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Horizontal transmission of *Rickettsia felis* between cat fleas, *Ctenocephalides felis*

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Abstract

Rickettsia felis is a rickettsial pathogen primarily associated with the cat flea, *Ctenocephalides felis*. Although laboratory studies have confirmed that *R. felis* is maintained by transstadial and transovarial transmission in *C. felis*, distinct mechanisms of horizontal transmission of *R. felis* among cat fleas are undefined. Based on the inefficient vertical transmission of *R. felis* by cat fleas and the detection of *R. felis* in a variety of haematophagous arthropods, we hypothesize that *R. felis* is horizontally transmitted between cat fleas. Towards testing this hypothesis, flea transmission of *R. felis* via a bloodmeal was assessed weekly for 4 weeks. Rhodamine B was used to distinguish uninfected recipient and *R. felis*-infected donor fleas in a rickettsial horizontal transmission bioassay, and quantitative real-time PCR assay was used to measure transmission frequency; immunofluorescence assay also confirmed transmission. Female fleas acquired *R. felis* infection more readily than male fleas after feeding on a *R. felis*-infected bloodmeal for 24 h (69.3% and 43.3%, respectively) and both *Rickettsia*-uninfected recipient male and female fleas became infected with *R. felis* after cofeeding with *R. felis*-infected donor fleas (3.3–40.0%). Distinct bioassays were developed to further determine that *R. felis* was transmitted from *R. felis*-infected to uninfected fleas during cofeeding and copulation. Vertical transmission of *R. felis* by infected fleas was not demonstrated in this study. The demonstration of horizontal transmission of *R. felis* between cat fleas has broad implications for the ecology of *R. felis* rickettsiosis.

Keywords

cat fleas; cofeeding; horizontal transmission; *Rickettsia felis*

Introduction

Rickettsia felis is an obligate intracellular Gram-negative bacterium belonging to the transitional group of *Rickettsia* (Gillespie *et al.* 2007) and is the causative agent of an emerging flea-borne rickettsiosis (Azad *et al.* 1997). Although *R. felis* has been identified by molecular means in a large number of blood-feeding arthropods, only cat fleas, *Ctenocephalides felis*, are associated with biological transmission of this pathogen (Azad *et*

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S.H. is a postdoctoral fellow, broadly interested in ecology of vector-borne pathogens and host-vector-pathogen interactions. K.R.M. is interested in the dynamics of rickettsial transmission by arthropods, molecular interaction between pathogen and vector, and mechanisms of vector competence.

Data accessibility: Raw data for gene copy numbers of *Rickettsia felis* 17-kDa and *Ctenocephalides felis* 18S rDNA in individual flea samples from rickettsial horizontal transmission bioassays are deposited at Dryad: doi:10.5061/dryad.vn88q.

al. 1992; Reif & Macaluso 2009). Cat fleas have a broad host range and, based on their ability to adapt to dynamic microenvironments, have a worldwide distribution. Consequently, the occurrence of *R. felis*-infected cat fleas has been reported on a near cosmopolitan scale, and confirmed cases of flea-borne rickettsiosis caused by *R. felis* have been reported in seventeen countries to date (Reif & Macaluso 2009; Ben-Zvi *et al.* 2010; Lindblom *et al.* 2010; Richards *et al.* 2010; Socolovsch *et al.* 2010; Williams *et al.* 2011).

Understanding the transmission of *R. felis* is essential to fully appreciate the epidemiology of this flea-borne rickettsiosis. The nature of interactions between different rickettsial species and their arthropod hosts is due in great part to the mode of transmission. This intricate relationship between *Rickettsia* and the arthropod host is best illustrated in the tick-associated spotted fever group of *Rickettsia* in which some rickettsial species not associated with vertebrate infection employ a maintenance strategy dependent on continued vertical, or inherited, transmission events (Niebylski *et al.* 1997). Conversely, virulent *Rickettsia* spp. with low efficiency of vertical transmission require frequent horizontal, or infectious, transmission events and often impose a negative fitness effect on the invertebrate host (Niebylski *et al.* 1999). Therefore, the pathogenicity of a particular rickettsial species is correlated to the necessity for successful horizontal transmission (Werren 1997). The complexity of the *Rickettsia*/arthropod relationship is highlighted by the dualistic nature of *R. felis* in cat fleas. Vertical transmission of *R. felis* has been described in laboratory-reared cat fleas (Azad *et al.* 1992; Wedincamp & Foil 2002). Although the efficiency has yet to be precisely determined, it is clear that there is no 100% transovarial transmission, as evidenced by varying incidence of infection in several research colonies of cat fleas (Higgins *et al.* 1994; Reif & Macaluso 2009). The variable vertical transmission of *R. felis*, coupled with compounding molecular and serological evidence of *R. felis* infection in a variety of arthropod and vertebrate hosts (Reif & Macaluso 2009), suggests that *R. felis* possesses the characteristics of a rickettsial pathogen that also favours horizontal transmission. In nature, it is not known if *R. felis* is maintained primarily through vertical or horizontal transmission events; in fact, clear evidence of horizontal transmission has not been described.

Specific mechanisms of horizontal transmission including cofeeding, mating and contact with contaminated insect faeces that are known to occur for *Rickettsia typhi*, the flea-borne rickettsial agent of endemic typhus, have not been previously demonstrated in the *R. felis*/arthropod model. Thus, the goal of the present study was to determine whether similar routes of transmission are involved in the spread of *R. felis* between fleas. Towards the objective, Rhodamine B (RB), which has previously been used as a biomarker for mammals, birds, fish and insects (Fisher 1999), was utilized to label cat fleas. Subsequently, bioassays were developed to assess horizontal transmission of *R. felis* between mixed populations of *R. felis*-infected donor and *Rickettsia*-uninfected recipient cat fleas. In addition to cofeeding infection of cat fleas, sexual transmission of *R. felis* was also demonstrated. The novel characterization of horizontal transmission of *R. felis* by cat fleas will further our understanding of this emerging rickettsiosis.

Materials and methods

Source of fleas and *Rickettsia*

Unfed, adult cat fleas (*C. felis*) were purchased from Elward II (EL) (Soquel, CA, USA). Several generations of adult fleas were provided a *Rickettsia*-uninfected bovine (Rockland) bloodmeal via an artificial dog (Wade & Georgi 1988), and eggs were reared to adults on sand with artificial diet (Lawrence & Foil 2002). Cat fleas from the EL Laboratory are reportedly free of *R. felis* infection (Pornwiroon *et al.* 2007; Reif *et al.* 2011); however, a portion of these experimental cat fleas (30 male and 30 female) were tested for *R. felis* infection by quantitative real-time PCR (qPCR) to ensure the fleas were *R. felis*-free. An

Ixodes scapularis-derived cell line (ISE6) maintained in modified L15B growth medium (Pornwiroon *et al.* 2006) was utilized to propagate *R. felis* (strain LSU). This rickettsial strain was originally isolated from the Louisiana State University cat flea colony (Pornwiroon *et al.* 2006). Cellular infection was examined by Diff-Quik (Dade Behring) staining as described previously by Pornwiroon *et al.* (2006); the BacLight viability stain kit (Invitrogen) was used to assess viability and enumerate rickettsiae as previously described (Sunyakumthorn *et al.* 2008).

Rhodamine B labelling of fleas

To differentiate *R. felis*-infected donor and uninfected recipient fleas in the rickettsial horizontal transmission bioassay, RB was evaluated as a biomarker in cat fleas. Newly emerged, unfed adult fleas were sexed, then male and female fleas, in separate feeding capsules, were allowed to prefeed with *Rickettsia*-uninfected heat-inactivated (HI) bovine blood for 24 h. Five hundred microlitres of a 0.1% solution of RB (Sigma-Aldrich) in HI bovine serum was added to 100 μ L of bovine blood and offered to prefed fleas for 24 h. Fleas were then fed on normal bovine blood for 30 days, and five of each RB-treated and control fleas were collected daily for RB detection by using a fluorescence stereomicroscope (Zeiss SteREO Lumar V12) at excitation wavelength 540 nm and emission wavelength 625 nm.

Detection of *Rickettsia* in fleas

All flea samples were cleaned by sequential surface washing with 10% bleach for 5 min, 70% ethanol for 5 min and three times (5 min each) sterile distilled water. Genomic DNA (gDNA) of each flea was extracted by a modified HotSHOT method (Truett *et al.* 2000; Reif *et al.* 2008). At least two negative environmental controls were performed during each DNA extraction process.

The standard plasmid, primers and reaction conditions for the qPCR used in this study have been described previously (Reif *et al.* 2008). Briefly, total 35 μ L of qPCR reaction contained 2 $\times$ of iTaq SYBR Green Supermix with ROX (BioRad), 100 nM of each primer, DNase/RNase-free dH₂O, and 5 μ L of gDNA from each sample was premixed in 96-well plates and aliquoted in triplicate 10- μ L reactions on 384-well plates. Amplification and detection were performed with an ABI 7900HT unit (Applied Biosystems). For each set of reactions, two negative environmental controls for gDNA extraction and a negative control for the qPCR (ultrapure sterile water instead of template) were included. Additionally, for a portion of *Rickettsia*-positive fleas, gDNA was utilized for amplification and DNA sequencing of a 434-bp section the *Rickettsia* genus-specific 17-kDa antigen gene using the primers and protocol described by Pornwiroon *et al.* (2006). The sequences obtained were analysed with BioEdit sequence alignment editor (Ibis Biosciences), and nucleotide similarity comparisons were made with GenBank.

An indirect immunofluorescence assay (IFA) to detect *R. felis* infection was performed on both *R. felis*-infected donor and recipient fleas, using a modified protocol for booklice as described by Thepparit *et al.* (2011). Slides containing formalin-fixed paraffin-embedded flea sections (4.5 μ m) were heated at 65 °C for 15 min and deparaffinized by repeated immersions in Hemo-De (Scientific Safety Solvents). Slides were rinsed with phosphate-buffered saline (PBS), and then antigen retrieval was performed by adding 10 μ g/mL proteinase K (Roche Diagnostics) in prewarmed buffer containing 1 M Tris and 0.5 M EDTA (pH 8) at 37 °C for 15 min. At room temperature, the treated slides were excessively washed with PBS prior to immunofluorescence staining. For *R. felis* detection, mouse polyclonal antisera to *R. felis* were generated as described by Sunyakumthorn *et al.* (2008). The pretreated slides were blocked with 3% bovine serum albumin (BSA) in PBS for 1 h. Then

slides were incubated 2 h with mouse polyclonal anti-*R. felis* at a dilution of 1:100 in 1% BSA in PBS followed by three 5 min washes with PBS containing 0.01% TritonX-100. The slides were then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (KPL Inc.) at a dilution of 1:200 for 1 h. After the slides were washed again with PBS/0.01% Triton X-100, flea tissues were counterstained with 0.1% Evan's blue in PBS at 37 °C for 30 min. Finally, slides were mounted using Hardset Vectashield with DAPI (Vector Laboratories) and visualized using a Zeiss fluorescence microscope. Flea sections in which no primary antibody was added served as a control for nonspecific binding of the FITC-conjugated antibody.

Rickettsial horizontal transmission bioassays

For all transmission bioassays, newly emerged, unfed adult fleas were sexed and male and female fleas, in separate feeding capsules, were prefed with *Rickettsia*-uninfected HI bovine blood for 24 h. Then, surviving fleas were starved of blood for 5–6 h prior to exposure to one of three different treatments: (i) *R. felis*-infected donor, (ii) RB-labelled uninfected recipient or (iii) uninfected recipient fleas. For the first treatment, an *R. felis*-infected bloodmeal was prepared by resuspending intact *R. felis*-infected cells containing 5×10^9 rickettsiae (passage 7) and collecting the cells by centrifugation at 13000 *g* for 10 min. Culture medium was removed, and the *R. felis*-infected cell pellet was resuspended in 600 µL of HI bovine blood. Both prefed male and female fleas in separate capsules were allowed to feed on the *R. felis*-infected bloodmeal for 24 h. For the second treatment, fleas were RB-labelled as described earlier. The third treatment consisted of HI bovine blood in the absence of rickettsiae or RB.

Four groups (I–IV) were assessed for rickettsial transmission between fleas utilizing fleas from *R. felis*-infected donor, RB-labelled uninfected recipient, and uninfected control and recipient flea feeding treatments (Fig. 1A). Group I consisted of 80 *R. felis*-infected donor male fleas placed in the same feeding capsule with 80 uninfected recipient female fleas. Group II was reciprocal and consisted of 80 uninfected recipient male fleas placed in the same feeding capsule with 80 *R. felis*-infected donor female fleas. In Group III, 80 each of *R. felis*-infected donor male and female fleas were placed in the same feeding capsule with 80 each of RB-labelled uninfected recipient male and female fleas. The control group (Group IV) was comprised of 80 each of uninfected male and female fleas in the same feeding capsule. Fleas in all four groups were fed on normal bovine blood, replenished every other day for 28 days, using the artificial feeding system. Ten male and female fleas were assessed for *R. felis* infection at days 1, 7, 14, 21 and 28 post-cofeeding. For Groups I, II and IV, fleas were collected and sexed, and the remaining fleas were placed back into their feeding capsules. For Group III, fleas were sexed and then RB-labelled uninfected recipient fleas were distinguished from *R. felis*-infected donor fleas by fluorescent microscopy. All fleas were kept at –20°C until gDNA was extracted. The remaining flea eggs with faeces in each feeding capsule on days 3, 7, 14, 21 and 28 were reared until F₁ adults emerged (approximately 20–25 days). Five each of male and female F₁ fleas from each experimental group were assessed for rickettsial infection by qPCR. Three separate trials were conducted, and data presented are a combination of the three trials.

To pinpoint the mechanisms of horizontal transmission, the contributions of cofeeding and mating were examined in two separate trials for each route. The data are presented as a combination of the two trials. Cofeeding bioassays (Fig. 1B) were executed by combining 80 each of *R. felis*-infected donor male and female fleas into a feeding capsule and allowing them to feed on 2 mL of uninfected bovine blood for 24 h. A 200 µL portion of the leftover blood was collected and assessed for *R. felis* infection by qPCR; meanwhile, the remaining blood was immediately offered to uninfected recipient female (Group V) and male (Group VI) fleas for 24 h. New, uninfected bovine blood (2 mL) was again provided twice more to

R. felis-infected donor fleas for 24 h and then, again, 200 μ L of leftover blood was processed by qPCR, and the remaining blood was offered to Group V and VI fleas. At all times, the uninfected recipient female (Group V) and male (Group VI) fleas were separated to prevent mating. Ten each of Group V and VI fleas were collected at 24, 48 and 72 h post-feeding on the initial leftover bovine blood and assessed for *R. felis* infection.

For the mating bioassays (Fig. 1C), newly emerged, unfed adult fleas were sexed and male and female fleas held in separate feeding capsules. Newly emerged male and female fleas do not mate prior to a bloodmeal, and feeding for approximately 24 h is required for successful mating, with most fleas mating by 48 h (Bi-song *et al.* 2002). Therefore, 80 each of virgin male and female fleas were pre-fed with *Rickettsia*-uninfected HI bovine blood for 24 h, starved for 5–6 h and then *R. felis*-exposed for 24 h. In Group VII, 80 *R. felis*-infected donor males were placed in the same feeding capsule with 80 uninfected recipient females. For Group VIII, 80 uninfected recipient males were placed in the same feeding capsule with 80 *R. felis*-infected donor females. To eliminate co-feeding transmission between *R. felis*-infected and uninfected fleas, both groups were allowed to mate in the absence of a bloodmeal. Ten male and female fleas from each group were assessed for *R. felis* infection at 12, 36 and 60 h post-mating.

Statistical analysis

Statistical analyses were performed using the GLM procedure of SAS® version 9.1.3 (SAS Institute Inc). Two-way analysis of variance ($_{ANOVA}$) was performed to examine potential differences of *R. felis* acquisition, transmission and density (ratio of *Rf* 7kDa/*Cf* 18S copy numbers). If overall significance was observed, Tukey's honestly significant difference post hoc test was used to determine pairwise differences of means of main effects. For significant interaction effects, pairwise comparisons were conducted with a *t*-test of least squares means. Differences among or between fleas and bioassay groups were considered statistically significant at $P < 0.05$.

Results

Rhodamine B-labelled fleas

All adult fleas fed RB-labelled serum were luminescent when examined by fluorescent microscopy. In comparison with unlabelled control fleas fed bovine blood alone, fleas exposed to RB-labelled bloodmeal appeared orange on day 1 post-feeding, with most of the body emitting signal (Fig. 2). At 7 days, and for up to 30 days, post-feeding on a single RB-labelled blood-meal, signal from the RB label was only detected in the eyes of fleas (Fig. 2, in the white circles). Survival of fleas fed RB-labelled bloodmeal was not different from control fleas. Using RB-labelled bloodmeal allowed for differentiation of fleas from unlabelled control fleas; thus, the RB-labelled fleas could be utilized to examine horizontal transmission of *R. felis* between fleas in the subsequent bioassay.

Infection of fleas with *R. felis* and transmission to previously uninfected fleas

A portion of EL colony of fleas (30 males and 30 females) were confirmed *R. felis* negative by qPCR prior to utilization in rickettsial transmission bioassays. Fleas were exposed to *R. felis*-infected bloodmeal, RB-labelled bloodmeal or bloodmeal alone for 24 h, and then divided into one of four groups. Fleas in each Group (I–IV) were allowed to co-feed for 28 days; *R. felis* infection was assessed by qPCR in individual fleas at days 1, 7, 14, 21 and 28 post-co-feeding (Table 1). Both donor male and female fleas were *R. felis* positive beginning 1 day after feeding on the *R. felis*-infected bloodmeal. The mean prevalence for all fleas ($\pm$ SEM) of *R. felis* acquisition by female fleas ($69.3 \pm 2.7\%$) exposed to the *R. felis* feeding treatment was significantly greater than male fleas ($43.3 \pm 4.7\%$).

Both uninfected recipient male and female fleas became positive for *R. felis* as evidenced by qPCR after cofeeding with *R. felis*-infected donor fleas (Table 1). Differences between sexes were not identified based on group assignment. In Group I, mean *R. felis* acquisition by recipient females over 28 days was 4.7% (7/150) after being combined with *R. felis*-infected donor males. A total of 16% (24/150) of uninfected recipient male fleas combined with *R. felis*-infected donor female fleas in Group II became positive for *R. felis* for the same period of time. For Group III, 10.3% (31/300) of both RB-labelled uninfected recipient male and female fleas became positive for *R. felis*. Over the course of cohabitation by *R. felis*-infected donor and recipient fleas, no significant difference in the mean percent transmission of *R. felis* to either recipient male ($13.7 \pm 2.7\%$) or female ($7.0 \pm 1.5\%$) fleas was identified; however, the mean prevalence of *R. felis* in recipient fleas was significantly lower than *R. felis*-infected donor male and female fleas. The transmission of *R. felis* from *R. felis*-infected donor fleas to uninfected recipient fleas in each Group (I–III) was confirmed by traditional PCR using the 17-kDa antigen gene primer sets (Williams *et al.* 1992; Pornwiroon *et al.* 2006) and sequencing amplicons from a representative subsample of *R. felis*-positive recipient fleas. Nucleotide sequences for the amplified fragments (394 bp) of 17-kDa antigen gene were identical to the published sequences for *R. felis* (LSU) (Pornwiroon *et al.* 2006) and *R. felis* URRWXC2 (Accession no. CP000053) (Ogata *et al.* 2005). All unlabelled, unexposed control male and female fleas (Group IV), fed normal bovine blood, were qPCR negative for *R. felis*.

Further confirmation of *R. felis* transmission by *R. felis*-infected donor fleas to uninfected recipient fleas was demonstrated by IFA. While IFA confirmed that uninfected recipient fleas became infected after being reared with *R. felis*-infected donor fleas (Fig. 3A–C), it was obvious that the recipient fleas had lower *R. felis* density (Fig. 3D,E), when compared with *R. felis*-infected donor fleas. Control (Group IV) fleas fed on normal bovine blood were IFA negative (inset rectangle in Fig. 3A).

Genomic equivalents of the *R. felis* 17-kDa antigen gene and the *C. felis* 18S rDNA gene were quantified, and the density of *R. felis* in individual fleas was determined by calculating the ratio of the logarithmically transformed *R. felis* 17-kDa to 18S rDNA copy number. When comparing the density of *R. felis* between *R. felis*-infected donor male and female fleas, the mean *Rf17kDa/Cf18S* ratio of *R. felis*-infected donor females (0.75) was significantly greater than the same ratio in *R. felis*-infected donor male (0.58) fleas (Fig. 4). When combining results from Groups I–III, no significant difference in mean *Rf17kDa/Cf18S* ratios between recipient male (0.32) and female (0.37) fleas (Fig. 4) was observed. There were significant differences in rickettsial density between fleas in the *R. felis*-infected donor cohort compared to those that became infected with *R. felis* after they were placed in the same feeding capsule (Fig. 4). Likewise, the mean *Rf17kDa/Cf18S* ratio of *R. felis*-infected donor fleas was significantly increased from day 1–7 post-cofeeding, remaining significantly greater at all other collection time points compared to day 1 post-cofeeding (Fig. 5). There were no significant differences in mean *Rf17kDa/Cf18S* ratios among recipient fleas collected at different time points (day 1–28 post-cofeeding) (Fig. 5). Total rickettsial infection load was also determined in both *R. felis*-infected donor and recipient fleas by quantifying the copy number of *Rf17kDa* per individual flea lysate. *Rickettsia felis*-infected donor fleas had very low *R. felis* infection load at day 1 (7.8×10^3), increasing significantly on day 7 through day 28 (7.4×10^6 – 1.3×10^7 , respectively). There was no significant difference in *R. felis* infection load among recipient fleas from day 1–28 (6.9×10^2 – 5.6×10^3 , respectively) after cofeeding with the *R. felis*-infected donor fleas.

Vertical transmission of *R. felis* to F₁ fleas was not detected

After *R. felis*-infected donor and uninfected recipient fleas were placed together in different horizontal transmission Groups (I–III) as previously described, female fleas from each

Group laid eggs. Their eggs, not separated from faecal material, from days 3, 7, 14, 21 and 28 were transferred into separate petri dishes containing sand and flea larval diet, and allowed to develop to the F₁ adult stage. A subsample of F₁ adults from each group were tested for *R. felis* infection by qPCR. Of 450 fleas examined, none of F₁ adult fleas were positive for *R. felis*.

Cofeeding and mating contribute to horizontal transmission of *R. felis* by fleas

During the previous rickettsial horizontal transmission bioassay, combining *R. felis*-infected donor and uninfected recipient fleas in the same feeding capsule allowed for both cofeeding and mating to occur. To further define the specific mechanisms of horizontal transmission, the contributions of cofeeding and mating were examined separately (Fig. 1). For cofeeding transmission potential, fleas were exposed to *R. felis* and subsequently exposed to new *R. felis*-free bloodmeal, from which a portion of each new bloodmeal was assessed for *R. felis* infection and offered to *Rickettsia*-uninfected fleas. *Rickettsia felis* DNA was detected in leftover blood at 24 and, again at 48 h post-exposure (3.3×10^3 and 3.0×10^2 rickettsiae per 200 μ L of leftover blood, respectively). Subsequently, 5.8% (7/120) of *Rickettsia*-uninfected fleas that fed on this leftover blood also became *R. felis* positive over 72 h of exposure. The number of *R. felis*-positive recipient females (five fleas) was greater than recipient males (two fleas). However, the mean *Rf*7kDa/*Cf*18S ratio in recipient males (0.38) was greater than females (0.31).

To determine the ability of fleas to transmit *R. felis* during mating, fleas were exposed to *R. felis* and subsequently combined with uninfected recipient fleas in a capsule in the absence of a bloodmeal, again separated out by sex, and 10 fleas each from Group VII (female) and Group VIII (male) were assessed for *R. felis* infection at 12, 36 and 60 h postmating. Over two trials, approximately 3.3% (4/120) of recipient fleas became *R. felis* positive after being combined with *R. felis*-infected donor fleas. Similar to cofeeding, the number of *R. felis*-positive recipient females (three fleas) was greater than males (one flea). Likewise, the mean *Rf*7kDa/*Cf*18S ratio in the recipient male (0.39) was greater than females (0.30).

Discussion

Epidemics of insect-borne rickettsial pathogens such as louse-borne *Rickettsia prowazekii* are typically associated with extreme living conditions including overcrowding, famine and war (Azad 1988). Throughout history, these pathogens have accounted for severe morbidity and mortality (Azad & Beard 1998). In the last 20 years, only one new insect-borne rickettsial pathogen, *R. felis*, has been described. A closer examination of the ecology and epidemiology of *R. felis* is needed to assess the public health importance of this emerging pathogen. Although *R. felis* is associated with a variety of arthropods including several blood-feeding arthropods, for example ticks, mites and multiple species of fleas (Reif & Macaluso 2009), cat fleas are the only known hematophagous vector of *R. felis* (Azad *et al.* 1992; Reif & Macaluso 2009). Interestingly, recent studies have identified and characterized a biological relationship between *R. felis* in non-hematophagous psocids (booklice) in which *R. felis* is a true endosymbiont (Perotti *et al.* 2006; Behar *et al.* 2010; Thepparit *et al.* 2011). The movement of *Rickettsia* species between widely distinct arthropods, conferring differential host-dependent fitness effects, is known to occur (Himler *et al.* 2011; Jiggins & Hurst 2011); thus, the infection of booklice is not unexpected. However, the symbiotic role of *R. felis* in psocids contrasts the horizontal transmission potential established with hematophagous arthropods associated with recent foci of human infection (Socolovsch *et al.* 2010; Williams *et al.* 2011) with clinical symptoms similar to other rickettsial diseases including fever, headache and general morbidity (Reif & Macaluso 2009). Epidemiological studies of this emerging insect-borne rickettsial pathogen have been hindered by the gap in

our understanding of the precise mechanisms of transmission to vertebrates and among invertebrate hosts.

Horizontal transmission of arthropod-borne *Rickettsia* occurs by multiple mechanisms including cofeeding, mating and contact with contaminated faeces (Hayes *et al.* 1980; Azad 1990; Matsumoto *et al.* 2005). In this study, we show that *R. felis* can be transmitted between cat fleas by both cofeeding and mating. Laboratory studies have indicated that *R. felis* is maintained in cat fleas by vertical transmission (transovarial and transstadial transmission). The efficiency of transmission is dependent on several still unknown factors, accounting for varying prevalence in colonized populations ranging from 0% to 100% (Reif & Macaluso 2009). Horizontal and vertical transmission of *R. felis* by wild-caught fleas has not been proven. The potential for horizontal transmission of *R. felis* to vertebrate hosts is supported by the identification of *R. felis* in the salivary glands of cat fleas (Macaluso *et al.* 2008), detection of *R. felis* DNA in the blood of cats exposed to *R. felis*-infected fleas, and subsequent host response to *R. felis* exposure by seroconversion (Wedincamp & Foil 2000). Despite this circumstantial evidence of arthropod vector-to-vertebrate host transmission cycle, transmission of *R. felis* between arthropods had not been demonstrated. This study is the first account of distinct horizontal transmission of *R. felis* between infected and uninfected fleas. The infectious dose used in current study was previously employed to generate an *R. felis*-infected cohort at incidence ranging 58–69% (Reif *et al.* 2011). Cat fleas exposed to *R. felis* via the artificial host system served as donor fleas and able to transmit the agent to uninfected recipient fleas when mixed together. Our results in the cofeeding bioassay demonstrated that *R. felis* was released from infected fleas to the bloodmeal during feeding. Moreover, uninfected fleas also became infected with *R. felis* after feeding on this leftover blood. While transmission of *R. felis* by bite of infected fleas is likely, as *R. felis* has been identified in salivary glands of the cat fleas (Macaluso *et al.* 2008), the kinetics and specific mechanisms of transmission via flea feeding require further investigation.

The previously undefined kinetics of horizontal transmission were examined in the current study demonstrating *R. felis* DNA in both donor and recipient fleas 1 day post-cofeeding in the same feeding capsule, suggesting a rapid expansion of infection to various tissues of the fleas. The transmission of *R. felis* to vertebrate hosts, in the absence of flea faeces, resulting in the induction of an antibody response has been demonstrated (Wedincamp & Foil 2000). Thus, while introduction of *R. felis* to vertebrate hosts via flea feeding has been shown, the acquisition of *R. felis* by naive cofed fleas was not known. The transmission of *R. felis* between arthropods via cofeeding on vertebrate blood has broad implications towards infection and potential transmission by cat fleas and other hematophagous arthropods. Upon exposure, if dissemination and infection of *R. felis* to flea ovaries or salivary glands occur, then cofeeding transmission of *R. felis* could greatly expand the number of potential vectors within foci. Conversely, if *R. felis* infection does not result in subsequent competent vectors, then surveillance of potential vectors of this pathogen should not be based solely upon detection of the rickettsiae in sampled arthropod populations. Over 19 arthropod species have been shown to be positive for *R. felis* DNA via PCR assay (Reif & Macaluso 2009); their role as vectors remains undefined.

Sexual transmission of *Rickettsia* species has been described for other hematophagous arthropods. For example, transmission of pathogenic *Rickettsia rickettsii* from infected male to uninfected female *Ixodes ricinus* ticks has been described (Hayes *et al.* 1980). In the current study in the absence for the potential for cofeeding infection, *R. felis* transmission from donor to recipient fleas at 12 and 36 h postmating was demonstrated. This may account for significant transmission of *R. felis* as cat fleas are known to have multiple mating events (Hsu & Wu 2000). Cat fleas mated on a cat host have significantly longer mating duration time when compared with off-host mating (Hsu & Wu 2001); thus, the observed low

transmission efficiency may be due to other factors including the density of rickettsiae and duration of mating as fleas were mated off-host (in the absence of bloodmeal). Consequently, the low transmission efficiency observed in the current study may explain why previous examination of transmission of *R. felis* by copulation or direct contact including cofeeding between infected and uninfected cat fleas failed to detect horizontal transmission events (Wedincamp & Foil 2002). As discussed previously, studies on the infection of ovaries or salivary glands in female fleas occurring subsequent to venereal transmission will be required to properly assess the importance of this route of horizontal transmission by cat fleas in the epidemiology of *R. felis*.

Even though vertical transmission is thought to be the primary mechanism of maintenance of *R. felis* by colonized cat fleas (Azad *et al.* 1992; Reif & Macaluso 2009), we did not detect vertical transmission of *R. felis* from fleas infected as adults to their respective progeny. As the primary objective of this study was to assess horizontal transmission, only a small proportion of F₁ fleas from the different experimental groups were examined for *R. felis* infection. For example, testing over 450 individuals, F₁ represented only 0.43% of the total F₁ produced; thus, missing a vertical transmission event is possible. A focused study to examine transovarial transmission efficiency by horizontally infected fleas should be conducted to complete the portrait of the *R. felis* transmission cycle.

In summary, this study demonstrates the first evidence of horizontal transmission of *R. felis* between cat fleas; both cofeeding and mating are mechanisms involved in the horizontal transmission of *R. felis*. In combination with known vertical transmission, maintenance and spread of *R. felis* between flea hosts are enhanced by horizontal transmission. The specifics of transmission are essential to understanding the ecology of *R. felis* in nature, and additional studies are needed to further evaluate other potential vectors of *R. felis*, as well as the mechanisms of transmission of *R. felis* to humans.

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References

- Azad, AF. Relationship to vector biology and epidemiology of louse and flea-borne rickettsioses. In: Walker, DH., editor. *Biology of Rickettsial Diseases*. CRC Press; Boca Raton, Florida: 1988. p. 52-62.
- Azad AF. Epidemiology of murine typhus. *Annual Review of Entomology*. 1990; 35:553–569.
- Azad AF, Beard CB. Rickettsial pathogens and their arthropod vectors. *Emerging Infectious Diseases*. 1998; 4:179–186. [PubMed: 9621188]
- Azad AF, Sacci JB Jr, Nelson WM, et al. Genetic characterization and transovarial transmission of a typhus-like rickettsia found in cat fleas. *Proceedings of the National Academy of Sciences, USA*. 1992; 89:43–46.
- Azad AF, Radulovic S, Higgins JA, Noden BH, Troyer JM. Flea-borne rickettsioses: ecologic considerations. *Emerging Infectious Diseases*. 1997; 3:319–327. [PubMed: 9284376]
- Behar A, McCormick LJ, Perlman SJ. *Rickettsia felis* infection in a common household insect pest, *Liposcelis bostrychophila* (Psocoptera: Liposcelidae). *Applied and Environmental Microbiology*. 2010; 76:2280–2285. [PubMed: 20139311]
- Ben-Zvi I, Meltzer E, Nogueras MM, Segura F, Bank I. First detection of human infection with *Rickettsia felis* in Israel. *American Journal of the Medical Sciences*. 2010; 340:343. [PubMed: 20881761]

- Bi-song W, Fang-dong Z, Qi-zhi S, Jing L. Mating behavior of the cat flea, *Ctenocephalides felis* Bouche (Siphonaptera: Pulicidae) and male response to female extract on an artificial feeding system. *Entomologia Sinica*. 2002; 9:29–43.
- Fisher P. Review of using Rhodamine B as a marker for wildlife studies. *Wildlife Society Bulletin*. 1999; 27:318–329.
- Gillespie JJ, Beier MS, Rahman MS, et al. Plasmids and rickettsial evolution: insight from *Rickettsia felis*. *PLoS One*. 2007; 2:e266. [PubMed: 17342200]
- Hayes SF, Burgdorfer W, Aeschlimann A. Sexual transmission of spotted fever group rickettsiae by infected male ticks: detection of *rickettsiae* in immature spermatozoa of *Ixodes ricinus*. *Infection and Immunity*. 1980; 27:638–642. [PubMed: 7380544]
- Higgins JA, Sacci JB Jr, Schriefer ME, Endris RG, Azad AF. Molecular identification of rickettsia-like microorganisms associated with colonized cat fleas (*Ctenocephalides felis*). *Insect Molecular Biology*. 1994; 3:27–33. [PubMed: 8069413]
- Himler AG, Adachi-Hagimori T, Bergen JE, et al. Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. *Science*. 2011; 332:254–256. [PubMed: 21474763]
- Hsu MH, Wu WJ. Effects of multiple mating on female reproductive output in the cat flea (Siphonaptera: Pulicidae). *Journal of Medical Entomology*. 2000; 37:828–834. [PubMed: 11126536]
- Hsu MH, Wu WJ. Off-host observations of mating and postmating behaviors in the cat flea (Siphonaptera: Pulicidae). *Journal of Medical Entomology*. 2001; 38:352–360. [PubMed: 11372958]
- Jiggins FM, Hurst GD. Microbiology. Rapid insect evolution by symbiont transfer. *Science*. 2011; 332:185–186. [PubMed: 21474745]
- Lawrence W, Foil LD. The effects of diet upon pupal development and cocoon formation by the cat flea (Siphonaptera: Pulicidae). *Journal of Vector Ecology*. 2002; 27:39–43. [PubMed: 12125871]
- Lindblom A, Severinson K, Nilsson K. *Rickettsia felis* infection in Sweden: report of two cases with subacute meningitis and review of the literature. *Scandinavian Journal of Infectious Diseases*. 2010; 42:906–909. [PubMed: 20735330]
- Macaluso KR, Pornwiroon W, Popov VL, Foil LD. Identification of *Rickettsia felis* in the salivary glands of cat fleas. *Vector-Borne and Zoonotic Diseases*. 2008; 8:391–396. [PubMed: 18399779]
- Matsumoto K, Ogawa M, Brouqui P, Raoult D, Parola P. Transmission of *Rickettsia massiliae* in the tick, *Rhipicephalus turanicus*. *Medical and Veterinary Entomology*. 2005; 19:263–270. [PubMed: 16134974]
- Niebylski ML, Schrupf ME, Burgdorfer W, et al. *Rickettsia peacockii* sp. nov., a new species infecting wood ticks, *Dermacentor andersoni*, in western Montana. *International Journal of Systematic Bacteriology*. 1997; 47:446–452. [PubMed: 9103635]
- Niebylski ML, Peacock MG, Schwan TG. Lethal effect of *Rickettsia rickettsii* on its tick vector (*Dermacentor andersoni*). *Applied and Environmental Microbiology*. 1999; 65:773–778. [PubMed: 9925615]
- Ogata H, Renesto P, Audic S, et al. The genome sequence of *Rickettsia felis* identifies the first putative conjugative plasmid in an obligate intracellular parasite. *PLoS Biology*. 2005; 3:e248. [PubMed: 15984913]
- Perotti MA, Clarke HK, Turner BD, Braig HR. *Rickettsia* as obligate and mycetomic bacteria. *FASEB Journal*. 2006; 20:2372–2374. [PubMed: 17012243]
- Pornwiroon W, Pourciau SS, Foil LD, Macaluso KR. *Rickettsia felis* from cat fleas: isolation and culture in a tick-derived cell line. *Applied and Environmental Microbiology*. 2006; 72:5589–5595. [PubMed: 16885313]
- Pornwiroon W, Kearney MT, Husseneder C, Foil LD, Macaluso KR. Comparative microbiota of *Rickettsia felis*-uninfected and -infected colonized cat fleas, *Ctenocephalides felis*. *ISME Journal*. 2007; 1:394–402. [PubMed: 18043659]
- Reif KE, Macaluso KR. Ecology of *Rickettsia felis*: a review. *Journal of Medical Entomology*. 2009; 46:723–736. [PubMed: 19645274]

- Reif KE, Stout RW, Henry GC, Foil LD, Macaluso KR. Prevalence and infection load dynamics of *Rickettsia felis* in actively feeding cat fleas. *PLoS One*. 2008; 3:e2805. [PubMed: 18665265]
- Reif KE, Kearney MT, Foil LD, Macaluso KR. Acquisition of *Rickettsia felis* by cat fleas during feeding. *Vector-Borne and Zoonotic Diseases*. 2011; 11:963–968. [PubMed: 21214386]
- Richards AL, Jiang J, Omulo S, et al. Human Infection with *Rickettsia felis*, Kenya. *Emerging Infectious Diseases*. 2010; 16:1081–1086. [PubMed: 20587178]
- Socolovsch C, Mediannikov O, Sokhna C, et al. *Rickettsia felis*-associated uneruptive fever, Senegal. *Emerging Infectious Diseases*. 2010; 16:1140–1142. [PubMed: 20587190]
- Sunyakumthorn P, Bourchookarn A, Pornwiroon W, et al. Characterization and growth of polymorphic *Rickettsia felis* in a tick cell line. *Applied and Environmental Microbiology*. 2008; 74:3151–3158. [PubMed: 18359823]
- Thepparit C, Sunyakumthorn P, Guillotte ML, et al. Isolation of a rickettsial pathogen from a non-hematophagous arthropod. *PLoS One*. 2011; 6:e16396. [PubMed: 21283549]
- Truett GE, Heeger P, Mynatt RL, et al. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and Tris (HotSHOT). *BioTechniques*. 2000; 29:52–54. [PubMed: 10907076]
- Wade SE, Georgi JR. Survival and reproduction of artificially fed cat fleas, *Ctenocephalides felis* Bouche (Siphonaptera: Pulicidae). *Journal of Medical Entomology*. 1988; 25:186–190. [PubMed: 3392715]
- Wedincamp J Jr, Foil LD. Infection and seroconversion of cats exposed to cat fleas (*Ctenocephalides felis* Bouche) infected with *Rickettsia felis*. *Journal of Vector Ecology*. 2000; 25:123–126. [PubMed: 10925806]
- Wedincamp J Jr, Foil LD. Vertical transmission of *Rickettsia felis* in the cat flea (*Ctenocephalides felis* Bouche). *Journal of Vector Ecology*. 2002; 27:96–101. [PubMed: 12125879]
- Werren JH. *Wolbachia* run amok. *Proceedings of the National Academy of Sciences, USA*. 1997; 94:11154–11155.
- Williams SG, Sacci JB Jr, Schrieffer ME, et al. Typhus and typhus like rickettsiae associated with opossums and their fleas in Los Angeles County, California. *Journal of Clinical Microbiology*. 1992; 30:1758–1762. [PubMed: 1629332]
- Williams M, Izzard L, Graves SR, Stenos J, Kelly JJ. First probable Australian cases of human infection with *Rickettsia felis* (cat flea typhus). *Medical Journal of Australia*. 2011; 194:41–43. [PubMed: 21449868]

Fig. 1. Diagrammatic representation of three rickettsial transmission bioassays. (A) The experimental design for rickettsial horizontal transmission bioassay consisted of four groups (I–IV); rickettsial transmission between fleas from *Rickettsia felis*-infected donor fleas, and Rhodamine B-labelled uninfected recipient or uninfected recipient fleas was assessed. (B) The experimental design for the co-feeding bioassay via the bloodmeal from *R. felis*-infected donor to uninfected recipient fleas. (C) Experimental design for the mating bioassay from *R. felis*-infected donor to uninfected recipient fleas.

Fig. 2. Fluorescence microscopy images of adult fleas after feeding on Rhodamine B (RB). A representative control flea, pictured in the top left column, fed on normal bovine blood. Other columns show RB-labelled fleas collected at days 1, 7, 14, 21 and 30 post-feeding on 0.1% Rhodamine B-labelled bloodmeal.

Fig. 3. Immunofluorescence detection of *Rickettsia felis* in donor and recipient fleas. Fleas were exposed to 5×10^9 rickettsiae and infection visualized at day 28 post-cofeeding at (A) 10 $\times$, (B) 40 $\times$ and (C) 100 $\times$. Recipient fleas cofed with *R. felis*-infected donor fleas also became infected as seen at (C) 40 $\times$ and (D) 100 $\times$. The inset rectangle in (A) is a control uninfected flea fed on normal bovine blood.

Fig. 4.

Overall mean *Rickettsia felis* infection density of male and female *R. felis*-infected donor and recipient fleas. Rickettsial densities in male and female *R. felis*-infected donor and recipient fleas in Groups I-III (combined) were assessed by qPCR. Donor female fleas had the greatest density of *R. felis*, compared with donor male fleas and both sexes of recipient fleas. Bars represent means ($\pm$ SEM) of *Rf*17kDa/*Cf*18S ratio; means with different letters are significantly different.

Fig. 5. Mean *Rickettsia felis* infection density of *R. felis*-infected donor and recipient fleas at different time points. Rickettsial densities in *R. felis*-infected donor and recipient fleas collected at days 1, 7, 14, 21 and 28 post-cofeeding were assessed by qPCR. In a temporal fashion, donor fleas typically had greater densities, compared with recipient fleas. Bars represent means ($\pm$ SEM) of *Rf*17kDa/*Cf*18S ratio; means with different letters are significantly different.

Table 1
***Rickettsia felis* infection of *R. felis*-infected donor and recipient fleas in rickettsial horizontal transmission bioassay**

Group	Day 1 (%)	Day 7 (%)	Day 14 (%)	Day 21 (%)	Day 28 (%)
I					
Donor males	10/30 (33.3)	15/30 (50.0)	14/30 (46.7)	13/30 (43.3)	8/30 (26.7)
Recipient females	2/30 (6.7)	1/30 (3.3)	2/30 (6.7)	0/30 (0.0)	2/30 (6.7)
II					
Donor females	22/30 (73.3)	23/30 (76.7)	26/30 (86.7)	22/30 (73.3)	23/30 (76.7)
Recipient males	2/30 (6.7)	4/30 (13.3)	1/30 (3.3)	5/30 (16.7)	12/30 (40.0)
III					
Donor males and females	27/60 (45.0)	34/60 (56.7)	25/60 (41.7)	38/60 (63.3)	38/60 (63.3)
Recipient males and females	4/60 (6.7)	2/60 (3.3)	8/60 (13.3)	5/60 (8.3)	12/60 (20.0)
IV					
Control males	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)
Control females	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)	0/30 (0.0)