

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

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## Abstract

Cat fleas (*Ctenocephalides felis*) are known as the primary vector and reservoir of *Rickettsia felis*, the causative agent of flea-borne spotted fever; however, field surveys regularly report molecular detection of this infectious agent from other blood-feeding arthropods. The presence of *R. felis* in additional arthropods may be the result of chance consumption of an infectious bloodmeal, but isolation of viable rickettsiae circulating in the blood of suspected vertebrate reservoirs has not been demonstrated. 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. 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 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 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* and furthers our understanding of this emerging rickettsiosis.

**Keywords:** cofeeding transmission, *Ctenocephalides felis*, *Rickettsia*, *Xenopsylla cheopis*

Received 16 July 2015; revision received 9 September 2015; accepted 22 September 2015

## 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) (Raoult *et al.* 2006). Presently, infections are encountered in populations living in unsanitary, crowded conditions (Brouqui & Raoult 2006; Raoult *et al.* 2006) as urban expansion into suburban

environments worldwide has generated ideal ecosystems for infectious disease outbreaks caused by these prevalent pathogens (e.g. re-emergence of flea-borne endemic typhus in southern California and Texas) (Gillespie *et al.* 2009; Blanton *et al.* 2015). 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 (Schriefer *et al.* 1994a; Zavala-Velazquez *et al.* 2000; Raoult *et al.* 2001; Parola *et al.* 2003; Zavala-Castro *et al.* 2009; Richards *et al.* 2010; Socolovschi *et al.* 2010; Parola 2011; Medianikov *et al.* 2013a,b; Edouard *et al.* 2014). Since the first

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human case reported from Texas in 1994, *R. felis* has been detected from every continent except Antarctica (Schriefer *et al.* 1994a; Parola 2011; Williams *et al.* 2011). The widespread range of *R. felis* corresponds to the cosmopolitan distribution of the primary haematophagous vector for this pathogen, the cat flea (*Ctenocephalides felis*) (Reif & Macaluso 2009). Cat fleas are arguably one of the most common flea species worldwide and lack true host specificity (Perez-Osorio *et al.* 2008); therefore, *R. felis* is essentially a household rickettsiosis in human populations where peri-domestic animals (e.g. cats, dogs and 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 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 (Eldridge & Edman 2000). It is the generalist blood-feeding behaviour of most arthropod vectors that increases the potential for emerging diseases by providing a novel infection route between animals and humans (Rosenberg & Beard 2011). 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 (Kilpatrick *et al.* 2006). Unless vertical transmission events are 100% efficient, then additional horizontal amplification is required for the maintenance of pathogens within host populations (Randolph 2011); 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 (Azad *et al.* 1992); however, this transmission route is shown to be highly variable with  $F_1$  infection rates ranging from 0 to 100% within commercial and institutional flea colonies (Reif & Macaluso 2009). 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 (Hirunkanokpun *et al.* 2011). 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) (Parola 2011). 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 and rats) (Williams *et al.* 1992; Schriefer *et al.* 1994b; Boostrom *et al.* 2002; Richter *et al.* 2002; Case *et al.* 2006; Hawley *et al.* 2007; Labruna *et al.* 2007; Bayliss *et al.* 2009). 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 (Williams *et al.* 1992; Bayliss *et al.* 2009). Moreover, *R. felis* has been identified by molecular detection from the blood, skin and internal organs of suspected reservoir hosts (Schriefer *et al.* 1994b; Abramowicz *et al.* 2011; Panti-May *et al.* 2014; Tay *et al.* 2014, 2015; Kuo *et al.* 2015), 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 (Dieme *et al.* 2015); however, naturally infected mammalian blood or tissues have never been shown to be a source of *R. felis* infection from vertebrate to arthropod host (Weinert *et al.* 2009). In addition, much debate surrounds the likelihood of freely circulating rickettsiae in the blood of vertebrates from nonfatal cases (Labruna & Walker 2014). Therefore, despite the demonstration of horizontal transmission in an artificial host system (Hirunkanokpun *et al.* 2011), 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 Randolph 2011). 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 noncompetent 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 (Jones *et al.* 1987). Similar results are observed for tick-borne encephalitis virus (family Flaviviridae), including cofeeding transmission with the use of both traditional (*Ixodes ricinus*) and nontraditional (*R. appendiculatus*) vector species (Alekseev & Chunikhin 1990; Labuda *et al.* 1993). Cofeeding transmission is not limited to tick-borne viruses and is a confirmed route for transmission of *Rickettsia conorii israelensis* between *Rhipicephalus sanguineus* ticks (Zemtsova *et al.* 2010). Also, as opposed to the long-term cofeeding transmission behaviour of ticks, experimental results revealed transfer of West Nile virus (family Flaviviridae) between intermittent cofeeding mosquito species (*Culex* and *Aedes* spp.) (McGee *et al.* 2007). 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 this 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. *X. 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 (Abramowicz *et al.* 2011). 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 ( $5 \times 10^9$  rickettsiae/mL) or high-dose ( $5 \times 10^{10}$  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. In addition, 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 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.

## Materials and methods

### *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 (Pornwiroon *et al.* 2006). Rickettsial infections within culture were monitored using the Diff-Quik staining procedure (Pornwiroon *et al.* 2006), and the number of rickettsiae was enumerated by the BacLight viability stain kit (Sunyakumthorn *et al.* 2008). 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 (Wade & Georgi 1988). Prior to exposure of their first bloodmeal, a portion of these experimental cat fleas was tested to verify the absence of *R. felis* infection with the use of quantitative real-time polymerase chain reaction (qPCR) analyses (Reif *et al.* 2008). The remaining cat fleas were allowed to feed on the bovine blood for 24 h without disturbance prior to use in bioassays. Rat fleas were generously provided by B. Joseph Hinnebusch (Rocky Mountain Laboratories, National Institutes of Health and Infectious Diseases, National Institutes of Health, Hamilton, MT, USA) and used in bioassays immediately following their arrival to LSU. Five-week-old, male, mouse strain C3H/HeJ was purchased from Jackson Laboratory as a murine model organism.

### 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 (Protocol Number: 13-034).

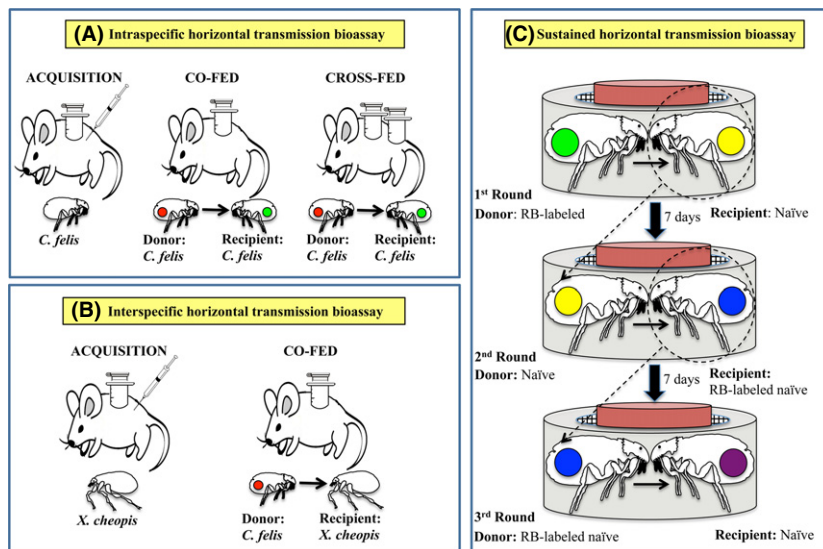
### Cat flea bloodmeal treatments in the artificial dog unit

Following the 24-h period of prefeeding on HI bovine blood, cat fleas were divided into three groups, starved for 5–6 h and given one of three bloodmeal treatments: *R. felis*-infected bloodmeal, Rhodamine B (RB)-labelled bloodmeal or control bloodmeal. Intact *R. felis*-infected cells were used following bacterial count and diluted to inoculation doses containing  $5 \times 10^9$  rickettsiae (low dose) or  $5 \times 10^{10}$  rickettsiae (high dose). *Rickettsia felis*-infected cells were pelleted by centrifugation at 13 000 g for 10 min and resuspended in 600  $\mu$ L of HI bovine blood. Cat fleas were allowed to feed on the *R. felis*-infected bloodmeal for 24 h, after which fleas fed on an uninfected

bloodmeal for an additional 48 h. To differentiate between cat fleas exposed or unexposed to a *R. felis*-infected bloodmeal, the biomarker RB was used as previously described (Hirunkanokpun *et al.* 2011). 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.

### 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), labelled with RB (recipient cat fleas) or unaltered (control cat fleas) to examine rickettsial transmission (Fig. 1A). 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 (Macaluso & Wikel 2001). To determine whether cat fleas could acquire *R. felis* from a vertebrate host, C3H/HeJ mice received an intradermal (ID) inoculation with  $5 \times 10^9$  rickettsiae in 100  $\mu$ L of SPG buffer (referred to as a bleb) and 10 cat fleas were placed into a feeding capsule adhered over the



**Fig. 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 h. 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) labelled 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).



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 (Hirunkanokpun *et al.* 2011); 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 (Fig. 1B). Identical to intraspecific bioassays, blebs were constructed to determine the 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-h period. After this 24-h 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. In addition, skin between capsules was collected from cross-fed animals, placed into RNAlater (Ambion) and stored at  $-80^{\circ}\text{C}$  for RNA extraction.

#### *Sustained rickettsial horizontal transmission bioassay*

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 (Fig. 1C). Following exposure to a high-dose *R. felis*-infected bloodmeal, donor cat fleas were housed with recipient cat fleas as previously described (Hirunkanokpun *et al.* 2011) 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 labelled 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%, 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 were used to create the new cage each week. The first round used 200 donor and recipient cat fleas, the second round used 165 donor and recipient cat fleas, and the third round used 85 donor and recipient cat fleas.

#### *Detection of Rickettsia in fleas and mice*

After the above experimentation, the collected fleas were washed with 10% bleach for 5 min, 70% ethanol for 5 min and sterile distilled water for 5 min (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  $\mu\text{L}$  PCR-grade  $\text{H}_2\text{O}$ . 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  $-20^{\circ}\text{C}$ . Quantitative PCR analyses used the plasmid pCR4-TOPO-Rf17kda + Cf18SrDNA as a standard template to create serial 10-fold dilutions ( $1 \times 10^9$  to 10 copies) as described previously (Reif *et al.* 2008). 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. In addition, 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.

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  $-80^{\circ}\text{C}$ . 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; Grasperge *et al.* 2012). Further sample lysis and wash steps were performed according to the manufacturer's instructions, and samples were eluted in 30  $\mu$ L 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<sup>®</sup> First-Strand Synthesis System (Invitrogen). 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 (Reif *et al.* 2011).

#### Histopathology and immunohistochemistry

After formalin fixation, skin samples were paraffin-embedded and sections were cut for both haematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) with a polyclonal anti-*Rickettsia* antibody (diluted 1/1000) as previously described (Grasperge *et al.* 2012). Skin sections were blindly examined by a board-certified veterinary anatomical pathologist, and dermatitis was categorized as absent (nonsignificant 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).

#### 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 vs. recipient cat fleas in the cofed and cross-fed bioassays, independence between the proportion of *R. felis* infections in recipient cat fleas vs. low- and high-infectious dosages in the cofed and cross-fed bioassays, as well as independence between *R. felis* infections in recipient cat fleas vs. recipient rat fleas in the high-dose cofed bioassays. Additional comparisons within bioassays were made by a Mann–Whitney *U*-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), 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 rickettsial vertebrate hosts. Given the absence of vertical transmission in our previous studies (Hirunkanokpun *et al.* 2011; Reif *et al.* 2011), 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 1). The transition rates for the stochastic simulation model are stated in Table 2. The framework for these 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 (Huvenne & Smagghe 2010) or 'contaminated' in the presence of at least one infectious flea ( $C_v$ ), independent of vertebrate systemic infection (Fig. 2). In addition, 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

**Table 1** Parameter values and definitions derived from experimental data or published literature for *Ctenocephalides felis*

Parameter (value)	Definition	References
$a$ (once daily)	The daily biting rate of fleas with vertebrates	Dryden & Gaafar (1991)
$b$ (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	Rust (1994)
$B$ (1000 fleas every 28 days)	The recruitment rate of new fleas	Set to maintain constant density of flea population
$\mu^{-1}$ (28 days)	The average lifespan of a flea	Personal observation utilizing the artificial membrane system

**Table 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	$U_v \rightarrow C_v$	$a*(I_f/N_f)*U_v$
Decontamination of a vertebrate through loss of all infectious fleas	$C_v \rightarrow U_v$	$f*(I_f/N_f)*C_v$

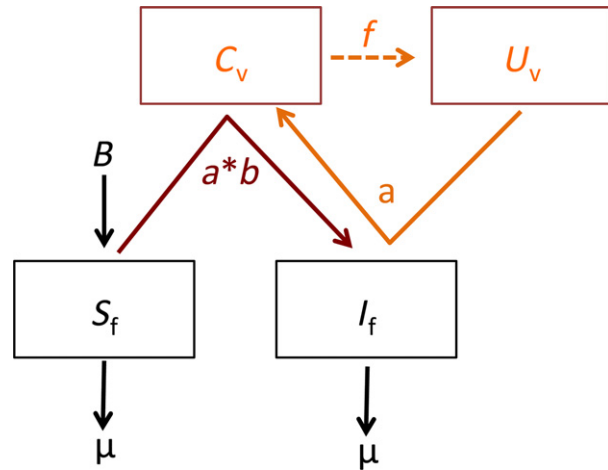
( $\mu^{-1}$ ) (Table 1). Stochastic realizations of the model were simulated using the tau-leap approximation to Gillespie's algorithm (Gillespie 2001). The model simulations ran for 280 days (equivalent to approximately 10 flea generations), and a time step of one of eight days was chosen for maximized computational efficiency and accuracy (Christofferson *et al.* 2014b). All model simulations were performed in R version 3.0.1.

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 centring all epidemic curves on the peak of transmission and averaging the variables at each centred time point to achieve a single, average epidemic curve. This enabled comparison of transmission dynamics by varying the probability of cofeeding transmission ( $b$ ) parameterized by the results from the experimental work in the current investigation.

## Results

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

To determine whether cat fleas could acquire *R. felis* infection from a murine host during feeding, an ID inoculation (or bleb) of  $5 \times 10^9$  rickettsiae in 100  $\mu$ L 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



**Fig. 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  $\mu$ m). 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 ( $f$ ).

(Fig. 1A). These acquisition bioassays generated *R. felis* infections in recipient cat fleas (10–20%) as evidenced by qPCR (Table 3), and rickettsial infection loads (determined by quantifying the copy number of *Rf17* kDa per individual flea lysate) ranged from  $5.8 \times 10^2$  to  $1.5 \times 10^3$  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 (Fig. 1A) were conducted in which donor cat fleas were exposed to either a low-dose ( $5 \times 10^9$  rickettsiae/mL) or high-dose ( $5 \times 10^{10}$  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% of the recipient cat fleas in all three trials (Table 3). The high-dose cofed bioassays generated *R. felis* infections in 100% of the donor cat fleas and yielded an infection prevalence of 16.7% in recipient cat fleas (Table 3). The low- and high-dose cross-fed bioassays resulted in an infection prevalence of 30% and 100% in donor cat fleas, respectively, and both dose experiments resulted in 10% acquisition of infection by recipient cat fleas for

**Table 3** Horizontal transmission of *Rickettsia felis* between cofeeding fleas on a vertebrate host

Group	<i>Ctenocephalides felis</i>		<i>Xenopsylla cheopis</i>	
	Prevalence (%)	Mean infection load ( $\pm$ SEM)	Prevalence (%)	Mean infection load ( $\pm$ SEM)
Acquisition				
Recipient fleas	4/30 (13.3)	$1 \times 10^3$ ( $\pm 2 \times 10^2$ )	5/30 (16.7)	$2.6 \times 10^4$ ( $\pm 1.8 \times 10^4$ )
Cofed: Low dose				
Donor fleas	5/30 (16.7)	$2.4 \times 10^{5*}$ ( $\pm 2.4 \times 10^5$ )	11/30 (36.7)	Not assessed
Recipient fleas	3/30 (10) <sup>†</sup>	$1.6 \times 10^3$ ( $\pm 1.4 \times 10^3$ )	0/30 (0)	NA
Cofed: High dose				
Donor fleas	30/30 (100)*	$2.7 \times 10^{6*‡}$ ( $\pm 4.4 \times 10^5$ )	30/30 (100)	$5.9 \times 10^6$ ( $\pm 1.3 \times 10^6$ )
Recipient fleas	6/30 (20) <sup>†</sup>	$1.6 \times 10^3$ ( $\pm 5.7 \times 10^2$ )	8/30 (26.7)	$1.4 \times 10^3$ ( $\pm 5 \times 10^2$ )
Cross-fed: Low dose				
Donor fleas	9/30 (30)*	$1.5 \times 10^4$ ( $\pm 8 \times 10^3$ )	Not assessed	Not assessed
Recipient fleas	1/30 (3.3) <sup>†</sup>	$7.5 \times 10^2$ (NA)	Not assessed	Not assessed
Cross-fed: High dose				
Donor fleas	30/30 (100)*	$9.7 \times 10^5$ ( $\pm 1.8 \times 10^5$ )	Not assessed	Not assessed
Recipient fleas	1/30 (3.3) <sup>†</sup>	$2 \times 10^2$ (NA)	Not assessed	Not assessed
Control				
Control fleas	0/30 (0)	NA	0/30 (0)	NA

Female cat fleas were given one of two infectious doses of *R. felis* during acquisition feeding (donor fleas) and subsequently cofed 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 *Rf17* kDa 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.

<sup>†</sup>A significant difference was detected in the prevalence between recipient fleas of cofed (low and high dose combined) and cross-fed bioassays (low and high dose combined).

<sup>‡</sup>A significant difference was identified in the infection load between donor fleas of low- and high-dose cofed bioassays; NA = Not applicable.

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. In addition, 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 3); whereas, mean *R. felis* infection load was significantly different between donor and recipient cat fleas in high-dose bioassays (Table 3). A significant difference in mean rickettsial load was demonstrated between donor cat fleas in low- and high-dose bioassays (Table 3); however, no significant difference was observed between mean rickettsial infection loads in recipient cat fleas of low- vs. high-dose bioassays (Table 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 (Hirunkanokpun *et al.* 2011), *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.

#### *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 (Reif & Macaluso 2009); of particular interest for this study is the detection of *R. felis* in rat fleas. 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 (Fig. 1B). Positive *R. felis* infections in recipient rat fleas (0–40%) were confirmed by qPCR (Table 3), and rickettsial infection load ranged from  $4 \times 10^2$  to  $9.8 \times 10^4$  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) (Fig. 1B) 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 3). The high-dose cofed bioassays generated an *R. felis* infection in 100% of the donor cat fleas and yielded an infection prevalence of 26.7% in recipient rat fleas (Table 3). No significant difference was observed between the number of *R. felis*-infected recipient cat fleas and recipient rat fleas (Table 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 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.

#### *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. Although 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 was negative; however, the amount of *R. felis* present between the two bioassays is likely disproportionate. During acquisition bioassays, a bleb ( $\sim 5 \times 10^9$  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.

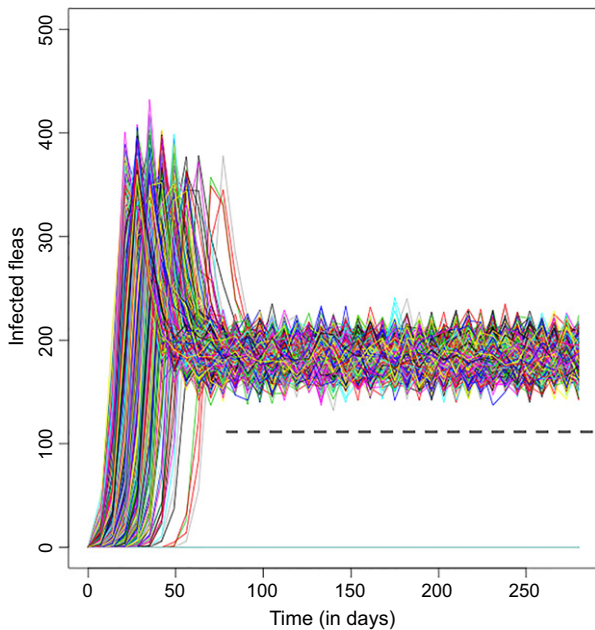
#### *Cofeeding transmission of R. felis is sustainable amongst cat flea populations*

To assess the 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 whether recipient cat fleas were infectious following 7 days of cofeeding transmission with *R. felis*-infected donor cat fleas (Fig. 1C). 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 labelled 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, 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. In addition, the average ( $\pm$ SEM) rickettsial load significantly decreased in recipient cat fleas from the first round of transmission bioassays ( $3.1 \times 10^4$ /flea lysate  $\pm 9 \times 10^3$ ) compared to the last round ( $6 \times 10^1$ /flea lysate  $\pm 1.1 \times 10^1$ ). Although rickettsial loads decreased following successive horizontal transmission bioassays, sustained transmission of *R. felis* was demonstrated.

#### *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%, 20% 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 Figs 3 and 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

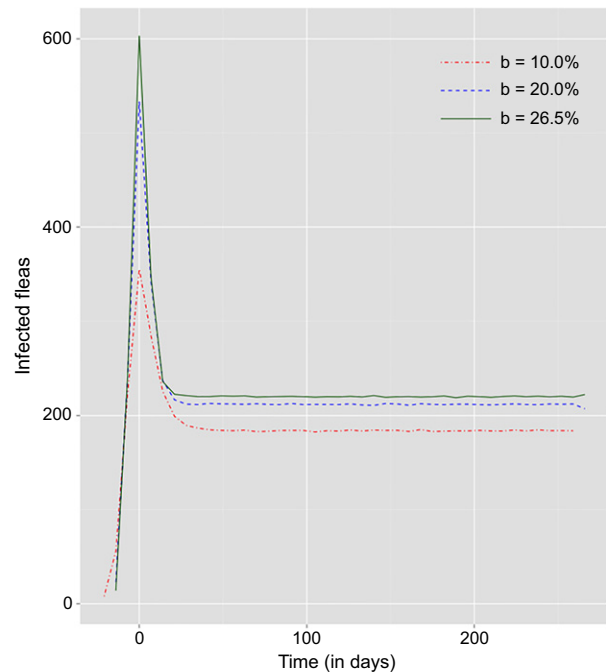
observed is no longer achievable. Interestingly, if transmission was achieved initially, there appeared to be no barriers to progression of the system towards equilibrium, that is persistent number of infected fleas at a value greater than zero (Fig. 3). While the probability of transmission and persistence was not affected by the probability of cofeeding transmission ( $b$ ), there were differences in the transmission dynamics. For  $b = 10\%$ , the time to peak was on average 3 weeks, while it was only 2 weeks for  $b = 20\%$  and  $b = 26.7\%$  (Fig. 4). In addition, the time to equilibrium was also affected by the value of  $b$ . For  $b = 10\%$ , the time to equilibrium was 6 weeks from peak (or 10 weeks from the onset of transmission after initial introduction event); for  $b = 20\%$  and  $b = 26.7\%$ , the time to equilibrium was 4 weeks (or 7 weeks from transmission onset) (Fig. 4). The per cent of fleas infected at equilibrium differed by  $\leq 4\%$  (approximately: 18.4% for  $b = 10\%$ , 21.1% for  $b = 20\%$ , and 22% 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 modelling demonstrates cofeeding as an important mechanism of pathogen maintenance and transmission within flea populations.



**Fig. 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.

## Discussion

Rickettsial transmission by arthropods can be vertical or horizontal; furthermore, transmission route and bacterial virulence are interdependent. Vertical transmission favours the evolution of benign associations, whereas frequent horizontal transmission between vectors favours virulent *Rickettsia* species (Werren 1997; Niebylski *et al.* 1999). Unique to *R. felis*, both transmission paradigms have been identified within cat flea populations and may coexist with no adverse cost to flea fitness (Azad *et al.* 1992; Wedincamp & Foil 2002; Hirunkanokpun *et al.* 2011). In addition to being a cosmopolitan flea-borne pathogen, *R. felis* is also a vertically maintained endosymbiont of nonhaematophagous booklice (psocids) (Thepparit *et al.* 2011). In the booklouse host, *R. felis* is an obligate mutualist required for the early development of the oocyte and is maintained 100% transovarially (Yusuf & Turner 2004; Thepparit *et al.* 2011). Unknown factors account for the variable prevalence of *R. felis* observed with vertical transmission amongst colonized populations of cat fleas (Hirunkanokpun *et al.* 2011). For *R. felis* to be maintained within and between arthropod populations, horizontal transmission must be utilized; however, a



**Fig. 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.

competent rickettsemic vertebrate host that can serve as a reservoir for *R. felis* is deemed either scarce or absent (Reif & Macaluso 2009). 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 faeces (Silverman *et al.* 1974; Azad 1990). In addition, 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 faecal transmission (Azad 1990). Similarities exist between transmission routes utilized by rickettsial pathogens; therefore, the ability of fleas to transmit *R. typhi* both horizontally and vertically suggests 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 (Hirunkanokpun *et al.* 2011). After a 24-h 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 (Hirunkanokpun *et al.* 2011). Using a comparable population of donor cat fleas on a live host produced positive *R. felis* infections in 10% 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% *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 (Randolph 2011). Cofeeding transmission in space is characteristic for most ectoparasite species because host-grooming behaviour often results in spatial aggregations on certain parts of the body (Randolph 2011). The highest percentage of cat fleas found on stray cats is on the smallest surface of the head and neck area, approximately 46% of feeding cat fleas are within a few centimetres of others (Hsu *et al.* 2002). 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%) when donor and recipient ticks were allowed to feed in close proximity, and transmission diminished (38%) when donor and recipient ticks were separated on nonimmune animals (Labuda *et al.* 1997). 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 haematophagous 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 (Labuda *et al.* 1993), as well as mosquito-transmitted West Nile virus (McGee *et al.* 2007) which is more applicable for this study given the similar short-term feeding behaviour of mosquitoes and fleas. Subsequently, viral infections resulted in potentially competent nontraditional 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 (Reif & Macaluso 2009). Serological-based studies have implicated several peri-domestic animals (e.g. cats, dogs, opossums, rats)

based on seropositive individuals independent from laboratory experiments (Williams *et al.* 1992; Schriefer *et al.* 1994b; Boostrom *et al.* 2002; Richter *et al.* 2002; Case *et al.* 2006; Labruna *et al.* 2007; Bayliss *et al.* 2009); 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* (Jordan *et al.* 2008; Grasperge *et al.* 2012). In the current study, all blood samples collected via cardiac puncture were qPCR negative for *R. felis* infection, indicating that experimental mice did not harbour a systemic infection. Although rickettsemia was not detected during our short-term study, other murine models for rickettsial species have observed disseminated infections at 1 day postinoculation (Walker *et al.* 1994; Dieme *et al.* 2015). 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 (Reif *et al.* 2008). 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 (Hirunkanokpun *et al.* 2011). 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, that is 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 (Reif & Macaluso 2009), 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 (Christofferson *et al.* 2014a). 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 modelling 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, that is a constant number of vertebrates in the system; also, flea density is assumed to be constant, that is 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, that is 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 (Labuda



*et al.* 1997; McGee *et al.* 2007), this assumption is made for numerous mosquito-borne disease models in that homogenous mixing of mosquitoes results in an equal chance of contact (Christofferson *et al.* 2014b). In addition, alternative forms of flea mortality, such as vertebrate grooming habits (Rust 1994), were not assessed, nor was seasonality of biting rate. Although these assumptions were required, support by modelling 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.

## Acknowledgements

We thank Dr. B Joseph Hinnebusch for providing rat fleas and Jacqueline Macaluso for her helpful comments. This research was supported by the National Institutes of Health (AI077784). RCC's contribution was supported by the National Institutes of Health NIGMS grant UO1GM097661.

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L.D.B., K.R.M. and L.D.F. conceived and designed the experiments. L.D.B., R.C.C. and K.H.B. performed the experiments. L.D.B., R.C.C. and F.D.P. analyzed the data. L.D.B., R.C.C. and K.R.M. wrote the paper.

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### Data accessibility

Raw data for gene copy numbers of *Rickettsia felis* 17-kDa in individual flea lysates from rickettsial horizontal transmission bioassays are deposited at Dryad (doi:10.5061/dryad.60hh0).