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Research Article

Acquisition of *Bartonella elizabethae* by Experimentally Exposed Oriental Rat Fleas (*Xenopsylla cheopis*; Siphonaptera, Pulicidae) and Excretion of Bartonella DNA in Flea Feces

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Abstract

Few studies have been able to provide experimental evidence of the ability of fleas to maintain rodent-associated *Bartonella* infections and excrete these bacteria. These data are important for understanding the transmission cycles and prevalence of these bacteria in hosts and vectors. We used an artificial feeding approach to expose groups of the oriental rat flea (*Xenopsylla cheopis* Rothschild; Siphonaptera, Pulicidae) to rat blood inoculated with varying concentrations of *Bartonella elizabethae* Daly (Bartonellaceae: Rhizobiales). Flea populations were maintained by membrane feeding on pathogen-free bloodmeals for up to 13 d post infection. Individual fleas and pools of flea feces were tested for the presence of *Bartonella* DNA using molecular methods (quantitative and conventional polymerase chain reaction [PCR]). The threshold number of *Bartonellae* required in the infectious bloodmeal for fleas to be detected as positive was 10^6 colony-forming units per milliliter (CFU/ml). Individual fleas were capable of harboring infections for at least 13 d post infection and continuously excreted *Bartonella* DNA in their feces over the same period. This experiment demonstrated that *X. cheopis* are capable of acquiring and excreting *B. elizabethae* over several days. These results will guide future work to model and understand the role of *X. cheopis* in the natural transmission cycle of rodent-borne *Bartonella* species. Future experiments using this artificial feeding approach will be useful for examining the horizontal transmission of *B. elizabethae* or other rodent-associated *Bartonella* species to naïve hosts and for determining the viability of excreted bacteria.

Key words: artificial feeding, flea, rat, *Bartonella*, transmission

Bartonella spp. are bacteria that commonly infect mammals in nature and may establish chronic infections in hosts (Harms and Dehio 2012). At least 30 *Bartonella* species have been described from mammalian hosts including carnivores, ungulates, rodents, bats, shrews, and other taxa. Despite recognized patterns of host specificity at various taxonomic levels, large gaps remain in our knowledge of how most of these *Bartonella* species are transmitted and maintained in host populations. A majority of described *Bartonella* species are found in rodents and some rodent-associated *Bartonella* species are recognized as emerging pathogens of humans and domestic animals, including *Bartonella doshiae*, *Bartonella elizabethae*, *Bartonella grahamii*, *Bartonella tribocorum*, *Bartonella vinsonii* subsp. *arupensis*, and *Bartonella washoensis* (Comer et al. 2001, Kosoy et al. 2003, Iralu et al. 2006, Bai et al. 2012, Buffet et al. 2013, Vayssier-Taussat et al. 2016). In the interest of protecting public health, we must understand more about the transmission of these bacteria in nature to quantify and mitigate the risk of spillover at the human–wildlife interface (Lloyd-Smith et al. 2009).

Flea-borne transmission has been suggested based on widespread presence of rodent-associated *Bartonella* species in fleas (Bown et al. 2004, Billeter et al. 2008, Gutiérrez et al. 2015, Withenshaw et al. 2016); however, other vectors may include ticks, lice, and mites (Billeter et al. 2008). Extensive experimental work has demonstrated vector competence of sand flies for *Bartonella bacilliformis* (Battistini 1929, 1931; Battisti et al. 2015), human body lice for *Bartonella quintana* (Swift 1920, Bruce 1921, Fournier et al. 2001), and cat fleas for *Bartonella henselae* (Chomel et al. 1996, Higgins et al. 1996, Finkelstein et al. 2002, Bouhsira et al. 2013b). Similar experiments have only been performed for a small number of rodent-associated *Bartonella* species and the important pathways of transmission between individual hosts and among host species remain unclear (Gutiérrez et al. 2015).

Krampitz (1962) showed that the oriental rat flea (*Xenopsylla cheopis*) was capable of transmitting an unidentified *Bartonella* species among bank voles (*Clethrionomys glareolus*). Bown et al. (2004) used a semi-natural experiment to demonstrate transmission of *B. taylorii* and *B. grahamii* to bank voles by the rodent flea *Ctenophthalmus nobilis*. The authors found no evidence of vertical transmission between parents and offspring or horizontal transmission between mating bank vole pairs in the absence of fleas (Bown et al. 2004).

Morick et al. (2013a,b,c) have been successful in replicating a natural rodent-associated *Bartonella* system in the laboratory. Their system, involving wild-caught Sundevall's jirds (*Meriones crassus*) and their naturally occurring flea species (*Xenopsylla ramesis*) and *Bartonella* strains (strain OE 1-1 closely related to *B. elizabethae*), has been used to demonstrate successful transmission of bartonellae from infected jirds to naïve fleas (Morick et al. 2011), then from infected fleas to naïve mice, which became persistently infected (Morick et al. 2013a). Additionally, the authors found some evidence of vertical transmission from infected female rodents to their offspring (Morick et al. 2013c), a result observed in other rodent-associated *Bartonella* systems (Kosoy et al. 1998, Boulouis et al. 2001), and nontransovarial transmission of bartonellae to larval fleas through infectious feces or gut voids (regurgitations of digested blood; Morick et al. 2013c), which has been documented in other *Bartonella* vectors (de Bruin et al. 2015, Leulmi et al. 2015). Therefore, multiple transmission pathways might be used in rodent-associated *Bartonella* systems.

We propose to evaluate the potential of the oriental rat flea (*X. cheopis* Rothschild; Siphonaptera, Pulicidae), which commonly infests rodents in nature (Ibrahim et al. 2006), to acquire the rodent-associated *B. elizabethae* Daly (Bartonellaceae:Rhizobiales) using an artificial blood feeding system and excrete these bacteria in their feces at different intervals postinfection. *B. elizabethae* is primarily associated with rats and mice and is a known human pathogen, causing symptoms ranging from fever to endocarditis (Daly et al. 1993, Comer et al. 2001, Kosoy et al. 2010). Our choice of the oriental rat flea is appropriate for this evaluation, as it has been shown to readily feed on humans. Studies in several countries have detected rodent-associated *Bartonella* species, including *B. elizabethae*, in *X. cheopis* populations feeding on wild rodents (Winoto et al. 2005, Reeves et al. 2007, Harrus et al. 2009, Morick et al. 2009, Bitam et al. 2012, Billeter et al. 2013, Kamani et al. 2013, Leulmi et al. 2014). Establishing that *X. cheopis* can acquire and excrete *Bartonellae* in their feces will provide further indication that they are possible vectors of these bacteria in rodents. In this case, transmission could occur through the contamination of the host's skin with infectious feces as is known to occur for flea-borne typhus (Azad 1990).

For this experiment, we fed naïve fleas with rat blood inoculated through a mouse skin membrane system containing *Bartonellae* at varying concentrations and then sampled these fleas for up to 13 d post infection. We hypothesized that 1) there would be a threshold concentration of bacteria necessary for fleas to be detected as positive, 2) fleas would only test positive until 11 d post infection, as seen in previous experimental infections of cat fleas (Kernif et al. 2014), and 3) fleas will continuously excrete *Bartonellae* in their feces. Our results will provide important information about the natural dynamics of *Bartonellae* in *X. cheopis*, investigate the likelihood of *Bartonella* transmission to hosts through contact with infectious flea feces, and guide future laboratory experiments focused on the ability of *X. cheopis* to transmit *Bartonellae* to rodents.

Materials and Methods

Artificial Flea Feeding

In total, 4,188 *X. cheopis* were taken from the CDC flea colony for this experiment. All fleas were taken from jars that were not currently being fed maintenance bloodmeals to ensure bacteria were not introduced, through feeding on live mice, between the prescreening tests and experimental use. Prior to the experiment and beyond the 4,188 *X. cheopis*, another initial sample of 240 fleas from jars were tested for the presence of *Bartonella* DNA; all were negative. Fleas for the experiment were separated into four treatment groups: a control group to be fed on pathogen-free, defibrinated rat blood (Bioreclamation IVT, Westbury, NY), a low concentration group, a medium concentration group, and a high concentration group. The experimental concentrations were on average 1.06×10^6, 5.94×10^7, and 1.24×10^9 colony-forming units per milliliter (CFU/ml) for all feedings for the low group, the medium group, and the high group, respectively. The experimental treatments were made by diluting a stock culture of *Bartonella* at a concentration of 1×10^{10} CFU/ml to the appropriate treatment concentration and inoculating 10 ml of pathogen-free, defibrinated rat blood (Bioreclamation IVT) with the corresponding treatment stock. The *Bartonella* strain used for all experiments was the type strain of *B. elizabethae* (strain F9251, ATCC 49927) originally isolated from a human patient (Daly et al. 1993).

Feeding was performed using Hemotek 6W1 artificial feeders (Hemotek Ltd., Blackburn, United Kingdom). Due to the idiosyncratic feeding behavior of *X. cheopis*, skin from a hairless mouse (strain SKH1, Charles River Laboratories, Inc., Wilmington, MA) was used instead of an artificial membrane to cover the blood reservoir. The mouse skins were harvested from mice sacrificed during regular maintenance feeding of the CDC flea colony and kept at −20°C until needed. Each group of fleas was allowed to feed on rat blood at the respective control or treatment concentrations for 1 h. Out of the initial 4,188 fleas, 969 fleas were exposed to pathogen-free blood, 924 fleas were exposed to the low *Bartonella* concentration, 1,018 to the medium concentration, and 1,277 to the high concentration during the first infectious feeding. Accounting for expected feed rates, flea mortality over the study period, and our sampling design for each timepoint, we estimated that we would need approximately 840 fleas per group. Any variation in flea number among groups beyond this number was due to differences in flea counting rate among the investigators and the inexact partitioning of fleas into groups during the sampling of fleas from the colony. We only kept fleas that took a bloodmeal during the first feeding to ensure that all fleas in the experiment had at least one exposure to the treatment concentration. This left 498 fleas (51.5%) from the control group, 550 (59.6%) from the low concentration group, 889 (88.4%) from the medium concentration group, and 654 (52.3%) from the high concentration group to be used during the rest of the experiment. The feeding process was repeated two more times, 3 and 7 d after the initial exposure.

After infection, all fleas were maintenance-fed three times on pathogen-free rat blood over a period of 13 d post infection (DPI; after the last infectious feed). Maintenance feedings took place at 3, 6, and 10 DPI. Fleas were partitioned into 12 separate conical tubes (three per group) to moderate flea densities. Aeration was provided by making holes in the tube caps using a sterile 26 gauge needle. A piece of damp filter paper was placed inside to maintain humidity and to provide substrate on which fleas could climb. All conical tubes were stored in a vented polycarbonate box at ~37°C and ~80% RH. Fleas still alive on each sampling day were placed into fresh conical tubes. Approximately 30 fleas from the control group and each treatment group were removed at 0, 5, 7, 9, 11, and 13 DPI for testing. Used tubes containing feces from each timepoint were also stored for later testing. The last sampling point (13 DPI) had variable sample sizes across treatments due to differences in

the size of initial flea groups and flea mortality over the six total feedings.

DNA Extraction and Molecular Detection in Fleas

Attempts to culture *Bartonellae* on agar from fleas or flea feces using previously published techniques (Billeter et al. 2012) were unsuccessful due to the overgrowth of *Bartonellae* by other bacteria and fungi. It is possible that some of these contaminating bacteria are associated with the flea gut. Thus, we decided to detect *B. elizabethae*-infected fleas entirely by molecular means (polymerase chain reaction [PCR] and quantitative real-time PCR [qPCR]).

A standard curve for qPCR was prepared by mixing one part brain heart infusion (BHI) broth with homogenized material from a single, negative flea with one part *Bartonella* culture (*B. elizabethae*) at a concentration of 1×10^{10} CFU/ml. This stock was then serially diluted 1:10 with BHI broth. DNA was extracted from dilutions using the QIAamp Blood Mini Kit and protocol (Qiagen, Valencia, CA). Sampled fleas were placed in individual 1.5 ml microcentrifuge tubes along with 4–6 glass beads (1.5 mm diameter) and 200 µl BHI broth and homogenized for 3 min at 12,000 rpm. The homogenate was then centrifuged for 3 min at 16,000 rpm and transferred into a clean 1.5 ml tube. Samples were then incubated at 95°C for 10 min to extract genomic DNA. Samples in BHI were then diluted 1:10 in AE extraction buffer (Qiagen) due to observed amplification interference in downstream qPCR and conventional PCR assays.

qPCR on flea samples and the standard dilutions was performed targeting the transfer messenger RNA (*ssrA*) gene using primers specific to the genus *Bartonella*: *ssrA*-F, 5′-GCTATGGTAATAAATGGACAATGAAATAA-3′ and *ssrA*-R, 5′-GCTTCTGTTGCCAGGTG-3′ (Diaz et al. 2012). All qPCR reactions were performed using a Bio-Rad C1000 thermocycler with the CFX96 Real-time System (Bio-Rad, Hercules, CA). For the standard curve, dilutions were tested in triplicate. Based on the qPCR results from these dilutions, we generated a linear model for the estimation of the number of DNA copies in experimental samples. The regression formula relating the cycle threshold (Ct) number to the log copy number was log (Copies) = $-0.2881 \times Ct + 12.815$ ($R^2 = 0.9949$).

Quantitative PCR results were confirmed using conventional PCR and sequencing of the 16S-23S intergenic spacer region (ITS) on all samples (Diniz et al. 2007). We used this target instead of the more commonly used citrate synthase (*gltA*) gene because ITS is more sensitive for *Bartonella* detection than *gltA* based on our experience and as demonstrated in previous studies (Kosoy et al. 2017). Conventional PCR reactions were performed using a Bio-Rad C1000 thermocycler (Bio-Rad). Amplification products were separated and visualized by 1.5% agarose gel electrophoresis with Biotium GelGreen stain (Biotium, Hayward, CA). For both qPCR and conventional PCR assays, positive (*B. doshiae*) and negative (RNase-free water) controls were included to verify the success of the protocol and contamination of reagents, respectively. Samples were only considered positive if both qPCR and conventional PCR tests yielded positive results with sequences matching *B. elizabethae*.

DNA Extraction and Molecular Detection in Flea Feces

A standard curve for qPCR was prepared by mixing one part BHI broth with homogenized feces taken from fleas fed uninoculated rat blood from a previous pilot study and one part *Bartonella* culture (*B. elizabethae*) at a concentration of 1×10^{10} CFU/ml. This stock was then serially diluted 1:10 with BHI broth. DNA was extracted from dilutions using a simple heat extraction (incubation at 95°C for 10 min). Tubes in which fleas were stored between feedings and after infection were retained after each sampling step when switched for clean tubes. These tubes were rinsed with 1,000 µl each with BHI broth and a flat cell scraper was used to clean any adhered fecal material from the sides of the tube. Feces samples were then incubated at 95°C for 10 min to extract DNA and diluted 1:10 in extraction buffer (Qiagen) as for fleas. qPCR on flea feces and the standard dilutions was performed using the same protocol as for fleas (*ssrA*) (Diaz et al. 2012). Standard curve dilutions were tested in triplicate and the resulting regression formula was log (Copies) = $-0.305 \times Ct + 13.13$ ($R^2 = 0.9931$). All fecal pools were tested with qPCR-targeting *ssrA* and conventional PCR-targeting ITS in triplicate.

Statistical Analysis

We calculated differences in the prevalence of *Bartonella* infection in fleas between treatment groups using two-sided chi-squared tests of proportions. We examined temporal trends in *Bartonella* prevalence in fleas using linear regression over the sampled timepoints. We assessed the significance of linear regressions using *F*-tests. Differences between infection levels in fleas and flea feces for different treatment groups as measured by qPCR copy number were evaluated using two-sided Welch's *t*-tests assuming unequal variance. Temporal trends in qPCR copy number in fleas and flea feces across the treatment groups were also examined via linear regression and *F*-tests. A figure depicting *Bartonella* prevalence was drawn using Clopper–Pearson exact binomial confidence intervals at each sampling timepoint (Clopper and Pearson 1934). Figures showing copy numbers from qPCR assays on fleas and flea feces were drawn with lines for the calculated mean log copy number for each treatment group and each sampling timepoint. All statistical analysis and plotting were performed in R (R Core Team 2018).

Results

Molecular Detection in Fleas

All control fleas that were fed pathogen-free blood tested negative. A single flea from low exposure group (1.06×10^6 CFU/ml) tested positive (Fig. 1). The single flea from the low group tested positive at 5 DPI with a Ct value of 42.83, corresponding to ~3 copies of the *Bartonella ssrA* gene in the 5 µl of sample DNA. Fleas in the groups fed 5.94×10^7 and 1.24×10^9 CFU/ml (medium and high group, respectively) were all positive on the day of the last infectious feed (0 DPI), but prevalence declined significantly to 60% ($\chi^2 = 12.6$; df = 1; $P < 0.001$) in the medium group and 67% ($\chi^2 = 9.72$; df = 1; $P = 0.0018$) in the high group by 5 DPI (Fig. 1a). Infection prevalence varied over 7–13 DPI between 29 and 42% in the medium group and between 30 and 49% for the high group; however, there was no evidence of a linear decline in prevalence from 5 to 13 DPI in either group (medium group: $F = 2.59$; df = 1, 3; $P = 0.21$; high group: $F = 0.54$; df = 1, 3; $P = 0.52$) and prevalence did not significantly differ between medium and high groups at any timepoint from 0 to 13 DPI (Fig. 1a).

Infection levels based on qPCR varied between exposure groups over time (Fig. 1b). Infection levels based on *ssrA* copies were greater for the high group than the medium group at 0 DPI ($t = 15.34$; df = 57.01; $P < 0.001$). The groups had statistically equivalent distributions of *ssrA* copies between 5 and 11 DPI, but the medium group had higher infection levels than the high group at the last timepoint ($t = 3.5$; df = 40.76; $P = 0.0011$). Based on our linear model calculated from the standard curve, fleas from medium and high groups were maintaining an average of 338.4 and 203.59 *Bartonellae* per

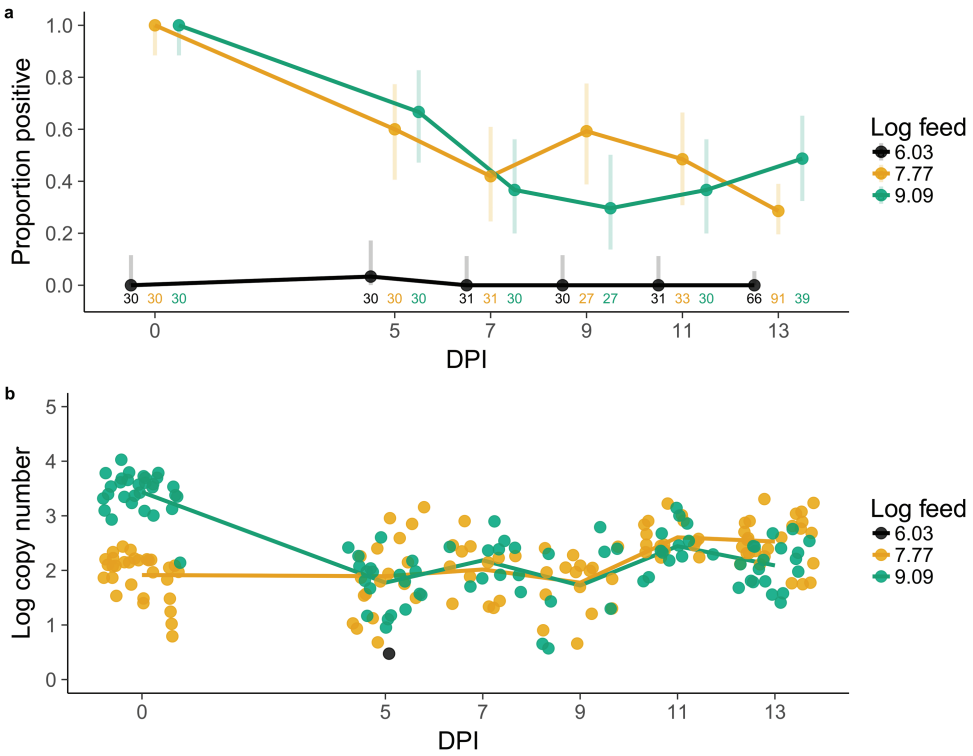

Fig. 1. *Bartonella* prevalence in groups of tested fleas and distribution of quantitative PCR results (log *Bartonella ssrA* copies) from individually tested positive fleas. Values along the bottom axis divide samples into time points: 0 to 13 d post infection (DPI). Colors separate samples into treatment groups fed on different concentrations of *Bartonella*-inoculated blood (log CFU/ml). (a) Prevalence was assessed using results from two tests: qPCR assay (*ssrA*) and a conventional PCR assay (ITS). Only samples that are positive by both assays were considered positive. Exact binomial confidence intervals were calculated using the Clopper–Pearson method. The number of tested samples is listed underneath each point. (b) The regression formula for converting cycle threshold (Ct) values to log copy numbers in fleas was log (Copies) = –0.2881 × Ct + 12.815 (R^2 = 0.9949). Lines run through the calculated mean log copies for each group at each time point.

flea from 5 to 13 DPI, respectively (Fig. 1b). Linear modeling demonstrated that there was an increase in the *ssrA* copies in both groups over this period (medium group: $F = 23.04$; df = 1, 87; $P < 0.001$; high group: $F = 5.38$; df = 1, 69; $P = 0.023$). Sequencing of the ITS region from PCR-positive fleas confirmed the presence of *B. elizabethae*.

Molecular Detection in Flea Feces

All fecal pools collected from control fleas fed pathogen-free blood tested negative for *Bartonella* DNA. Only a single fecal pool a single fecal pool from the low exposure group tested positive at 11 DPI with a Ct value of 39.42, corresponding to ~13 *Bartonella* copies in the 5 µl of sample DNA. All fecal pools from medium and high-exposure groups were positive at 0 DPI and at all following timepoints (Fig. 2), with the exception of the medium group at 11 DPI where all three test samples from that timepoint were negative. For the high group, excretion of *Bartonella* DNA in feces appeared to peak at 3 DPI with an average Ct value of 31.73, corresponding to an average of 2911.57 *ssrA* copies in the 5 µl of sample DNA. For the medium group, excretion peaked at 5 DPI with an average Ct value of 35.4, corresponding to an average of 228.06 copies. The number of *ssrA* copies was greater in the high group compared to the medium group at 0 DPI ($t = 7.6$; df = 2.53; $P = 0.0083$) and 3 DPI ($t = 19.06$; df = 3.12; $P < 0.001$), but were statistically similar at 5 and 9 DPI; comparisons at 7, 11, 13 DPI could not be made due to an insufficient number of positive qPCR measurements for one of the exposure groups at each timepoint. Excretion of *Bartonella* DNA declined after these peak timepoints, but the decline was only significant for the high-exposure group (medium group: $F = 4.19$; df = 1, 10; $P = 0.068$; high group: $F = 27.72$; df = 1, 10; $P < 0.001$). *Bartonella* DNA was still present in feces at 13 DPI in both exposure groups. Sequencing of *Bartonella* DNA in positive fecal pools confirmed the presence of *B. elizabethae*.

Discussion

Our experiment involved artificial feeding of oriental rat fleas (*X. cheopis*) with rat blood inoculated with *B. elizabethae* bacteria at varying concentrations. The experiment sought to establish a threshold concentration of bacteria necessary to colonize the flea gut and to measure the duration of maintenance in the gut and fecal shedding of bacteria by fleas. Our results demonstrated that *X. cheopis* can acquire *Bartonellae* from an infectious bloodmeal and presumably maintain and excrete viable *B. elizabethae* for a minimum of 13 d post infection as indicated by detection of DNA from this bacterium in flea feces. Additionally, it appears that fleas must ingest a threshold concentration of bacteria, around 10^6 CFU/ml, from rat bloodmeals to become infected and be detected as positive using our PCR assays.

The infectious threshold of 10^6 CFU/ml is likely attributable to the size of the bloodmeal taken by individual fleas. Specifically, *X. cheopis* fleas generally take on average 0.5 µl of blood per flea per feeding (Douglas and Wheeler 1943). Based on that size of bloodmeal, fleas fed a concentration of 10^6 bacteria would only consume 500 bacteria on average with each feeding, which may not be a sufficient inoculum for *Bartonellae* to invade the flea gut lumen. The presence of an infectious threshold agrees with past studies of *Bartonella* acquisition and excretion in cat fleas (*Ctenocephalides felis*). Kernif et al. (2014) found that *C. felis* artificially fed with blood inoculated with 3.6×10^5 *B. quintana*

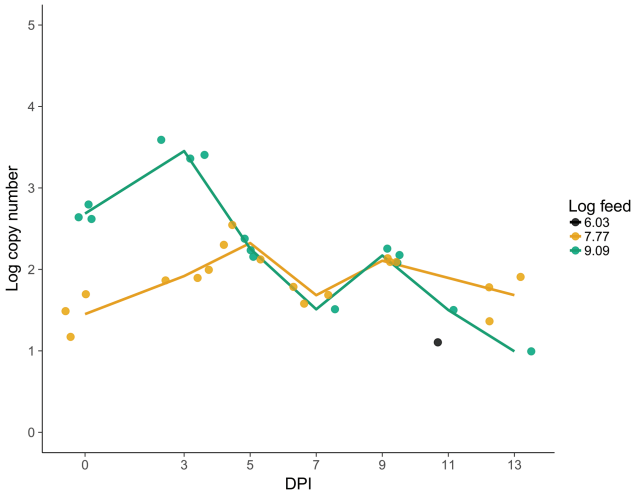

Fig. 2. Distribution of quantitative PCR results (log *Bartonella ssrA* copies) from pools of flea feces. Values along the *x*-axis divide samples into time points: 0 to 13 d post infection (DPI). Colors separate samples into treatment groups fed on different concentrations of *Bartonella*-inoculated blood (log CFU/ml). The regression formula for converting cycle threshold (Ct) values to log copy numbers in feces was log (Copies) = –0.305 × Ct + 13.13 (R^2 = 0.9931). Lines run through the calculated mean log copies for each group at each time point.

bacteria (1.64 × 10^5 CFU/ml for 200 µl of inoculate in 2 ml of blood) or below did not develop detectable infections. The slightly lower infectious threshold in the study by Kernif et al. may be attributable to differences in feeding behavior of the separate species or the sensitivity of the different qPCR assays used in the previous study.

For the groups that were exposed to higher concentrations of bacteria (10^7 or 10^9 CFU/ml), the fleas showed initial differences in the level of infection on the last day of exposure (0 DPI), but the groups showed similar levels of infection from 5 to 11 DPI and only a small yet statistical significant difference at 13 DPI. The relatively constant levels of infection in exposed *X. cheopis* were similar to results from experimental exposure of *C. felis* to blood inoculated with *B. henselae* performed by Bouhsira et al. (2013b), although in this study the authors tested pools of 20 fleas rather than individual fleas, so changes in prevalence of infected individuals in the exposed groups were not examined. In our study, we found by testing fleas individually that infection prevalence declined in the population rapidly between 0 and 5 DPI, but that prevalence did not differ significantly between exposure groups between 5 and 13 DPI and in fact prevalence increased slightly over time for both groups. *Bartonella* DNA was also detectable in flea feces for all timepoints except at 11 DPI for the medium exposure group. We had initially hypothesized that *Bartonella* DNA would only be detectable in fleas up to 11 DPI based on a previous study by Kernif et al. (2014) of *B. quintana* infection in *C. felis*. However, another study by Bouhsira et al. (2013b) found that *C. felis* exposed to *B. henselae* could remain infected up to 13 DPI and excrete bacteria in their feces up to 12 DPI. The authors of this study used a sampling strategy where they tested pools of 20 fleas and a standardized mass of 20 mg of feces on each sampling day, so there were timepoints where pools of fleas and feces tested negative between 1 and 13 DPI. By testing individual fleas and pools of feces from the entire population, our study was able to measure infections more consistently. Indeed, the presence of relatively constant levels of detectable *B. elizabethae* DNA in the fleas tested in our study suggest that a stable *Bartonella* infection was established and maintained in the guts of the fleas examined for at least the duration of our study.

We note that our experiment did not assess the effects of *Bartonella* infection status or other experimental parameters, such as flea density in tubes, on flea mortality or susceptibility of fleas to infection over the course of the study. While we observed no qualitative difference in mortality between flea groups, we did not take a full count of deceased fleas at each timepoint during the experiment that would have allowed a more quantitative analysis. However, previous studies have demonstrated that *Bartonella* infection status does not have an effect on mortality in experimentally infected *C. felis* (Kernif et al. 2014) or *X. ramesis* fleas (Morick et al. 2013b). Furthermore, infected and uninfected *X. ramesis* individuals did not differ in bloodmeal size, metabolic rate during digestion, fecundity, or fertility (Morick et al. 2013b).

One of the necessary assumptions we made in this study is that detectable *Bartonella* DNA in feces corresponds to active bacterial replication in the flea gut and that bacteria excreted in flea feces is viable. We did not attempt to localize *Bartonella* bacteria within the gut of infected fleas, however other studies of *C. felis* fleas have done so using an immunofluorescent antibody assay targeting *B. henselae* (Higgins et al. 1996) or immunohistochemistry targeting *B. quintana* (Kernif et al. 2014), suggesting that these bacteria are capable of invading and replicating in the cells of the flea gut. Our DNA detection results are in agreement with this model. The levels of *B. elizabethae* infection in fleas remained constant from 5 to 13 DPI despite fleas being fed with blood containing no *Bartonella* bacteria, suggesting that bacteria had successfully invaded the gut of exposed fleas. Additionally, *Bartonella* DNA was detectable in pooled flea feces throughout this same period, suggesting that live bacteria were shed from the flea gut. Further attempts to localize *B. elizabethae* bacteria in the gut of *X. cheopis* and culture *Bartonellae* in feces (particularly from individual fleas or standardized pools of fleas) are warranted to clarify these results and confirm that bacteria are viable.

In this study, we only examined excretion of *Bartonellae* in flea feces. Contamination of scratch or bite wounds with ectoparasite feces containing *Bartonellae* has been proposed as the main route of vector-borne transmission (Chomel et al. 1996, Foil et al. 1998). This suggestion seems reasonable based on existing literature and the fact that transmission of the etiologic agent of flea-borne typhus (*Rickettsia typhi*) occurs primarily by this means (Azad 1990). Our study did not explore other forms of vertical and horizontal

transmission involving fleas, but these may be important for the maintenance of *Bartonella* infection in natural systems and should be further investigated. For example, it is unclear whether fleas may transmit bacteria during the feeding process through their saliva or in regurgitated bloodmeals. Bouhsira et al. (2013a) demonstrated regurgitation and contamination of fresh blood with *B. henselae* DNA during feeding by *C. felis* and Morick et al. (2011) found two out of five regurgitations from *X. ramesis* carrying *Bartonella* sp. OE 1-1 to be *Bartonella*-positive, suggesting that this may represent an alternative route for horizontal transmission to naïve vertebrate hosts. Furthermore, exposure of flea larvae (*X. ramesis*) to gut voids and feces from infected adults in a separate experiment led to infection in 16% of larvae (Morick et al. 2013c), suggesting that this nontransovarial form of vertical transmission may be an important route for the maintenance of *Bartonella* infection in flea populations. Transovarial and transstadial transmission have not been demonstrated in *X. ramesis* (Morick et al. 2011) or any other flea species, however these routes of vertical transmission in fleas should be studied further.

Finally, additional experiments that demonstrate the onward horizontal transmission of *Bartonellae* from *X. cheopis* to naïve hosts could help to determine the important routes of infection and the potential of *X. cheopis* to be a competent vector for other *Bartonella* species. In these experiments, determining whether *Bartonella*-contaminated flea feces are sufficient to cause infection in naïve rodents (and at what bacterial concentration) would be useful in understanding the kinetics of the transmission process. *X. cheopis* collected from rats (*Rattus* spp.) and mice (*Mus* spp.) are known to harbor a variety of *Bartonella* species, including strains related to *B. elizabethae*, *B. grahamii*, *B. tribocorum*, *B. rochalimae*, *B. rattimassiliensis* (Winoto et al. 2005, Reeves et al. 2007, Harrus et al. 2009, Morick et al. 2009, Bitam et al. 2012, Billeter et al. 2013, Kamani et al. 2013, Leulmi et al. 2014). Additional studies demonstrating acquisition and excretion of other *Bartonella* species by *X. cheopis* in single or mixed cultures using an artificial feeding system may be able to replicate more realistic features of natural systems, where individual rodents may be infected by multiple *Bartonella* strains simultaneously and pass these infections to ectoparasites.

The results of this study indicate that *X. cheopis* is capable of acquiring *B. elizabethae* bacteria during artificial feeding on inoculated blood. Exposed fleas remained infected for a minimum of 13 d after exposure and continuously excreted *Bartonella* DNA in their feces. Fleas required a minimum bacterial concentration of 10^6 CFU/ml to establish infection in the flea gut. These results will help to guide future experimentation aiming to demonstrate onward transmission of *B. elizabethae* or other rodent-associated *Bartonella* species in naïve rodents. *Rattus* spp. or *Mus* spp. might be ideal candidates for these experiments due to their known infection with multiple *Bartonella* species, including *B. elizabethae*, and the ready availability of these species as laboratory animals.

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