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Effect of *Rickettsia felis* Strain Variation on Infection, Transmission, and Fitness in the Cat Flea (Siphonaptera: Pulicidae)

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Abstract

Rickettsia felis is a human pathogen transmitted by the cat flea, *Ctenocephalides felis* (Bouché) (str. LSU), as well as an obligate symbiont of the parthenogenic booklouse *Liposcelis bostrychophila* (Badonnel) (str. LSU-Lb). The influence of genetic variability in these two strains of *R. felis* on host specialization and fitness and possible resulting differences on infection and transmission kinetics in *C. felis* is unknown. Utilizing an artificial host system, cat fleas were exposed to a *R. felis* str. LSU-Lb-infected bloodmeal and monitored for infection at 7-d intervals for 28 d. Quantitative real-time PCR was used to determine rickettsial load and infection density in newly exposed cat fleas, and transmission frequency between cat fleas. The effect of persistent *R. felis* infection on cat flea F_1 progeny was also assessed. At 7 d postexposure 76.7% of the cat fleas successfully acquired *R. felis* str. LSU-Lb. In *R. felis* str. LSU-Lb-exposed cat fleas, the mean infection load (6.15×10^6), infection density (0.76), and infection prevalence (91/114) were significantly greater than *R. felis* str. LSU infection load (3.09×10^6), infection density (0.68), and infection prevalence (76/113). A persistent *R. felis* str. LSU-Lb infection was detected for 28 d in adult cat fleas but neither female:male ratio distortion nor vertical transmission was observed in F_1 progeny. While infection kinetics differed, with higher intensity associated with *R. felis* str. LSU-Lb, no distinct phenotype was observed in the F_1 progeny.

Key words: *Rickettsia felis* strain, *Ctenocephalides felis*, cat flea, *Liposcelis bostrychophila*, infection kinetics

Insect-borne *Rickettsia* encompasses several agents of human disease across the globe, including *Rickettsia felis*, which is recognized as an emerging human pathogen in distinct regions of the world (e.g., sub-Saharan Africa and Southeast Asia; Richards et al. 2010, Socolovschi et al. 2010, Parola 2011, Maina et al. 2012). Originally identified in the cat flea, *Ctenocephalides felis* (Bouché) (Adams et al. 1990), the presence of *R. felis* and related genotypes has been reported from a wide range of arthropod hosts, including at least 40 additional species of fleas, mosquitoes, ticks, and mites (Brown and Macaluso 2016). Although there has been molecular detection in numerous hematophagous arthropods, identification of competent biological transmission vectors has been limited (Dieme et al. 2015, Brown and Macaluso 2016). Consequentially, the occurrence of *R. felis* in most hematophagous arthropods is credited to ingestion of an *R. felis*-infected bloodmeal via cofeeding transmission between infected and naïve vectors (Brown et al. 2015).

In addition to molecular detection in multiple hematophagous hosts, a novel strain of *R. felis* (str. LSU-Lb) has been identified within the non–blood-feeding booklouse, *Liposcelis bostrychophila* (Badonnel) (Insecta: Psocoptera) (Yusuf and Turner 2004, Behar et al. 2010, Thepparit et al. 2011). Although considered a facultative parasite of cat fleas, as an obligate mutualist in the booklouse host *R. felis* is required for the early maturation of the oocyte, maintained 100% transovarially, and is associated with a parthenogenic phenotype (Perotti et al. 2006). Previous studies demonstrated that the loss of *R. felis* from *L. bostrychophila*, either as larvae by increased temperature or as adults via antibiotics, results in diminished longevity and fecundity, as well as the production of nonviable eggs (Yusuf and Turner 2004, Perotti et al. 2006). Currently, no measurable effect on the fitness of fleas infected with *R. felis* has been recognized, and transovarial transmission is inconsistent (ranging from 0 to 100% in laboratory colonies; Higgins et al. 1994,

Hirunkanokpun et al. 2011, Reif et al. 2011). Therefore, heterogeneity within each *R. felis* genotype may influence the transmission routes utilized for sustained infection among distinct arthropod populations.

Several genotypes of *R. felis* have been found in colonized and wild-caught cat fleas and propagated using various cell lines (Horta et al. 2006, Pornwiroon et al. 2006), including the reference strain Marseille-URRWXCal2 derived from fleas of California origin (La Scola et al. 2002). A recent study showed genomic diversity across *R. felis* strains, and identified several factors that differentiate the *R. felis* isolated from cat fleas (str. URRWXCal2 and str. LSU) from that of booklice (str. LSU-Lb; Gillespie et al. 2015). In addition to the pRF plasmid, common to all *R. felis* genomes, *R. felis* str. LSU-Lb also contains the pLbaR plasmid. Furthermore, phylogenomics analysis demonstrated that the booklouse strain of *R. felis* diverged from the flea-associated strains. Despite the recent detailed report of the phylogenetic relationship between strains of *R. felis*, little is known about the biology or transmission phenotype of such strains.

In an effort to conclude if genetic variability in *R. felis* underlies host specialization and fitness, and results in strain-specific infection and transmission kinetics we examined: 1) the capacity of cat fleas to acquire *R. felis* str. LSU-Lb via an infectious bloodmeal; 2) the prevalence and infection load dynamics of *R. felis* str. LSU-Lb in cat fleas; 3) the horizontal transmission of *R. felis* str. LSU-Lb between cat fleas; 4) the vertical transmission of *R. felis* str. LSU-Lb in the cat flea; and 5) the effect of *R. felis* str. LSU-Lb on the development and reproductive fitness of the cat flea.

Materials and Methods

Source of Fleas and *Rickettsia*

Freshly emerged, unfed cat fleas were obtained from Elward II (El-Labs, Soquel, CA). Cat fleas from this colony have previously been described as being negative for *R. felis* (Pornwiroon et al. 2007). Fleas were given a bloodmeal using an artificial dog (Wade and Georgi 1988), and eggs were raised to adults on sand with artificial diet as previously described (Lawrence and Foil 2000). *Rickettsia felis* str. LSU (Pornwiroon et al. 2006) and *R. felis* str. LSU-Lb (Thepparit et al. 2011), were isolated from LSU colonies of cat fleas and booklice (*L. bostrychophila*), respectively, and were grown in ISE6 cells as described previously (Pornwiroon et al. 2006).

Rickettsia felis-Infected Bloodmeal and Flea Infection

Bloodmeals for infection were created by resuspending ISE6 cells infected with *R. felis* str. LSU or *R. felis* str. LSU-Lb. The *R. felis* exposure dose was prepared using the *Bac*Light viability stain kit (Molecular Probes, Carlsbad, CA) to assess viability and enumerate rickettsiae (Sunyakumthorn et al. 2008). For the infection kinetics experiment, concentrations of *R. felis* (passage 6) were adjusted to 5×10^9 rickettsiae in 600 µl of heat-inactivated, defibrinated bovine blood (HemoStat Laboratories, Dixon, CA). For the horizontal transmission experiment, the concentration of *R. felis* str. LSU-Lb (passage 6) was adjusted to 2.5×10^{10} rickettsiae in 600 µl of heat-inactivated, defibrinated bovine blood (Hirunkanokpun et al. 2011).

Sample Preparation and Rickettsial Quantification by PCR

All flea samples were washed with 10% bleach for 5 min, 70% ethanol for 5 min and three times sterile distilled water (5 min). Fleas were then placed in 1.5-ml tubes and crushed with sterile plastic pestles in a liquid nitrogen bath. Extraction of genomic DNA (gDNA) was accomplished using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD) according to the manufacturer's instructions for tissue samples and eluted into 25 µl of UltraPure DNAse/RNAse-free distilled water (dH_2O; Invitrogen, Grand Island, NY). A negative environmental control was utilized for each DNA extraction process.

Fleas were assessed for the presence of *R. felis* str. LSU and *R. felis* str. LSU-Lb by qPCR amplification of a 157-bp portion of the 17-kDa antigen gene (Reif et al. 2008). The absence of rickettsial infection before exposure to *R. felis*, was shown by testing a subset of fleas by qPCR. The qPCR was performed with a LightCycler 480 Real-Time PCR system (Roche, Indianapolis, IN) and *R. felis* infection load was quantified as the copy number of *Rf*17kDa per individual flea lysate. Quantitative PCR analyses used serial 10-fold dilutions of the plasmid pCR4-TOPO-Rf17kda + Cf18SrDNA to generate a standard curve and quantify *R. felis* gene copies for individual flea lysates (Reif et al. 2008). *Rickettsia felis*-infection density was calculated as the ratio of log transformed *Rf*17kDa and log transformed *Cf*18SrDNA copy numbers (*Rf*17kD/*Cf*18SrDNA) per each flea (Reif et al. 2008).

Infection of *R. felis* str. LSU-Lb Through Infectious Blood

In two trials, both containing two experimental groups and one uninfected (control) group, fleas were challenged for 24 h to a *R. felis* str. LSU, *R. felis* str. LSU-Lb, or an uninfected (control) bloodmeal. After exposure to the blood, cat fleas were sustained on defibrinated bovine blood that was replaced every 2–3 d for the entirety of the trial. Fleas were examined every 7 d postexposure (dpe) for acquisition (ability to acquire *R. felis* from an infected bloodmeal), persistence (detection of *R. felis* in fleas over 28 d period), prevalence (no. positive/no. tested), infection load (copy number of Rf17kDa per individual flea lysate), and infection density (ratio of Rf17kDa/Cf18S per flea). At each time point (7, 14, 21, and 28 dpe), gDNA was extracted from 10 female and 5 male viable fleas and *R. felis* infection was tested by qPCR. For each trial the prevalence of *R. felis* infection, *R. felis* infection load per flea lysate, and *R. felis* infection density were compared between treatments at each time point.

Horizontal Transmission of *R. felis* str. LSU-Lb

To distinguish *R. felis*-infected donor and uninfected recipient fleas in the bioassay, rhodamine B (RB) (Sigma-Aldrich, St. Louis, MO) was used as a biomarker (Hirunkanokpun et al. 2011). The donor fleas were fed a *R. felis* str. LSU-Lb infected bloodmeal as described earlier. The RB labeled bloodmeal was created with 500 µl of a 0.1% solution of RB in heat-inactivated bovine serum added to 100 µl of heat-inactivated defibrinated bovine blood and fed to recipient fleas for 24 h, as previously described (Hirunkanokpun et al. 2011). *Rickettsia felis* str. LSU-Lb-infected donor fleas ($n = 70$) were placed in the same cage with RB-labeled uninfected recipient fleas ($n = 70$). Fleas were then fed on normal defibrinated bovine blood and assessed for *R. felis* str. LSU-Lb infection at days 1, 7, 14, and 21 post cofeeding.

Vertical Transmission of *R. felis* str. LSU-Lb to Flea Progeny and F_1 Sex Ratio

In both trials, eggs from fleas exposed to *R. felis* str. LSU and *R. felis* str. LSU Lb-infected bloodmeals were collected every 5 or 10 d post parental bloodmeal contact, and reared to adults in an incubator maintained at 27°C and 90% relative humidity. Adult fleas were

sexed and resulting female-to-male sex ratio determined. Total gDNA was then extracted from individual (LSU $n = 80$, LSU-Lb $n = 79$, Control $n = 15$) or pools of 10 (LSU $n = 1{,}050$, LSU-Lb $n = 840$, Control $n = 30$) F_1 cat fleas and the presence of *R. felis* was determined by qPCR as described previously.

Statistical Analysis

The ratio of *Rf*17kD/*Cf*18SrDNA was assessed after the logarithmic transformation of the quantity of the genes of interest (*Rf*17kD and *Cf*18SrDNA). Data were analyzed with one-way analysis of variance (ANOVA) to examine potential differences between *R. felis* str. LSU and str. LSU-Lb infection load (copy number of Rf17kDa per individual flea lysate), and infection density (ratio of Rf17kDa/Cf18S per flea) over the course of the experiment (IBM SPSS Statistics for Windows, Version 23.0, Armonk, NY). When overall significance was found, Tukey's honestly significant difference (HSD) post hoc test was used to examine pairwise differences of means (Figs. 2 and 3). An independent samples t-test was used for general comparisons of grouped means (Fig. 1). Differences in the proportion (prevalence) of infected fleas in *R. felis* str. LSU vs. *R. felis* str. LSU-Lb (Table 1) and donor vs. recipient fleas (Table 2) were analyzed using Fisher's Exact test (Brown et al. 2015). Differences in sex ratios in the F_1 generations (Table 3) were analyzed using Fisher's exact test (Morick et al. 2013). For all comparisons, a P value of < 0.05 was considered significantly different.

Results

Acquisition of *R. felis* str. LSU-Lb and Infection in Cat Fleas Exposed to an Infectious Bloodmeal

The *C. felis* colony tested negative for *R. felis* as had been shown in previous accounts (Pornwiroon et al. 2007, Reif et al. 2011, Thepparit et al. 2013). In two trials, both groups fed on an infected bloodmeal had positive fleas for 4 wk postexposure, with a 67.3% and 79.8% mean prevalence of infection in *R. felis* str. LSU and *R. felis* str. LSU-Lb, respectively. The total Day 21 infection prevalence for *R. felis* str. LSU-Lb-infected fleas (26/30) was significantly higher than that of *R. felis* str. LSU-infected fleas (14/30). In addition, the overall total infection prevalence for *R. felis* str. LSU-Lb-infected fleas (91/114) was significantly higher than that of *R. felis* str. LSU-infected fleas (76/113) (Table 1). The prevalence of infection ranged from 46.7% to 82.6% and 73.3% to 86.7% within *R. felis* str. LSU- and *R. felis* str. LSU-Lb-exposed fleas, respectively.

The overall mean (±SEM) infection load per flea lysate was significantly higher in *R. felis* str. LSU-Lb-exposed fleas ($6.15 \times 10^6 \pm 7.40 \times 10^5$), compared to fleas exposed to *R. felis* str. LSU ($3.09 \times 10^6 \pm 4.80 \times 10^5$; Fig. 1A). The range of infection load for *R. felis* str. LSU was 2.65×10^2 to 1.73×10^7 rickettsiae per flea lysate. Similarly, the infection load for the *R. felis* str. LSU-Lb-exposed fleas ranged from 2.65×10^2 to 2.92×10^7 rickettsiae per flea lysate. When comparing the density of *R. felis* infection in fleas by calculating the mean (±SEM) *Rf*17kD/*Cf*18S ratio for each collection point, the overall mean ratio was significantly higher in *R. felis* str. LSU-Lb (0.76 ± 0.02) than in *R. felis* str. LSU (0.68 ± 0.02) exposed cat fleas (Fig. 1B). The ranges of the infection density were 0.29 to 0.93 and 0.29 to 0.96 in *R. felis* str. LSU and *R. felis* str. LSU-Lb-exposed fleas, respectively.

Assessing infection dynamics at weekly intervals over the course of infection, significant differences in the mean (±SEM) quantities of *R. felis* str. LSU and *R. felis* str. LSU-Lb at 21 and 28 dpe were observed, with a significant increase in the rickettsial load in *R. felis* str. LSU-Lb-exposed fleas after 14 dpe (Fig. 2A). These differences

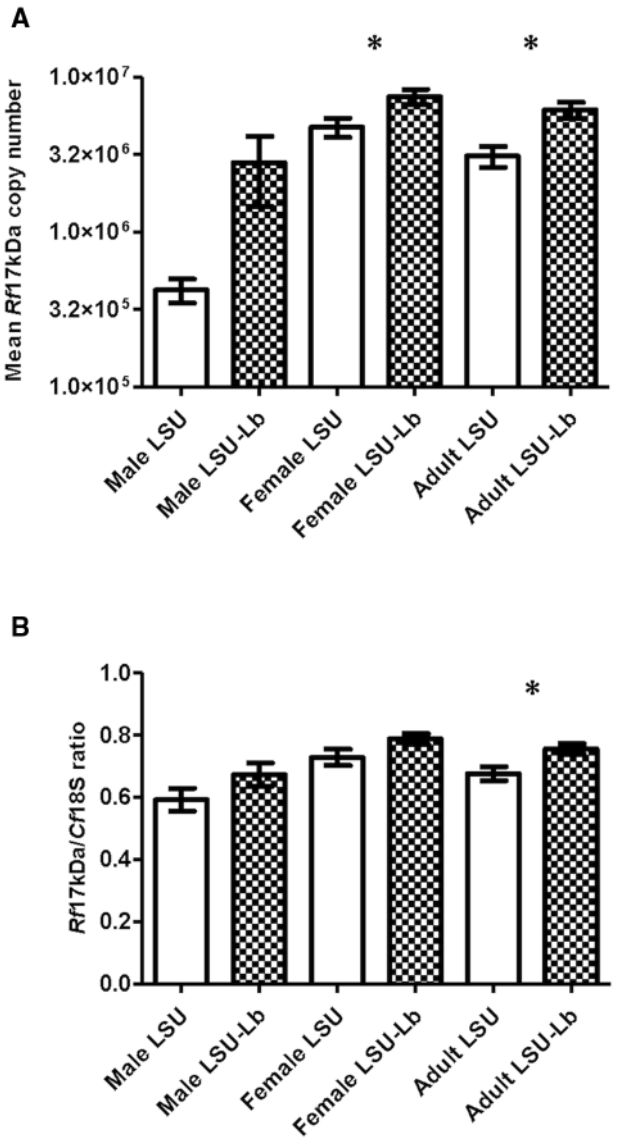

Fig. 1. Overall mean *Rickettsia felis* str. LSU-Lb infection in fleas. (**A**) Overall Rickettsial load in *R. felis* str. LSU Lb-infected fleas compared to *R. felis* str. LSU. (**B**) Overall Rickettsial infection density in *R. felis* str. LSU Lb-infected fleas. Overall *R. felis* str. LSU Lb-infected fleas had significantly greater rickettsial loads (females and mixed sex adults) and densities (mixed sex adults), compared with *R. felis* str. LSU-infected fleas. Bars represent means (±SEM) of *Rf*17kDa copy number and *Rf*17kDa/*Cf*18S ratio. Significant differences ($P < 0.05$) between strains are marked with an asterisk.

were not associated with sex of the host, except for males exposed to *R. felis* str. LSU-Lb at 28 dpe (data not shown). In contrast, the mean infection density of *R. felis* str. LSU and *R. felis* str. LSU-Lb did not significantly differ after 7 dpe (Fig. 2B). The increase in rickettsial density at the 7dpe time point is associated with a significant difference observed in female fleas exposed to *R. felis* str. LSU-Lb (data not shown).

Horizontal Transmission of *R. felis* str. LSU-Lb

Horizontal transmission of *R. felis* str. LSU-Lb to uninfected cat fleas occurred after 1 to 21 d of cofeeding with infected donor cat fleas with a total of 29.7% (19/64) of RB-labeled recipient cat fleas becoming positive for *R. felis* str. LSU-Lb. The total infection prevalence for *R. felis* str. LSU-Lb infected donor fleas (31/36) was significantly

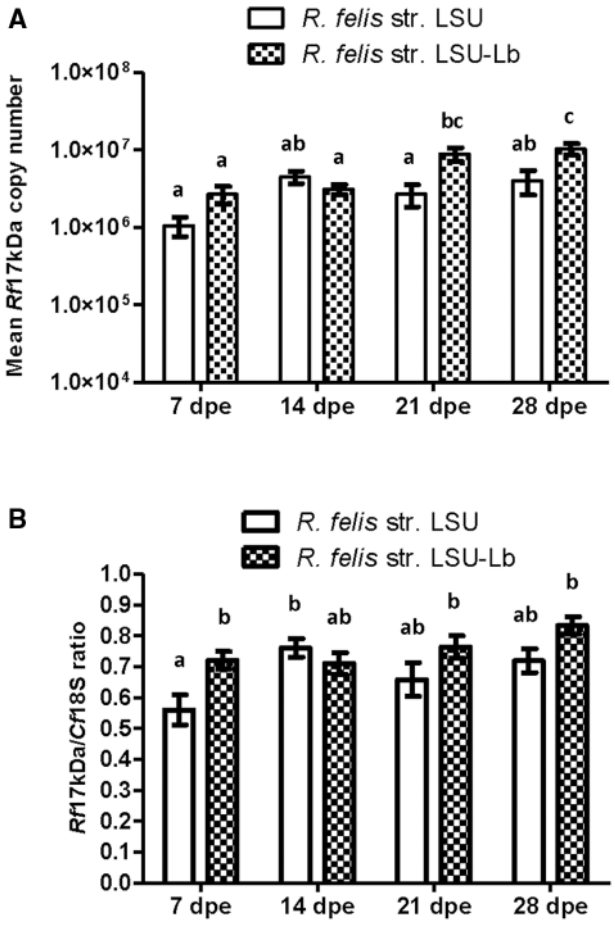

Fig. 2. Rickettsial load and infection density in fleas exposed to a *R. felis* str. LSU- or *R. felis* str. LSU-Lb-infected bloodmeal 7–28 d postexposure (dpe). (**A**) Rickettsial load in adult fleas infected with *R. felis* str. LSU or *R. felis* str. LSU-Lb collected at different time points. (**B**) Rickettsial infection density in adult fleas infected with *R. felis* str. LSU or *R. felis* str. LSU-Lb collected at different time points. Bars represent means (±SEM) of *Rf*17kDa copy number and *Rf*17kDa/*Cf*18S ratio. Means with different letters are significantly different ($P < 0.05$).

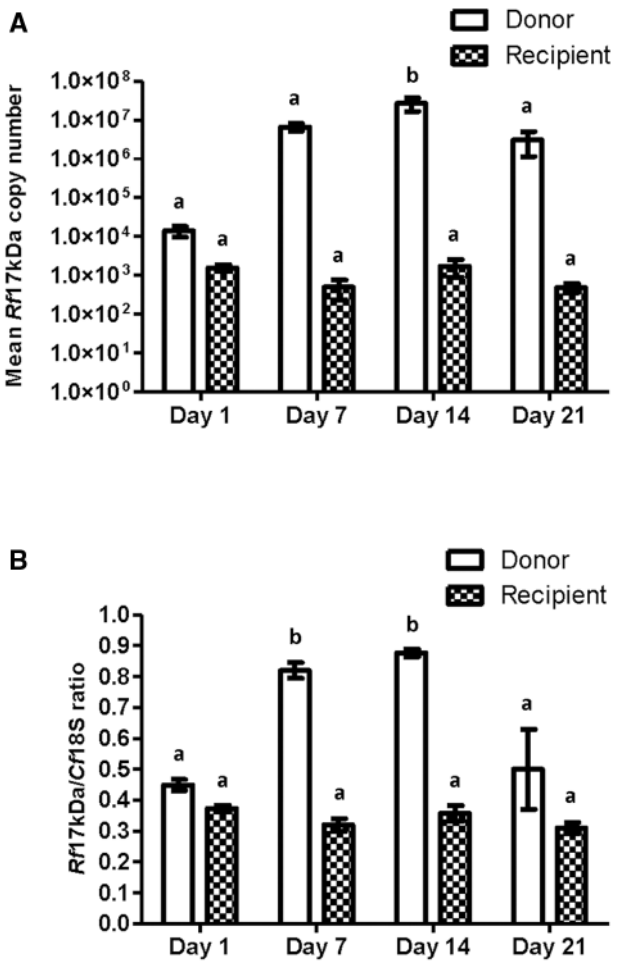

Fig. 3. Mean *Rickettsia felis* str. LSU-Lb infection in donor and recipient fleas at different time points. (**A**) Rickettsial load in *R. felis* str. LSU Lb-infected donor and recipient fleas collected at days 1, 7, 14, and 21 post cofeeding. (**B**) Rickettsial infection density in *R. felis* str. LSU Lb-infected donor and recipient fleas collected at days 1, 7, 14, and 21 post cofeeding. Donor fleas typically had greater rickettsial loads and densities, compared with recipient fleas. Bars represent means (±SEM) of *Rf*17kDa copy number and *Rf*17kDa/*Cf*18S ratio. Means with different letters are significantly different ($P < 0.05$).

higher than that of recipient fleas (19/64) (Table 2). The mean (±SEM) rickettsial load for *R. felis* str. LSU-Lb-infected donor fleas ($3.76 \times 10^6 \pm 1.69 \times 10^6$) was significantly higher than that of infected recipient fleas ($1.17 \times 10^3 \pm 2.94 \times 10^2$). Likewise, the mean (±SEM) infection density (*Rf*17kDa/*Cf*18S ratio) for *R. felis* str. LSU-Lb infected donor fleas (.54 ± .04%) was significantly higher than that of infected recipient fleas (.34 ± .01%). The mean (±SEM) load of *R. felis str.* LSU-Lb infected donor fleas on day 14 ($2.69 \times 10^7 \pm 1.03 \times 10^7$) was significantly higher than infected recipient cat fleas at all time points and donor fleas at days 1, 7, and 21 (Fig. 3A). The mean (±SEM) infection density of *R. felis str.* LSU-Lb infected donor fleas on day 7 (.82 ± .03) and day 14 (.88 ± .01) were significantly higher than *R. felis str.* LSU-Lb infected recipient fleas at all time points and other donor fleas on days 1 and 21 (Fig. 3B).

Vertical Transmission of *R. felis* str. LSU-Lb to Flea Progeny and F_1 Sex Ratio

Eggs from cat fleas ($n = 200$) exposed to *R. felis* str. LSU and *R. felis* str. LSU Lb-infected bloodmeals were collected every 5 or 10 d post parental bloodmeal exposure, and reared to adults. The *R. felis* str. LSU-infected fleas produced 542 females and 512 males ($n = 1{,}054$). The *R. felis* str. LSU-Lb-infected fleas produced 416 females and 432 males ($n = 848$). The uninfected fleas produced 514 females and 502 males ($n = 1{,}016$). The difference between the F_1 sex ratios in the *R. felis* str. LSU-infected fleas and the *R. felis* str. LSU-Lb-infected fleas was not significant. Additionally, neither of the F_1 sex ratios in the *R. felis* str. LSU-infected fleas and the *R. felis* str. LSU-Lb-infected fleas were significantly different from the uninfected flea sex ratio (Table 3). Thus, *R. felis* str. LSU-Lb did not influence F_1 sex determination as it does in *L. bostrychophila* (Yusuf and Turner 2004).

Total gDNA was then extracted from individual (LSU $n = 80$, LSU-Lb $n = 79$, Control $n = 15$) or pools of 10 (LSU $n = 1{,}050$, LSU-Lb $n = 840$, Control $n = 30$) F_1 cat fleas and the presence of *R. felis* was assessed by qPCR. Despite *R. felis* str. LSU and *R. felis* str. LSU-Lb infections in adults, infection from either genotype was not detected in any F_1 progeny.

Discussion

Several genotypes of *R. felis* have been isolated from colonized and wild-caught arthropods, with unique genotypes for specific host

Table 1. Prevalence (no. positive/no. tested) of *R. felis* str. LSU and *R. felis* str. LSU-Lb in cat fleas exposed to an infected bloodmeal

Fleas	Day 7 (%)		Day 14 (%)		Day 21 (%)		Day 28 (%)		All Days (%)	
	R. felis str. LSU	*R. felis* str. LSU-Lb	*R. felis* str. LSU	*R. felis* str. LSU-Lb	*R. felis* str. LSU	*R. felis* str. LSU-Lb	*R. felis* str. LSU	*R. felis* str. LSU-Lb	*R. felis* str. LSU	*R. felis* str. LSU-Lb
Female	13/20 (65.0)	16/20 (80.0)	15/20 (75.0)	17/20 (85.0)	7/20 (35.0)	19/20 (95.0)	12/13 (92.3)	13/14 (92.9)	47/73 (64.4)	65/74 (87.8)
Male	8/10 (80.0)	7/10 (70.0)	7/10 (70.0)	5/10 (50.0)	7/10 (70.0)	7/10 (70.0)	7/10 (70.0)	7/10 (70.0)	29/40 (72.5)	26/40 (65.0)
Total	21/30 (70.0)	23/30 (76.7)	22/30 (73.3)	22/30 (73.3)	14/30* (46.7)	26/30* (86.7)	19/23 (82.6)	20/24 (83.3)	76/113* (67.3)	91/114* (79.8)

*A significant difference was detected in the total prevalence between strains.

Table 2. Donor and recipient fleas (no. positive/no. tested) infected with *R. felis* str. LSU-Lb in horizontal transmission bioassay

Fleas	Day 1 (%)	Day 7 (%)	Day 14 (%)	Day 21 (%)	All days (%)
Donor	20/21 (95.2)	3/5 (60.0)	3/5 (60.0)	5/5 (100.0)	31/36* (86.1)
Recipient	5/21 (23.8)	3/15 (20.0)	6/14 (42.9)	5/14 (35.7)	19/64* (29.7)

*A significant difference was observed in the prevalence between recipient and donor fleas.

populations (Gillespie et al. 2015, Brown and Macaluso 2016). Aside from the number of plasmids, there is minimal genomic divergence between *R. felis* str. LSU (isolated from a cat flea colony) and *R. felis* str. LSU-Lb (isolated from a booklice colony; Gillespie et al. 2015). Because of the niche overlap of *C. felis* and *L. bostrychophila*, it is hypothesized that the host-specific strain of *R. felis* from the nonhematophagous booklouse arose via the lice feeding on infectious flea detritus (Gillespie et al. 2015). The capability of *R. felis* str. LSU-Lb to infect vertebrates is unknown, but rickettsial DNA has been detected in dust samples (containing *L. bostrychophila* and other dust-associated arthropods) collected from the beds of *R. felis*-infected patients in Senegal (Mediannikov et al. 2014). While not confirmed, it is postulated that these individuals may have acquired *R. felis* by inhalation or dermal inoculation of booklouse feces (Parola et al. 2015). Therefore, although *R. felis* str. LSU-Lb is considered an obligate mutualist of *L. bostrychophila*, certain qualities suggest that the booklouse strain has the potential to be a human pathogen. As such, it is critical to determine whether *R. felis* str. LSU-Lb can infect cat fleas, which would provide an additional route for human exposure. To determine whether vector competence varies as a function of the genetic characteristics of the infecting strain, we aimed to determine if cat fleas could acquire, maintain, and transmit *R. felis* str. LSU-Lb.

In principle, the normal feeding behavior of cat fleas may lead to the acquisition of any number of microorganisms present in the bloodstream of a vertebrate host (Mehlhorn 2012); however, the rapid turnover of midgut contents in actively feeding cat fleas generally disrupts progression to an established microbial infection (Bland and Hinnebusch 2016). For example, cat fleas are competent vectors for the plague bacterium (*Yersinia pestis*), but the frequency with which cat fleas feed results in bacterial clearance and disruption of biofilm accumulation (Bland and Hinnebusch 2016). As cat fleas are not associated with a diverse range of other rickettsial species, and transmission biology for *R. felis* genotypes in cat fleas has not been assessed, the present study demonstrated acquisition and maintenance of *R. felis* str. LSU-Lb by cat fleas after feeding on an infectious bloodmeal. Molecular analyses revealed that more than 75% of the cat fleas exposed to *R. felis* str. LSU-Lb were positive for rickettsial DNA after 1 wk, and more than 80% of the cat fleas were infected at 4 wk post exposure. Interestingly, the overall mean infection load and density, as well as infection prevalence, of *R. felis* str. LSU-Lb within cat fleas was significantly higher when compared to the flea-associated strain of *R. felis*. This observation perhaps highlights the underlying genetic factors that differentiate infecting strains of *R. felis* as facultative parasites of fleas from obligate mutualists of booklice. Indeed, short-term infection of cat fleas by either strain of *R. felis* demonstrates a distinct transcriptional profile compared to culture conditions, suggesting that vector adaptation occurs (Verhoeve et al. 2016). Although less studied in rickettsial species, the efficiency of vector infection is known to differ among viral strains within a genotype (e.g., dengue virus), which often results in variable transmission phenotypes (Armstrong and Rico-Hesse 2003). The selective factors that induce host-specific genetic variability of *R. felis*, including *R. felis* interaction with host microbiota, require further study.

Acquisition of a pathogen by an arthropod is not sufficient alone to confirm its transmission potential to other hosts. Given that rickettsial transmission by arthropods can be vertical or horizontal, bioassays were generated to examine the transmission of *R. felis* str. LSU-Lb from adult cat fleas to their progeny (vertical), as well as transmission between infected and uninfected cofeeding cat fleas (horizontal). Similar to recent experiments with *R. felis* str. LSU (Hirunkanokpun et al. 2011, Reif et al. 2011), vertical transmission of *R. felis* str. LSU-Lb was not observed in cat fleas, nor was there an impact of *R. felis* str. LSU-Lb infection on the development or reproductive fitness of cat fleas. Additional studies are required to characterize the dissemination of horizontally acquired *R. felis* str. LSU-Lb in cat fleas and other factors that might influence vertical transmission to progeny. Remarkably, using comparable experimental parameters as with *R. felis* str. LSU (Hirunkanokpun et al. 2011, Brown et al. 2015), horizontal transmission of *R. felis* str. LSU-Lb was observed between cofeeding cat fleas in an artificial host system. Rickettsial DNA was detected in both donor and recipient fleas 1-day post cofeeding on a shared bloodmeal, and transmission continued over the course of 21 d. Regardless of whether infection load was quantified as total rickettsiae per flea or as a ratio of *R. felis* to *C. felis* genes, rickettsial load was significantly decreased between donor and recipient fleas. In addition, infection prevalence was also significantly higher in donor fleas than in recipient fleas, similar to previous work with *R. felis* str. LSU (Hirunkanokpun et al. 2011, Brown et al. 2015). The transmission of *R. felis* str. LSU-Lb between cat fleas via cofeeding on vertebrate blood has broad implications toward the potential exposure risk for humans. The ability of fleas to transmit different genotypes may contribute to the high proportion of *R. felis* infections detected from patients and vertebrate hosts (Maina et al. 2016).

Table 3. Sex ratio (male: female) of F_1 adult fleas (no. males/no. females)

Group	Sex ratio m:f (n male/n female)						
	Days eggs collected post infection						
	1–5	6–10	11–15	16–20	21–30	31–40	Total
R. felis str. LSU	1:1.05 (20/21)	1:1.07 (196/209)	1:1.06 (165/175)	1:1.06 (50/63)	1:0.92 (75/69)	1:0.83 (6/5)	1:1.06 (512/542)
R. felis str. LSU-Lb	1:2.78 (9/25)	1:0.89 (130/115)	1:0.75 (166/124)	1:1.56 (48/75)	1:0.96 (73/70)	1:1.17 (6/7)	1:0.96 (416/432)
Uninfected	1:0.78 (9/7)	1:1.13 (192/216)	1:1.02 (127/129)	1:1.01 (102/103)	1:0.82 (62/51)	1:0.73 (11/8)	1:1.02 (502/514)

Although these *R. felis* isolates possess unique genotypes, there were no profound consequences for their subsequent infection of and transmission by the cat flea vector. Intriguingly, *R. felis* str. LSU-Lb actually infected cat fleas more efficiently despite adaptation in the booklouse host. Rickettsial strain variation in infection density is consistent with the previous observation of higher density of *R. felis* str. LSU-Lb, compared to strain LSU, in booklouse and flea hosts, respectively (Thepparit et al. 2011). However, the higher load did not result in a distinct flea biological phenotype, as seen with *R. felis* str. LSU-Lb infection of booklice. The results of the current study suggest that although genetic variability determines *R. felis* host specialization, cat fleas maintain a universal ability to acquire and transmit horizontally multiple genotypes of *R. felis*. Additional flea transmission studies examining novel *R. felis* isolates are needed to elucidate the role of other *R. felis* genotypes in the epidemiology of flea-borne spotted fever.

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