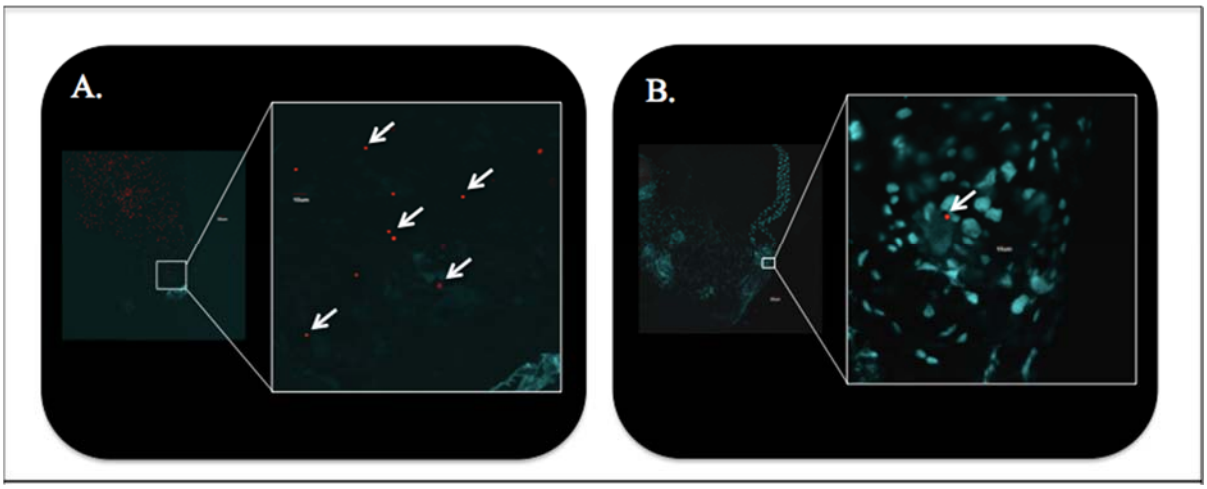

Figure 3.6. Dissections of cat flea midguts exposed to fluorescent latex beads**.** (A) Donor cat flea with fluorescent beads (arrows) after 1 day post-exposure to an "infectious" bloodmeal. (B) Recipient cat flea with fluorescent beads (arrow) after 1 day of cofeeding with donor cat fleas.

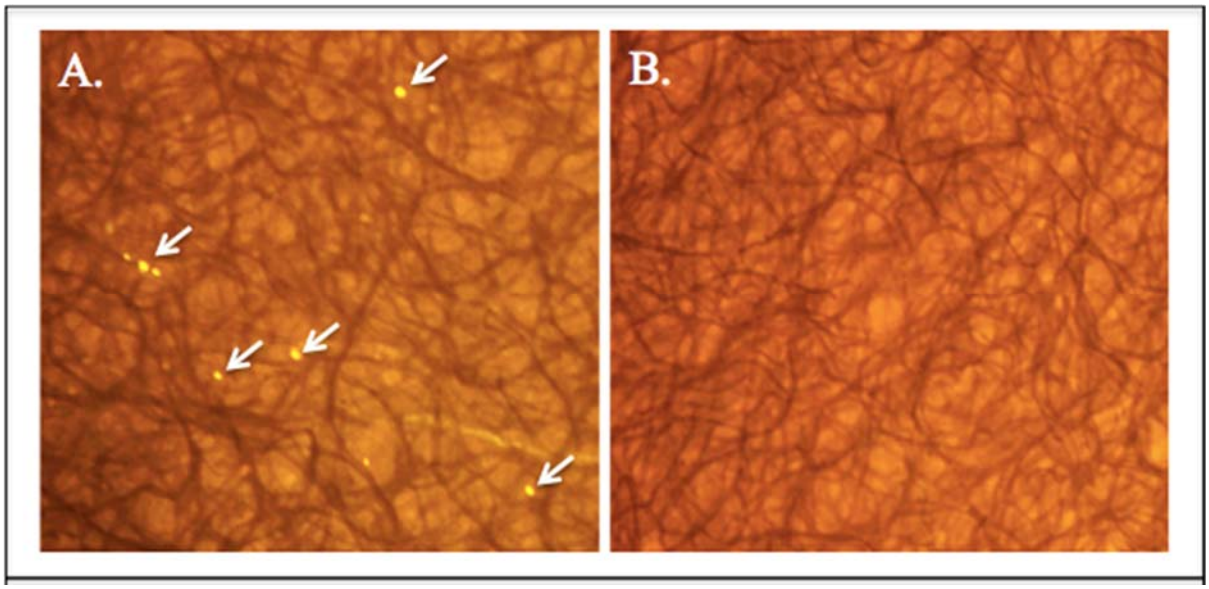

Figure 3.7. Whatman™ FTA cards placed in cat flea cages at 24 hpe to fluorescent latex beads in blood. (A) Cat fleas deposited beads (arrows) onto cards following surface sterilization and no access to blood. (B) Whatman™ FTA card exposed to non-experimental cat fleas with no access to blood.

3.4. Discussion

In principle, there are biological, morphological, and behavioral aspects of fleas that are favorable for the transmission of any microorganism that has entered the bloodstream of a vertebrate host; yet, there are proven significant differences between distinct vector species and their efficacy rate in transmitting a given agent of disease [40]. For example, among the 30 flea species confirmed as competent vectors of *Y. pestis* in North America, *X. cheopis* showed the highest proportion of pathogen acquisition (70 – 100%) and transmission efficiency rates (30 – 70%) compared to the other 29 species of fleas [24]. However, *X. cheopis* requires a long EIP (12–16 dpe) before subsequent transmission of *Y. pestis* to others, and persistent *Y. pestis* infection is typically followed by death [24]. Although *X. cheopis* is perceived as the most efficient vector of *Y. pestis*, this species of fleas transmits the plague bacterium inefficiently. Consequently, the EIP of a pathogen within a given arthropod is considered one of the most important factors affecting vector efficacy. The cat flea has demonstrated proficiency in both pathogen acquisition (30 – 100%) and transmission efficiency rates (10 – 30%) for *R. felis* in previous laboratory studies [16, 21, 22], but the length of time needed from ingestion to later transmission of *R. felis* by cat fleas was not assessed. Furthermore, although *R. felis* is widely disseminated throughout the cat flea host (including the midgut epithelial cells, muscle cells, fat body, tracheal matrix, ovaries, epithelial sheath of testes, and salivary glands), a correlation between rickettsial distribution in flea tissues and distinct transmission routes has not been determined [23]. Given that molecular detection of *R. felis* from numerous wild-caught arthropod species suggests the potential for other competent vectors, it is imperative to assess the vectorial capacity as well as the transmission mechanisms of a known biological vector to fully understand the

epidemiology of this emerging rickettsiosis, particularly as it applies to the vulnerability of susceptible vertebrate hosts, including humans.

The current study employed horizontal transmission bioassays to measure temporal dynamics of *R. felis* between cofeeding cat fleas, including exposure time (1, 3, 6, and 12 hrs) to produce infectious donor fleas and association time (1, 3, 6, and 12 hrs) to transmit infection to recipient fleas. Our results demonstrated that donor cat fleas are infectious as early as 6 hpe to an *R. felis*-infected bloodmeal, but do not transmit *R. felis* if the association time with recipient fleas is 12 hrs or less. Interestingly, although more than 50% of the donor cat fleas were infected with *R. felis* at 1 and 3 hpe, cofeeding transmission to naïve fleas was not observed until 6 and 12 hpe in these bioassays. The initial assumption for the observed delay was that perhaps rickettsial loads within each donor flea group (1, 3, 6, and 12 hpe) influenced *R. felis* cofeeding transmission (*i.e.* transmission events were dose-dependent), but the only significant difference between rickettsial loads of donor cat fleas was at 3 and 6 hpe. This difference is not considered substantial because there was no transmission at 1 hpe and the highest proportion of transmission events occurred at 12 hpe; yet, rickettsial loads of donor cat fleas from 1 and 12 hpe were not significantly different from other time points. Surprisingly, cofeeding transmission of *R. felis* to recipient fleas was not observed at any association time points (1, 3, 6, and 12 hrs) even though more than 60% of the donor cat fleas were infected in all groups with comparable rickettsial loads. Therefore, similar to earlier work [22], cofeeding transmission of *R. felis* between cat fleas is not dose-dependent; however, there is an incubation period required before transmission may occur for reasons not currently understood.

Transmission of flea-borne pathogens may occur through several possible mechanisms, including: contaminated feces (*e.g. R. typhi, B. henselae*); soiled mouthparts (*e.g.* viral pathogens); regurgitation of gut contents (*e.g. Y. pestis*); and infectious saliva via infected salivary glands (*e.g. R. felis*) [24]. Similarities often exist between transmission routes utilized by rickettsial pathogens, but the flea-associated *Rickettsia*, *R. typhi* and *R. felis,* exhibit rather dissimilar transmission routes. Jointly, infection in the flea is initiated when ingested rickettsiae enter and replicate within the epithelial cells of the midgut. For *R. typhi*, the rickettsiae within the midgut cells are released into the gut lumen for excretion with feces at 10 dpe to an infectious host [26]. For *R. felis*, the rickettsiae migrate from the midgut cells to the salivary glands for inoculation into hosts with flea saliva, roughly a 7-14 day migration from the moment of arthropod ingestion [29]. Since the kinetics of bloodmeal-acquired *R. felis* in cat fleas was demonstrated [29], interpretation of other studies now suggests that transmission of *R. felis* by cofeeding cat fleas may occur prior to salivary gland infection [21, 22]. Similar to the kinetics account [29], the current study did not detect *R. felis* in the salivary glands of cat fleas following a short-term exposure event (24 hpe) by qPCR or IFA analyses; nevertheless, transmission of *R. felis* between cofeeding cat fleas occurrs at 24 hpe or less based on past and the present studies [21, 22]. Rickettsial DNA was, however, detected by qPCR in 10% and 4% of the dissected flea heads (encompassing the mouthparts) at 24 and 48 hpe, respectively. Although no definitive organisms were detected from the heads via IFA, this may be due to the lower specificity of IFA when compared to qPCR analyses. Currently, the survival of *R. felis* on the external mouthparts of cat fleas is unknown, but it is possible that bacteria present in residual blood on the posterior portion of

the flea mouthparts (or anterior pharynx) could survive environmental elements from within the flea's head capsule [30].

The dissimilar transmission routes of flea-borne rickettsial species may also reflect differences between the feeding behavior of each vector, with *C. felis* and *X. cheopis* as the recognized biological vectors for *R felis* and *R. typhi*, respectively. Because *X. cheopis* feed so infrequently, once every 1-3 days [41], there is ample opportunity for *Rickettsia* to replicate and escape the midgut cells before defecation on a host. In addition to fecal transmission, further studies revealed that *X. cheopis* infected for >21 days were capable of transmitting *R. typhi* to hosts by bite; however, oral transmission of *R. typhi* is the result of regurgitation of excess *Rickettsia* present in the gut lumen of fleas rather than through salivary secretions [42]. Due to the rapid feeding behavior (roughly 14 hours total daily of intermittent feeds) and high turnover rate of gut contents [43], *R. felis*-infected cat fleas are not known to regurgitate excess bacteria from the midgut during successive bloodmeal acquisition. A more likely scenario for transmission of *R. felis* prior to salivary gland infection is that cat flea mouthparts harbor residual blood along the grooved surfaces that form the food and salivary canals [30]. The general feeding behavior of many arthropods with piercing-sucking mouthparts is performed by a series of brief probes to locate capillaries within the vertebrate [44]. During these probing events, bacteria present in the salivary grooves distal to the salivary pump would be driven into the bite site [30, 44]. Our results demonstrated that *R. felis* is released from soiled mouthparts of cat fleas following exposure to an infectious bloodmeal as evidenced by nucleic acid isolation from Whatman™ FTA cards. Additionally, residual blood was deposited between intermittent feeds by probing cat fleas as visualized on these cards when access to blood was granted. Given that flea

mouthparts were unable to penetrate through the card due to the thickness of the paper, the presence of these blood droplets is significant because it demonstrates the potential for remaining blood in the salivary canal to transfer with saliva into the next bite site. Due to the opposing hydrodynamic forces of the food and salivary canals, regurgitation of excess blood blocked before the prestomach by probing cat fleas seems unlikely [30]. Furthermore, no visual evidence of probing was demonstrated when cat fleas were surface sterilized prior to placement with Whatman™ FTA cards and given no access to blood, yet *R. felis* was still detected using the same techniques; thus, highlighting that bacteria soiling the salivary grooves seems most prone to transmission during probing expeditions.

In the strictest sense of the delineation between a biological and mechanical mechanism, transmission of *R. felis* by cat fleas with no discernable EIP (*e.g.* transfer of *R. felis* before disseminated arthropod infection) would be classified as a mechanical mechanism. Moreover, the potential for declining transmission efficiency with additional bloodmeals (*e.g.* the proportion and infection load of *R. felis* in the head region of cat fleas decreased between 24 and 48 hpe) indicates that the source for early *R. felis* transmission is not sufficient for multiplication and persistence of the bacteria (another qualifier for a mechanical mechanism). However, early-phase transmission of *R. felis* is not instantaneous, which is not compatible with a mechanical mechanism. A minimal incubation period is required before *R. felis* transmission may occur, but this interval is not dependent on the amount of *Rickettsia* ingested or replication of the bacteria within the flea. Several authors [30, 45, 46] have proposed that the mechanical vs. biological dichotomy is oversimplified, and suggested two other possible mechanisms of vector-borne transmission: ingestion-salivation and ingestion-egestion. Although currently classified as non-biological, these two

mechanisms depend on adherence of the pathogen to the interior surfaces of the vector before subsequent inoculation during the next feeding event. The present study used fluorescent latex beads, which possess no biological capacity for vector-borne transmission, to demonstrate that early-phase transmission of *R. felis* by cat fleas is accomplished by a mechanical mechanism. The release of latex beads from feeding and/or probing cat fleas, as demonstrated through cofeeding bioassays and Whatman™ FTA cards, supports the notion that early-phase transmission is mechanical; however, mechanical, ingestion-salivation, and ingestion-egestion mechanisms may not be mutually exclusive. The minimal theoretical conditions required for mechanical transmission are (i) high parasitemia in donor vertebrate hosts, (ii) high density of potential mechanical arthropod vectors, (iii) high receptivity and susceptibility of a major part of potential recipient vertebrate hosts, and (iv) close contact between recipient and donor vertebrate hosts [34]. Although systemic vertebrate infections with *R. felis* remain an occasional phenomenon with highly variable frequency and impact, these minimal conditions for mechanical transmission are met when the cat flea is considered the biological vector and reservoir host for this pathogen.

Utilization of both biological and mechanical mechanisms may be extremely advantageous depending on the transmission cycle of a pathogen. The majority of our current understanding of *R. felis* transmission is derived from cat flea colonies maintained on live cats or in an artificial host system. Remarkably, exploitation of both mechanisms by *R. felis* coincides with the general ecology of cat fleas associated with domestic cats. For example, on-host longevity of cat fleas is approximately 8 days due to the grooming efficiency of cats [47]. Thus, if the EIP for biological transmission of *R. felis* by cat fleas is roughly the same amount of time as the average lifespan of the vector, then a mechanical mechanism must be

used to safeguard the probability of pathogen transmission. Moreover, only about 5% of cat fleas transfer from one cat host to another every 7 days [48]. An immediate transfer to a second host is favorable for mechanical transmission, but weekly transfer rates of cat fleas was previously demonstrated as sufficient for the maintenance and persistence of *R. felis* within cat flea populations [22]. Intriguingly, the current study revealed that *R. felis*-infected cat fleas must cofeed with naïve fleas for more than 12 hours in order for transmission to occur. This rather lengthy association time needed to ensure *R. felis* transmission might reflect a crucial component in the vectorial capacity of cat fleas for this pathogen, such as the long-term persistent feeding behavior of cat fleas on the same vertebrate host compared to transient blood-feeding arthropods. Furthermore, migration to the salivary glands must be required for sustained transmission given that the presence of *R. felis* on the mouthparts of cat fleas declined between 24 and 48 hpe with the advent of an uninfected bloodmeal. Similar results were found in a previous study when a portion of fresh blood was assessed for rickettsial DNA at 24 and 48 hpe to *R. felis*-infected cat fleas ($3.3x10^3$ and $3.0x10^2$ rickettsiae per 200 μL of blood, respectively) [21]. Likely, intermittent feeding by cat fleas on the same host consists of both infected and uninfected bloodmeals because cofeeding transmission of *R felis* is dependent upon the close proximity (within a few centimeters) of infected and uninfected vectors [22]. Therefore, *R. felis* does not appear to thrive, multiply or persist in a transmissible state under a mechanical mechanism alone.

The primary role of cat fleas in the transmission biology of *R. felis* has been well established; yet, transmission mechanisms utilized by *R. felis* within cat flea populations for sustaining enzootic cycles are less understood. In summary, our results demonstrate that cat fleas are infectious following a brief exposure to an *R. felis*-infected bloodmeal, and

transmission of *R. felis* prior to dissemination within cat flea tissues is accomplished by a mechanical mechanism. The *R. felis*/*C. felis* relationship is truly unique in that most noncirculative, nonpersistent pathogens are generally not vector species-specific [44]; however, the demonstration of mechanical transmission may incriminate other human-biting vectors in the transmission cycle of this pathogen. Although *R. felis* has been detected molecularly in numerous arthropod species, there exists the potential for arthropods that have just consumed an *R. felis*-infected bloodmeal to appear positive for infection, despite being a noncompetent vector. Therefore, vector competence must be assessed, and additional studies will be required to discern the biological significance of *R. felis* infection in these various arthropod hosts.

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CHAPTER 4
DISCUSSION OF RESULTS AND FUTURE DIRECTIONS

4.1. Discussion of Results and Future Directions

Rickettsia felis is an emerging insect-borne rickettsial pathogen and the causative agent of flea-borne spotted fever [1-3]. First described as a human pathogen from the United States in 1991 [4], *R. felis* is now identified throughout the world and considered a common cause of fever in Africa [5]. The cosmopolitan distribution of this pathogen is credited to the equally widespread occurrence of cat fleas (*Ctenocephalides felis*), the primary vector and reservoir of *R. felis* [3]. Although *R. felis* is a relatively new member of the pathogenic *Rickettsia*, limited knowledge of basic *R. felis* biology continues to hinder research progression of this unique bacterium. Currently, no vertebrate *R. felis*-infection models are available to study *R. felis* pathogenesis. Plus, the role of vertebrates as reservoirs of *R. felis* and their impact on the maintenance of this pathogen in nature are unknown. Determination of the *R. felis* transmission cycle is essential to fully understand the vulnerability of susceptible vertebrate hosts, including humans, to flea-borne spotted fever.

Most transmission cycles of pathogenic *Rickettsia* include transovarial and transstadial passages in their arthropod hosts as well as transmission to new hematophagous vectors through the infectious blood of vertebrate amplifying hosts [6]. The continuous molecular detection of *R. felis* from other blood-feeding vectors supports the notion of infectious transmission cycles [5]; however, naturally infected mammalian blood or tissues have never been shown to be a source of *R. felis* infection from vertebrate to arthropod host. Therefore, despite the demonstration of horizontal transmission in an artificial host system [7], the principal route of rickettsial pathogens from systemically infected vertebrates to uninfected arthropods may not be applicable to the *R. felis* transmission cycle. The broad

hypothesis of this dissertation research is that horizontal transmission of *R. felis* occurs independent of a rickettsemic vertebrate host. To address this hypothesis the following areas of *R. felis* transmission biology were examined: horizontal transmission of *R. felis* via vector cofeeding on a vertebrate host; the extrinsic incubation period of *R. felis* within cat fleas; and the mechanisms utilized for horizontal transmission of *R. felis* by cat fleas. Although several basic questions concerning the ecology and epidemiology of *R. felis* remain unanswered (refer to section 1.5), the experiments conducted in this document will serve as a platform for future studies.

Cat fleas are known as the primary vector and reservoir of *R. felis*; however, field surveys regularly report molecular detection of this infectious agent from other blood-feeding arthropods [3, 5]. The presence of *R. felis* in additional arthropods may be the result of chance consumption of an infectious bloodmeal [3], but isolation of viable rickettsiae circulating in the blood of suspected vertebrate reservoirs has not been demonstrated [8-13]. Successful transmission of pathogens between actively blood-feeding arthropods in the absence of a disseminated vertebrate infection has been verified, referred to as cofeeding transmission [14]. Therefore, the principal route from systemically infected vertebrates to uninfected arthropods may not be applicable to the *R. felis* transmission cycle. Here, we show both intra- and interspecific transmission of *R. felis* between cofeeding arthropods on a vertebrate host. Analyses revealed that infected cat fleas transmitted *R. felis* to naïve cat fleas and Oriental rat fleas (*Xenopsylla cheopis*) via fleabite on a nonrickettsemic vertebrate host. Also, cat fleas infected by cofeeding were infectious to newly emerged uninfected cat fleas in an artificial host system. Furthermore, we utilized a stochastic model to demonstrate that cofeeding is sufficient to explain the enzootic spread of *R. felis* amongst populations of the

biological vector. Our results implicate cat fleas in the spread of *R. felis* amongst different vectors, and the demonstration of cofeeding transmission of *R. felis* through a vertebrate host represents a novel transmission paradigm for insect-borne *Rickettsia*.

While we established that rat fleas could acquire *R. felis* during cofeeding transmission events, the role of rat fleas as vectors for this pathogen remains undefined. Demonstration of subsequent transmission of acquired *R. felis* infection to others is needed before rat fleas are considered a competent vector for flea-borne spotted fever. Examination of tissue dissemination of horizontally acquired *R. felis* is needed to determine if *R. felis* infection in rat fleas is restricted to the midgut or spreads to other tissues (*e.g.* reproductive tissues or salivary glands). Lack of *R. felis* dissemination within rat flea tissues may reveal barriers to infection that limits the vectorial capacity of this flea species. Distinguishing vector competency is difficult with bacterial infections because the parasite form does not change between infectious and noninfectious vectors [15]. Rather the ability to transmit may be implied by propagation, accumulation, and dissemination within the arthropod.

Vector-borne pathogens must overcome arthropod infection and escape barriers (*e.g.* midgut and salivary glands) during the extrinsic incubation period (EIP) before subsequent transmission to another host [15]. This particular timespan is undetermined for the etiological agent of flea-borne spotted fever (*R. felis*). Acquisition of *R. felis* through blood-feeding on an artificial host by cat fleas revealed dissemination to the salivary glands after 7 days [16]; however, this length of time is inconsistent with cofeeding studies that produced infectious cat fleas within 24 hours of infection [7, 17]. In the current study, we demonstrated that an alternative mechanism is responsible for the early-phase transmission that typifies the spread of *R. felis* within laboratory experiments. Cofeeding transmission bioassays were constructed

to assess temporal dynamics of *R. felis* amongst cat fleas, including exposure time to produce infectious fleas and association time to transmit infection to naïve fleas. Additional experiments examined the proportion of *R. felis*-exposed cat fleas with contaminated mouthparts, as well as the likelihood for cat fleas to release *R. felis* from soiled mouthparts following exposure to an infectious bloodmeal. The potential for mechanical transmission of *R. felis* by cofeeding cat fleas was further examined using fluorescent latex beads, as opposed to a live pathogen, which would not require a biological mechanism to achieve transmission. Analyses revealed that *R. felis*-infected cat fleas were infectious to naïve fleas less than 24 hours after exposure to the pathogen, but showed no rickettsial dissemination to the salivary glands during this early-phase transmission. Additionally, the current study revealed that *R. felis*-infected cat fleas must cofeed with naïve fleas for more than 12 hours in order for early-phase transmission to occur. Further evidence supported that contaminated flea mouthparts may be the source of the bacteria transmitted early, and experimental trials demonstrated that *R. felis* is released from these soiled mouthparts during brief probing events. Moreover, the use of fluorescent latex beads supports the notion that early-phase transmission of *R. felis* may be due to a mechanical mechanism.

In terms of early-phase transmission, the infectious dose of *R. felis* for flea infection needs to be determined as well as the survival of *R. felis* on the external mouthparts of cat fleas. Along with identifying the infectious dose, the following variables need to be considered when determining early-phase transmission efficiency of *R. felis*: quantity of *R. felis* in exposure bloodmeal; period of time cat fleas are 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 require ingestion of only a few organisms to acquire infection and subsequently transmit *R. typhi* to a new host [18, 19]. Additionally, transmission of *R. felis* by fleas during the first week after the infectious bloodmeal is considered to be mechanical; however, there is an incubation period required before early-phase transmission occurs that is currently of unknown biological relevance. Thus, designation of a mechanical mechanism for early-phase transmission of *R. felis* may be oversimplified, and further studies are needed identify the anatomical site in the flea that is the source of the bacteria transmitted early.

It is postulated that there are *Rickettsia*- and flea-derived factors critical for the infection of cat fleas and ultimately responsible for the transmission of *R. felis* to naïve cat fleas [7, 16, 17, 20, 21]; however, very little is known about cat flea molecular responses to *R. felis* infection and the biology underlying their vector competency. Blood-feeding arthropods produce a variety of secreted peptides in their salivary glands during bloodmeal acquisition, and parasites may utilize these molecules to maximize their transmission to a new host [22]. Given that salivary glands provide another important barrier to pathogen transfer, a competent vector of *R. felis* would likely possess unique molecules essential for successful horizontal biological transmission. Although cat fleas have been identified as a competent vector for *R. felis*, host-dependent molecules essential for rickettsial transmission have not been examined. Future research should aim to designate whether there are salivary gland-associated molecules specific to cat fleas that influences *R. felis* horizontal transmission.

Like most vector-borne pathogens, the parameters of *R. felis* transmission are broad and multifaceted. The research described in this dissertation sought to understand the ecology

of *R. felis* transmission by examining the diverse interaction between the pathogen and vector. The combination of intraspecific and interspecific cofeeding transmission of *R. felis* on a vertebrate host, sustained transmission of *R. felis* between cofeeding cat fleas in an artificial host system and support by modeling demonstrated that cofeeding is an important mechanism of pathogen maintenance and transmission within flea populations. Furthermore, the results of these studies indicated that not only are *R. felis*-exposed cat fleas infectious following a brief incubation period, but utilization of a mechanical mechanism may also explain the rapid rate of spread that typifies *R. felis* flea-borne transmission within experimental and computational models. The results of these studies provide the basis for future work as several areas of research still remain concerning the biology and ecology of *R. felis*.

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APPENDIX A
COMMONLY USED ABBREVIATIONS

cDNA – Complementary DNA

*Cf*18S – Portion of *C. felis* 18S rRNA gene

DNA – Deoxyribonucleic acid

dpe – Days post-exposure

EL – Elward II Laboratories

EIP – Extrinsic incubation period

gDNA – Genomic DNA

HI – Heat inactivated

H&E – Hematoxylin and eosin

hr(s) – Hour(s)

hpe – Hour(s) post-exposure

IHC – Immunohistochemistry

IFA – Indirect immunofluorescent assay

ID - Intradermal

ISE6 – *Ixodes scapularis* cell line

LSU – Louisiana State University

PCR – Polymerase chain reaction

qPCR – Quantitative Real-time PCR

*Rf*17kDa – Portion of *R. felis* 17-kDa antigen gene

RFLO – *Rickettsia felis*-like organism

RB – Rhodamine B

RNA – Ribonucleic acid

RT-PCR – Reverse transcription PCR

SEM – Standard error of the mean

APPENDIX B
PERMISSION

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Title: Cofeeding intra- and interspecific transmission of an emerging insect-borne rickettsial pathogen

Author: Lisa D. Brown,Rebecca C. Christofferson,Kaikhushroo H. Banajee,Fabio Del Piero,Lane D. Foil,Kevin R. Macaluso

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VITA

Lisa Diane Brown is the daughter of Mr. Thomas L. Brown and Mrs. Karen L. Brown. She was born in Tyler, Texas in 1985, and is the older sister of Ms. Lauren M. Brown. Lisa graduated with a Bachelor of Science degree from the University of Texas at Tyler in 2009. While pursuing her undergraduate degree, Lisa conducted funded research at her *alma mater* and Texas A&M University at Corpus Christi as a National Science Foundation REU intern. After college, Lisa earned a Master of Science degree at the University of Louisiana at Monroe with Dr. John L. Carr in 2012. Lisa's thesis research focused on aquatic herpetofaunal communities in a regenerating bottomland hardwood forest in northern Louisiana. During her time as a master's student, Lisa had the opportunity to conduct international research on the ectoparasites of native turtles in Colombia. It was during her time in Colombia that Lisa decided to pursue a research career in vector-borne diseases. Lisa began her dissertation research in the Department of Pathobiological Sciences at the School of Veterinary Medicine and Louisiana State University under the guidance of Dr. Kevin R. Macaluso. At Louisiana State University, Lisa studied the transmission mechanisms of an emerging flea-borne rickettsiosis known as *Rickettsia felis*. Lisa is a candidate to receive the degree of Doctor of Philosophy in biomedical and veterinary medical sciences with a concentration in pathobiological sciences in May, 2016. After graduation, Lisa will continue her studies of vector-borne diseases at Vanderbilt University in Nashville, Tennessee.