Host distribution and pathogen infection of fleas (Siphonaptera) recovered from small mammals in Pennsylvania

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ABSTRACT: The number of recognized flea-borne pathogens has increased over the past decade. However, the true number of infections related to all flea-borne pathogens remains unknown. To better understand the enzootic cycle of flea-borne pathogens, fleas were sampled from small mammals trapped in central Pennsylvania. A total of 541 small mammals were trapped, with white-footed mice (*Peromyscus leucopus*) and southern red-backed voles (*Myodes gapperi*) accounting for over 94% of the captures. Only *P. leucopus* were positive for examined blood-borne pathogens, with 47 (18.1%) and ten (4.8%) positive for *Anaplasma phagocytophilum* and *Babesia microti*, respectively. In addition, 61 fleas were collected from small mammals and tested for pathogens. *Orchopeas leucopus* was the most common flea and *Bartonella vinsonii* subspecies *arupensis*, *B. microti*, and a *Rickettsia felis*-like bacterium were detected in various flea samples. To the best of our knowledge, this is the first report of *B. microti* DNA detected from a flea and the first report of a *R. felis*-like bacterium from rodent fleas in eastern North America. This study provides evidence of emerging pathogens found in fleas, but further investigation is required to resolve the ecology of flea-borne disease transmission cycles. ***Journal of Vector Ecology* 45 (1): 32-44. 2020.**

Keyword Index: Fleas, rodents, *Peromyscus leucopus*, *Myodes gapperi*, pathogenic bacteria, Pennsylvania.

INTRODUCTION

Ectoparasites can spread various pathogens among host populations in nature. Ticks in particular play an important role in zoonotic pathogen transmission, including *Borrelia* spp., *Babesia* spp., *Anaplasma* spp., *Ehrlichia* spp., *Rickettsia* spp., and others which can cause important human and animal diseases (de la Fuente et al. 2008). Many species of wild mammals, including rodents, serve as reservoir hosts for tick-borne pathogens in North America (Baneth 2014). However, along with ticks, fleas are commonly recovered from small mammals and may play a role in pathogen transmission cycles.

Among the disease agents that fleas transmit to humans and domestic animals are *Bartonella henselae*, transmitted by the cat flea *Ctenocephalides felis,* causing cat-scratch disease (Chomel et al. 1996, Higgins et al. 1996), *Rickettsia typhi*, the pathogen responsible for murine typhus that is re-emerging in Texas (Blanton et al. 2015), and *Rickettsia felis*, an emerging human pathogen in the United States (Brown and Macaluso 2016, Legendre and Macaluso 2017). While multiple pathogens may be transmitted by fleas to humans, only plague (*Yersinia pestis*) is a nationally reportable disease in the U.S. Therefore, the actual number of disease cases from all flea-borne pathogens is underreported.

Pathogens primarily isolated from ticks have been reported in fleas. For example, *Anaplasma phagocytophilum* was identified from fleas recovered from red foxes (Torina et al. 2013), and *Borrelia burgdorferi* has been identified from fleas recovered from small mammals (Lindsay et al. 1991, Netušil et al. 2013). Pathogens not typically associated with fleas may be ingested during blood meals on infected reservoir hosts. Alternatively, there is the possibility of transfer from a feeding infected ixodid tick to an adjacent feeding naïve arthropod, known as co-feeding transmission (Gern and Rais 1996, Patrican 1997, Belli et al. 2017). The potential for new and re-emerging pathogens in fleas as well as the potential for fleas to play a role in natural transmission cycles of tick-borne pathogens is of interest given the historical and current significance of interactions among small mammals, fleas, and flea-borne pathogens and the current expansion of tick-borne pathogens. Moreover, documentation of natural infection of fleas with unexpected pathogens needs to be followed up by experimental vector competence studies to assess the

potential impact on enzootic pathogen transmission.

Fleas are widely distributed on small mammal hosts in the United States (Holland and Benton 1968, Clark and Durden 2002, Ritzi and Whitaker 2007, Nims et al. 2008, Storm and Ritzi 2008). However, as with ticks (Brunner and Ostfeld 2008), distribution and recovered flea species are often dependent on host species and individual hosts (Kiffner et al. 2014). Level of infestation on hosts has been found to be male-biased (male hosts typically have more fleas) with some species but not all; thus, physiological determinants such as sex and body mass are not universal (Kiffner et al. 2013). The distribution of parasites across potential hosts has important consequences for the ecology of vector-borne diseases as host species have variable reservoir competence for zoonotic pathogens.

Vector-borne disease cases in humans have tripled in the United States in a little over a decade (Rosenberg et al. 2018). Thus, there is a need to better understand the ecology of vectors in important pathogen-endemic areas. To better address this gap, the goal of this study was to describe flea associations with small mammals and document pathogen infection in the fleas recovered from the small mammals in the commonwealth of Pennsylvania, U.S.A. The objectives were to (1) determine the diversity of fleas on small mammal hosts, (2) describe the associations of flea burdens to host physical characteristics and habitat, and (3) detect pathogens in hosts and fleas from central Pennsylvania.

MATERIALS AND METHODS

Small mammal trapping and ectoparasite sampling

Small mammal trapping occurred in State Game Lands (SGL) 92 (41.000 N, -77.769 W) and 103 (40.999 N, -77.893 W) in Centre County, PA, U.S.A. with one trapping site in SGL 92 and two trapping sites in SGL 103 (Figure 1). A total of 150 traps (50 per site) were set weekly, with 25 traps each per site in trapping transects separated into "edge" and "wooded" habitat. Traps in edge habitats were placed alongside rights-of-way or gravel roads and included brushy vegetation dominated by scrub oak (*Quercus ilicifolia*), Allegheny blackberry bramble (*Rubus allegheniensis*), and hay-scented fern (*Dennstaedtia punctilobula*). Wooded habitat transects were at least 100 m from the edge habitat and vegetation included striped maple (*Acer pensylvanicum*), witch hazel (*Hamamelis virginiana*), mountain laurel (*Kalmia latifolia*), and chestnut oak (*Quercus montana*).

Small mammals were trapped using perforated Sherman traps (7.62 x 8.89 x 22.86 cm, Sherman Trap Company, Tallahassee, FL) baited with peanut butter, oats, sunflower seeds, and apples. Cotton balls were provided for protection and warmth as well as nesting material. Traps were placed in the late afternoon and opened the following morning. Traps were placed approximately 10 m apart in suitable rodent habitat, usually under wooded or brushy areas and along trees and logs (Barry and Francq 1980). Trapping occurred once a week between May-August, 2018.

Upon capture, small mammals were anesthetized with isoflurane via an induction chamber using a bell jar for further processing (Fish et al. 2011). Isoflurane was placed directly into the chamber and a removable platform protected the mammal from direct contact with the anesthetic. A unique identifying ear tag was applied (Stoetling Company, Wood Dale, IL), mice were sexed, and measurements were taken of body mass, body length from tail base to nose tip, right foot length, and right ear length. Captured rodents recovered from the isoflurane in their respective traps or in a recovery cage with handwarmers, depending on outside temperatures. Once recovered, they were released at the location of their capture.

Blood samples were taken using submandibular puncture and blood was spotted on filter paper and stored in a centrifuge tube at 4° C. Rodent blood spot filter papers were sent to the Centers for Disease Control and Prevention (CDC) for testing of *A. phagocytophilum*, *Borrelia miyamotoi*, and *Babesia microti*. Fleas were collected using louse combs and forceps, preserved in 70% ethanol, and stored at 4° C. Fleas were identified using keys from Benton (1983) and Holland (1985), and then sent to the Walter Reed Biosystematics Unit (WRBU) in Suitland, MD for pathogen detection. All procedures were conducted according to the Pennsylvania State University Institutional Animal Use and Care Committee Protocol (#201800036).

Small mammal blood pathogen detection

Nucleic acids were isolated from rodent blood samples as follows. First, 400 µl of lysis buffer (376 µl ATL; 20 µl proteinase K; 2 µl Reagent DX; and 2 µl Carrier RNA, 1 µg/µl) (Qiagen, Valencia, CA) was added to each tube containing the blood sample and incubated for 20 min at 56° C. Following the incubation step, 300 µl lysate of the blood sample was processed using the KingFisher DNA extraction system and the MagMAX™ Pathogen RNA/DNA Kit (ThermoFisher Scientific, Houston, TX).

The subsequent multiplex TaqMan PCR reactions included in-house primer and probe master mixes M73 and M74, targeting *A. phagocytophilum*, *B. miyamotoi, B. microti*, and rodent GAPDH for blood samples (Table 1). The rodent GAPDH target (Applied Biosystems® TaqMan® Rodent GAPDH Control Reagents kit; Thermo Fisher Scientific) was included as a PCR and DNA purification control. PCR reactions for M73 and M74 were performed in 15 µl solutions with 7.5 µl iQ Multiplex Powermix (BioRad, Hercules, CA), 5 µl DNA extract, primers/probes (see Table 1 for concentrations) and water (Courtney et al. 2004, Hojgaard et al. 2014, Graham et al. 2016). With 88 blood samples, the PCR included six negative extraction controls, one water negative for the PCR reaction, and a PCR positive control comprised of recombinant plasmids.

The TaqMan PCR cycling conditions for M73 consisted of DNA denaturation at 95° C for 3 min followed by 40 cycles at 95° C for 10 s and 60° C for 30 s on a C1000 Touch thermal cycler with a CFX96™ real time system (Bio-Rad). The TaqMan PCR cycling conditions for M74 consisted of DNA denaturation at 95° C for 3 min followed by 40 cycles of 95° C for 10 s and 65° C for 30 s. All PCR samples were analyzed using CFX Manager 3.1 software (Bio-Rad) with the

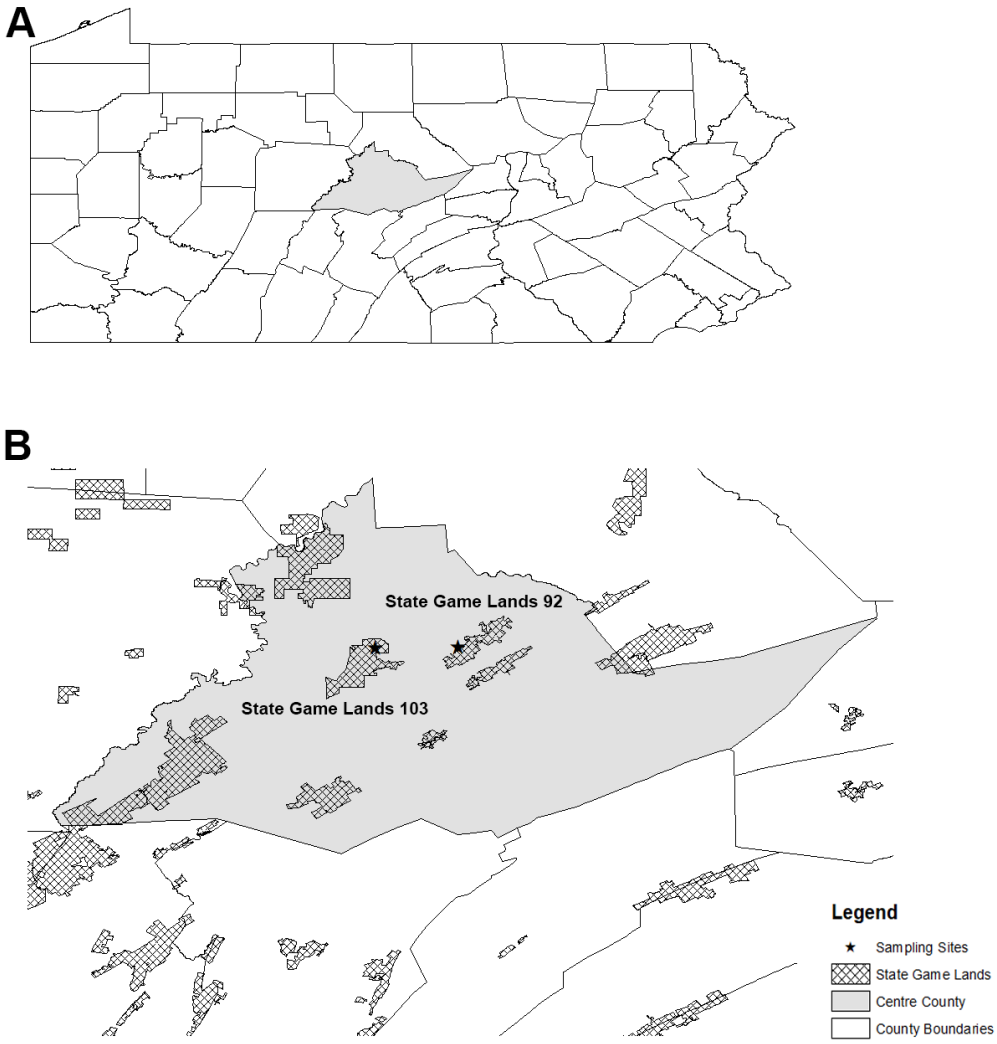

Figure 1. (A) Trapping sites in Pennsylvania. Centre County, PA is shaded in grey. (B) Small mammal trapping occurred in State Game Lands 92 and 103, represented by stars.

quantitation cycle (Cq) determination mode set to regression. Based on Graham et al. (2018), only Cq values <40 were considered indicative of a target being present in the tested sample.

Flea pathogen detection

Trace ethanol was removed from specimens using a phosphate buffered saline (PBS) rinse prior to disruption using 5 mm stainless steel beads. Samples were homogenized in 200 µl PBS with a Qiagen TissueLyzer and 5 mm stainless steel bead. Bead beating was performed at 30 Hz for 5 min. DNA was extracted on the Autogen 950E platform (AutoGen Inc., Holliston, MA) using the "Mouse Tail" protocol provided by the manufacturer. DNA was re-suspended in TE buffer and stored at -20° C until analysis. The ABI7500 Fast Real-time PCR System (Applied Biosystems, Foster City, CA) was used to amplify the template. A reaction volume of 10 µl included 0.3 µM forward primer, 0.3 µM reverse primer, 2.5 mM $MgCl_2$, 0.5 mM DNTPs, 1 µl template DNA, 0.6 µl Bioline BIOLASE DNA polymerase, and 3% DMSO. Running conditions began with a 5 min 95° C denaturation, followed by 35 cycles of a 30 s denaturation at 95° C, 45 s annealing at X° C (see Table 2 for values), and 60 s extension at 72° C; short products were extended by a final 5 min extension. PCR products were visualized on a 1.5% agarose gel. Primers and DNTPs were removed using the Thermofisher (Waltham, MA) EXO/sap-IT enzyme mix and bidirectional cycle sequencing reactions were performed with the Thermofisher Big Dye Terminator V3.1 kit. Sequencing products were filtered through a sephadex gel, and excess water was evaporated by incubation at 95° C. Sequencing was performed at the Smithsonian Laboratory of Analytical Biology and then processed for pathogen species identification verification through DNA barcoding at the WRBU, Museum Support Center, Smithsonian Institution, Suitland, MD. Sequences from PCR products were edited in Sequencer v.5.4.1 (Genes Codes Co, Ann Arbor, MI). Partial sequences were compared with available sequences in GenBank using Nucleotide BLAST search engine (National Center for Biotechnology Information, Bethesda, MD).

Statistical analysis

A chi-squared test of independence determined an association between rodent species and trapping location. The unpaired two-sample Wilcoxon test was used to determine

Table 1. Primers and probes included in the in-house multiplex PCR master mixes to detect pathogens in mouse blood. Two mixes of primers and probes were used for PCR reactions to detect *A. phagocytophilum*, *B. miyamotoi*, and *B. microti*. The PCR reactions were performed in 15 µl solutions with 7.5 µl iQ Multiplex Powermix (BioRad, Hercules, CA), 5 µl DNA extract, primers/probes (concentrations listed below), and water.

Target	Sequence 5' → 3'	Size (bp)	Reference	Final concentration (mM)
M73 mix				
Anaplasma phagocytophilum (*msp2* gene)	F - ATGGAAGGTAGTGTTGGTTATGGTATT	77	Courtney et al. 2004	0.3
	R – TTGGTCTTGAAGCGCTCGTA		Courtney et al. 2004	0.3
	Probe - FAM-TGGTGCCAGGGTTGAGCTTGAGATTG-BHQ1		Courtney et al. 2004	0.2
Borrelia miyamotoi (*purB* gene)	F - TCCTCAATGATGAAAGCTTTA	121	Graham et al. 2016	0.3
	R – GGATCAACTGTCTCTTTAATAAAG		Graham et al. 2016	0.3
	Probe - CalRD610-TCGACTTGCAATGATGCAAAACCT-BHQ2		Graham et al. 2016	0.2
Babesia microti (*sa1* gene)	F - ACAGAATGCAGTCGGTGAAG	115	Hojgaard et al. 2014	0.3
	R – ATCAAGGAGAGTGGATAGGTTTG		Hojgaard et al. 2014	0.3
	Probe - Q705-CCATTGACGCTGTTGTTGCTCACA-BHQ3		Hojgaard et al. 2014	0.2
GAPDH		Rodent DNA	TaqMan® Rodent GAPDH Control Reagents kit	0.2/0.2

Table 1 (continued).

Primers and probes	Sequence 5' → 3'	Size (bp)	Reference	Final concentration (mM)
M74 mix				
Anaplasma phagocytophilum (*msp4* gene)	F - TATATCCAACTTCAACTTCCACTC	93	Hojgaard et al. 2014	0.3
	R – CATTCAAGTTCGCTAAGAGTTTAC		Hojgaard et al. 2014	0.3
	Probe - HEX-CTCCGCCAATAGCATAGCCAGTTG-BHQ1		Hojgaard et al. 2014	0.2
Borrelia miyamotoi (*glpQ* gene)	F - GACCCAGAAATTGACACAACCACAA	108	Graham et al. 2016	0.3
	R – TGATTTAAGTTCAGTTAGTGTGAAGTCAGT		Graham et al. 2016	0.3
	Probe - CalRd610-CAATCGAGCTAGAGAAAACGGAAGATATTACG-BHQ2		Graham et al. 2016	0.2
Babesia microti (*18S* gene)	F - CGACTACGTCCCTGCCCTTTG	99	Hojgaard et al. 2014	0.3
	R – ACGAAGGACGAATCCACGTTTC		Hojgaard et al. 2014	0.3
	Probe - Q705-ACACCGCCCGTCGCTCCTACCG-BHQ3		Hojgaard et al. 2014	0.2

differences in the number of fleas found on small mammals by species, sex, and trapping location. A linear regression determined if weight was a contributing factor to the number of fleas found on a host.

RESULTS

Seven species of small mammals and 541 individuals were trapped during the field season (Table 3). White-footed mice (*Peromyscus leucopus*) accounted for 82.8% of the total collections. Southern red-backed voles (11.5% of total, *Myodes gapperi*) and Eastern chipmunks (3.7%, *Tamias striatus*) were also live-trapped. Because *P. leucopus* and *M. gapperi* comprised over 94.0% of the samples and no fleas were recovered from other trapped rodents, analyses focused on these two species of rodents and their fleas. Small mammal captures peaked in June, with over 32.7% of the small mammals trapped in that month (Figure 2). Although *P. leucopus* and *M. gapperi* tended to be trapped more commonly in edge habitats (257 mice and 42 voles) than wooded habitats (191 mice and 20 voles), there was no statistically significant association between rodent abundance and habitat ($p = 0.15$, $\chi^2 = 2.032$).

A total of 255 *P. leucopus* and five *M. gapperi* blood samples were processed for pathogen testing. Blood samples from *P. leucopus* tested positive for *A. phagocytophilum* (n=47; 18.4%) and *B. microti* (n=9; 3.5%), but none were positive for *B. miyamotoi*. Furthermore, co-infection of *A. phagocytophilum* and *B. microti* was evident in three *P. leucopus*. The blood samples from *M. gapperi* were not positive for any tested pathogen.

Overall percent parasitism of *P. leucopus* and *M. gapperi* by fleas was 9.4%. Out of 61 fleas that were collected and submitted for identification and pathogen testing, the most common flea was *Orchopeas leucopus* (82.0% of total fleas) (Table 4). Other species of fleas included *Ctenophthalmus pseudagyrtes*, *Peromyscopsylla hesperomys*, and *Stenoponia americana*. *Orchopeas leucopus* was more commonly found on *P. leucopus* than *M. gapperi*. However, *C. pseudagyrtes* was found more often on *M. gapperi*. Two flea species, *P. hesperomys* and *S. americana,* were exclusively found on *P. leucopus*. Fleas were most numerous during June and July, with 51 collected specimens recovered during these months (Figure 2). In general, more fleas were collected from male mice than females, but the number of fleas found per sex was not statistically significant ($p = 0.65$, $U_{31, 24} = 25436$, Figure 3A). Most fleas were collected from *P. leucopus* and *M. gapperi*, with the former accounting for 90.2% of the collected fleas (Figure 3B). However,the number of fleas collected from *P. leucopus* and *M. gapperi* was not statistically different ($p = 0.08$, $U_{55, 6} = 13024$). Mouse weight was not correlated with the number of fleas found on a mouse (Figure 3C, $R^2 = 0.0003$).

Pathogenic bacteria and parasites were detected in 21 flea samples (33.3% of total fleas collected) (Table 4). The number of fleas positive for pathogens was not significantly different between *P. leucopus* and *M. gapperi* ($p = 0.41$, $U_{19, 3} = 61.5$). Co-infections were not recorded in any fleas. *Bartonella vinsonii* subspecies *arupensis* was detected in 19 flea samples, with 16 samples coming from *O. leucopus* and three samples from *C. pseudagyrtes*. *Orchopeas leucopus* fleas that were positive for *B. vinsonii* were found on different *P. leucopus*, while three positive *C. pseudagyrtes* fleas came from a single *M. gapperi*. *Babesia microti* was detected in one *O. leucopus* that originated from a *P. leucopus*. Another *P. leucopus* had an *O. leucopus* flea that tested positive for a *Rickettsia spp.* The PCR amplicon of the 23S-5S intergenic spacer was 366 bp in length and was a 96% match to *R. felis* sensu stricto (GenBank HM222603.1). Other *Rickettsia* spp. matches included *Rickettsia australis* (89% similarity), *Rickettsia akari* (89%), and *Rickettsia monacensis* (86%). A 197-bp sequence of *B. microti* COI was recovered from one *Orchopeas leucopus t*hat was found to be 99% identical to *B. microti* strain R (GenBank LN871603.1). No pathogenic bacteria were detected in *P. hesperomys* or *S. americana* fleas. Overall, *O. leucopus* was the greatest contributor of pathogens, with 84.2% of the positive samples for *B. vinsonii* (GenBank JN402328.1) and the only species to have *B. microti* and a *R. felis*-like bacterium detected.

Mouse infection with *B. microti* was not related to flea infection with this pathogen. The one flea that was positive for *B. microti* was recovered from a *P. leucopus* which was negative for *B. microti*. The original host that was infected with *B. microti* is unknown.

Fleas were found on hosts collected in both edge and wooded habitats. The overall number of fleas found on hosts in edge habitat was not significantly different from those found in wooded habitat ($p = 0.57$, $U_{36, 25} = 32270$). A total of 36 fleas were found on *P. leucopus* collected from edge habitat, while 19 fleas from *P. leucopus* were collected in wooded habitat. All six fleas collected from *M. gapperi* were found in wooded habitat. Most pathogens were detected in fleas from edge habitats. *Bartonella vinsonii* was detected in 11 fleas from the edge habitats and eight fleas from the wooded habitats. Of those found on hosts in the wooded habitat, three *C. pseudagyrtes* positive for pathogenic bacteria were found on one *M. gapperi* and five positive *O. leucopus* were collected from five *P. leucopus*. Both the *R. felis*-like bacterium and *B. microti* were detected in *O. leucopus* that were collected from two separate *P. leucopus* from the edge habitat.

DISCUSSION

Fleas were consistently found on *P. leucopus* and *M. gapperi*, which were the most common small mammals captured during this study. The small mammal distributions found in this study reflected a study conducted in Maryland, where *P. leucopus* and *Microti spp.* were commonly trapped (Kuchinsky et al. 2019). Fleas were found more often on *P. leucopus* than *M. gapperi* which is consistent with Kollars et al. (1997) and Clark and Durden (2002). The most abundant flea was *O. leucopus,* which was most commonly found on *P. leucopus*. This finding supports previous literature identifying *P. leucopus* as the main host for *O. leucopus* (Holland and Benton 1968, Amin 1976). More *C. pseudagyrtes* were collected from *M. gapperi*, but this flea is also known to parasitize *P. leucopus* and other small mammals (Holland and

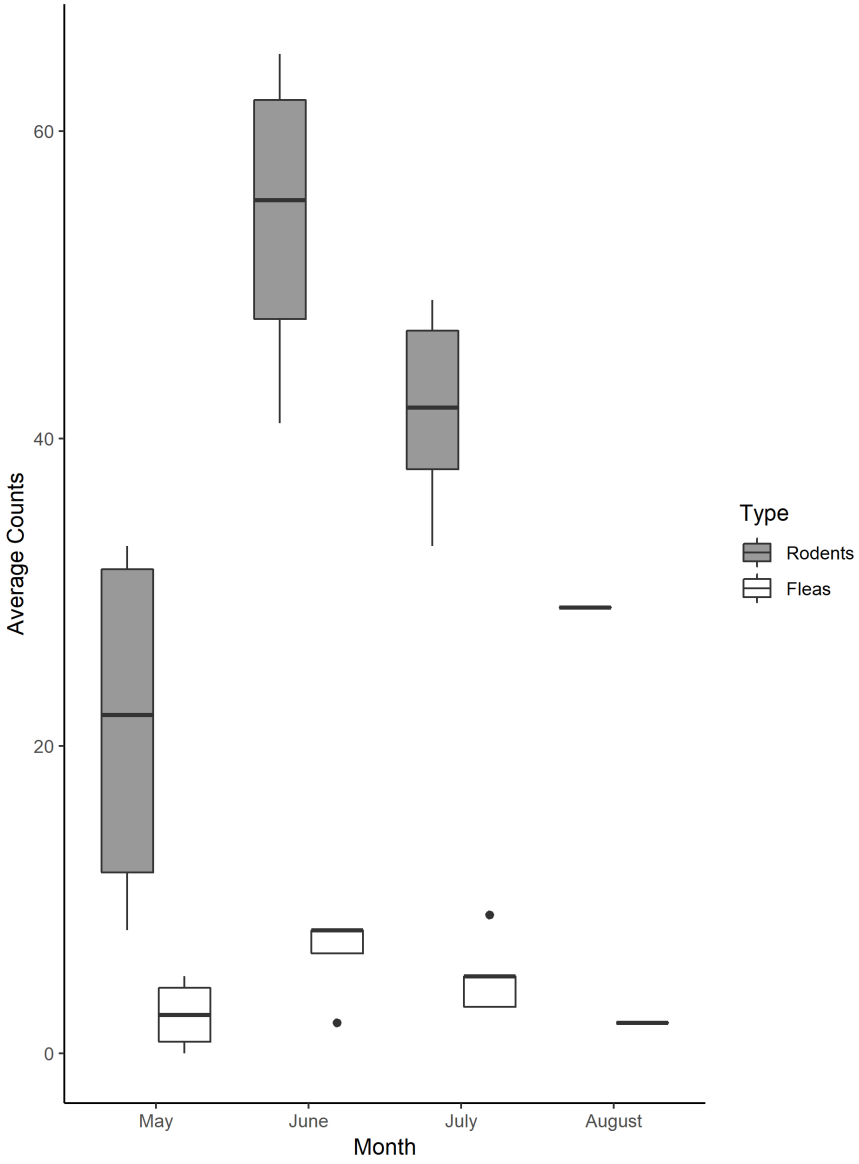

Figure 2. Boxplot of weekly capture counts for rodents (*P. leucopus* and *M. gapperi*) and fleas in Centre County, PA in 2018, averaged per month. The counts for rodents are shaded in gray and counts for fleas are represented in white. The two outliers in June and July refer to the outlier of fleas sampled during those months.

Benton 1968, Durden et al. 2012).

Similar to sex and body mass of the host, habitat distribution of the host was not statistically significant. However, differences in habitat preferences for *P. leucopus* and *M. gapperi* found in the literature may suggest differences in flea and flea-borne pathogen distribution. While both rodent species were captured in edge and wooded habitats, *P. leucopus* was more often captured along edge habitats; however, this finding was not statistically significant. *Peromyscus leucopus* can be found ubiquitously in many environments and is considered a generalist in terms of habitat preferences and would be expected to thrive in transitional landscapes due to the absence of competitors in these areas (Nupp and Swihart 2000). Conversely, *M. gapperi* is likely to be found in moist areas that are in forested areas (Gunderson 1959, Main et al. 1982, Mills 1995). Interestingly, this study found more *M. gapperi* located in edge habitat. The unique habitat preferences for each rodent species may explain the distributions of the flea-borne pathogens detected in this study. More *P. leucopus* that had fleas positive for pathogenic bacteria were found in edge habitats, while *M. gapperi* with fleas positive for pathogenic bacteria were found in forested habitats, potentially suggesting that increased pathogen transfer occurs in fleas with higher host populations. While sex and body mass of the host did not correlate with flea parasitism, the distribution of *P. leucopus* and *M. gapperi* differed, which may contribute to differences in flea and flea-borne pathogen distribution.

Three pathogens were detected in flea specimens including DNA from *B. microti* in one *O. leucopus* flea found on a *P. leucopus* host. To our knowledge, this is the first report of *B. microti* DNA detected in a flea. *Peromyscus leucopus* is a known reservoir for *B. microti* (Spielman et al. 1981) and 3.5% of the tested *P. leucopus* blood samples tested positive for *B. microti* DNA. While *P. leucopus* is considered to be the natural reservoir, *Ixodes* spp. ticks are considered enzootic vectors of *B. microti* (Westblade et al. 2017). Given that some flea species may co-parasitize small mammal hosts with ticks, horizontal transmission of pathogens may occur via a vertebrate host (Sprong et al. 2009). Similar to *Babesia vogeli*

Table 2. Primers included in the in-house multiplex PCR master mixes to detect pathogens in fleas. The PCR reactions were performed in 10 µl solutions with 0.3 µM forward primer, 0.3 µM reverse primer, 2.5 mM $MgCl_2$, 0.5 mM DNTPs, 1 µl template DNA, 0.6 µl Bioline BIOLASE DNA polymerase and 3% DMSO. Unique annealing temperatures are provided for each target.

Target	Sequence 5' → 3'	Size (bp)	Reference
Tick/Flea Cytochrome Oxidase I	F - TACTCTACTAATCATAAAGACATTGG	660	Barrett and Hebert 2005
	R - CCTCCTCCTGAAGGGTCAAAAAATGA		
Borrelia spp 16S	F - AGCCTTTAAAGCTTCGCTTGTAG	148	Parola et al. 2011
	R - GCCTCCCGTAGGAGTCTGG		
Babesia spp COI	F - ACCTGTCAARTTCCTTCACTAAMTT	150	Qurollo et al. 2017
	R - TCTTAACCCAACTCACGTACCA		
Babesia microti COI	F - TTGCGATAGTAATAGATTTACTGC	230	Qurollo et al. 2017
	R - TCTTAACCCAACTCACGTACCA		
Ehrlichia/Anaplasma 16S	F - GGTACCYACAGAAGAAGTCC	409	Nazari et al. 2013
	R - TAGCACTCATCGTTTACAGC		
Bartonella spp 16S-23S intergenic spacer	F - CCTCAGATGATGATCCCAAGCCTTCTGGCG	419-586	Oteo et al. 2017
	R - AATTGGTGGGCCTGGGAGGACTTG		
Rickettsia 23S	F – GATAGGTCRGRTGTGGAAGCAC	337	Jado et al. 2006
	R - TCGGGAYGGGATCGTGTGTTTC		

Table 3. Common and scientific names of small mammals trapped in May-August, 2018 in Centre County, PA.

Common Name	Scientific Name	Number Trapped
White-footed mouse	*Peromyscus leucopus*	448
Southern red-backed vole	*Myodes gapperi*	62
Eastern chipmunk	*Tamias striatus*	20
Eastern harvest mouse	*Reithrodontomys humulis*	2
Meadow jumping mouse	*Zapus hudsonius*	2
Short-tailed shrew	*Blarina brevicauda*	1
Woodland jumping mouse	*Napaeozapus insignis*	1
Unknown	--	5

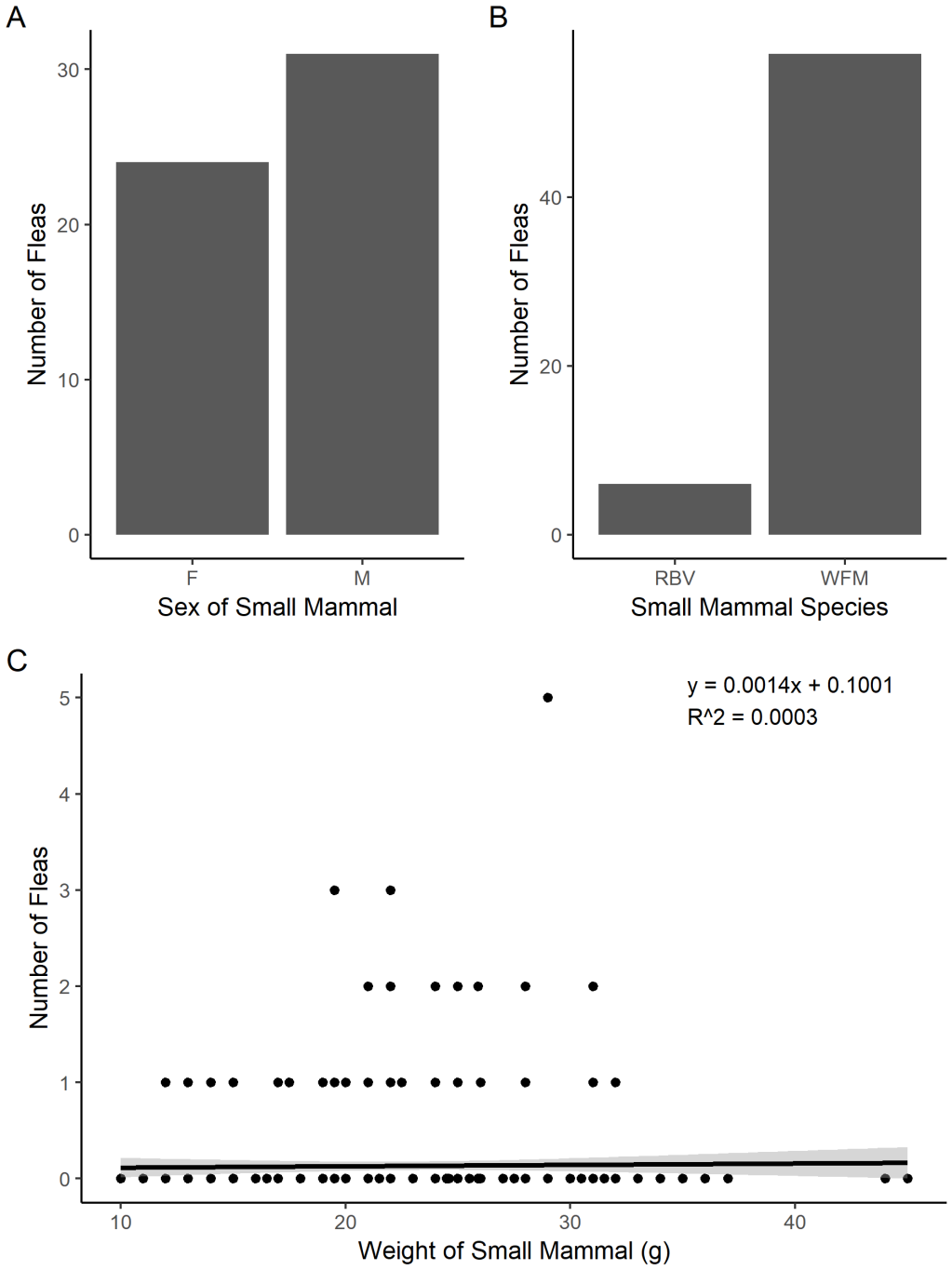

Figure 3. Distribution of fleas collected from rodents by host sex (A), species (B), and weight (C) during May-August 2018 in Centre County, PA (M, Males, F, Females, RBV, Red-backed vole, WFM, White-footed mouse).

Table 4. Flea species and associated bacteria and parasites found on *Peromyscus leucopus* and *Myodes gapperi* during May-August 2018 in Centre County, Pennsylvania.

Fleas and Bacteria and Parasites Detected	*P. leucopus*	*M. gapperi*
Orchopeus leucopus	49	1
Bartonella vinsonii subsp. *arupensis*	16	0
Rickettsia sp. (*Rickettsia felis* relative)	1	0
Babesia microti	1	0
Ctenophthalmus pseudagyrtes	1	5
Bartonella vinsonii subsp. *arupensis*	0	3
Peromyscopsylla hesperomys	4	0
Stenoponia americana	1	0
Total parasitism (prevalence, %)	12.3%	9.7%
Total flea count	55	6
Overall parasitism (%) of both rodent species		9.4%

recently detected in cat fleas (Kamani et al. 2018), this study cannot conclude that fleas are able to transmit the pathogen, but this warrants further study. Interestingly, the flea sample that tested positive for *B. microti* came from a host negative for this pathogen, although the host may have had a resolving infection that was not detected. While this flea may have transferred from an infected mouse to the uninfected mouse host it was collected from, there is a possibility that the flea acquired *B. microti* by co-feeding adjacent to an infected tick. While this has not been empirically shown between ticks and other hematophagous arthropods, the phenomenon has been demonstrated between infected and naïve ticks (Gern and Rais 1996, Patrican 1997, Belli et al. 2017). Conversely, assuming that fleas can transmit *B. microti*, detectable infection may not occur in a host if a flea recently fed on its host, since peak parasitemia may take one to two weeks post-infection via intraperitoneal injection in laboratory mice (Ruebush and Hanson 1979). While *P. leucopus* can become parasitemic, hosts can have less than 0.1% of their erythrocytes infected, which affects detection of *B. microti* (Etkind et al. 1980, Spielman et al. 1981). Similarly, if the host was older, then peak parasitemia can be further delayed (Habicht et al. 1983). Overall, studies on vector competence of fleas for tick-borne pathogens and potential co-feeding between ticks and fleas may warrant further investigation (Westblade et al. 2017).

Bacteria detected in fleas included *B. vinsonii* subsp. *arupensis* and a *R. felis*-like bacterium. *Bartonella vinsonii* subsp. *arupensis* was first identified in 1994 and can cause endocarditis in humans (Welch et al. 1999, Fenollar et al. 2005). *Bartonella vinsonii* subsp. *arupensis* was the most common pathogen detected in fleas during the study. The bacterium was detected in *O. leucopus* and *C. pseudagyrtes*, which were found on *P. leucopus* and *M. gapperi*, respectively. Exclusive infection of *B. vinsonii* subsp. *arupensis* in *O. leucopus* found on *P. leucopus* and *C. pseudagyrtes* on *M. gapperi* could be explained by host preferences of the flea species (Holland and Benton 1968). The findings presented in this study suggest that the transmission cycle of *B. vinsonii* subsp. *arupensis* may include fleas as a possible vector, but vector competency studies are needed to confirm if fleas can transmit the bacterium to a naïve host. While the reservoir status of *P. leucopus* and *M. gapperi* for this bacterium was not investigated in this study, previous studies in Wisconsin and Minnesota have identified *P. leucopus* as the main reservoir in the transmission cycle (Hofmeister et al. 1998, Welch et al. 1999, Chomel et al. 2005). Future work including vector and reservoir competence studies for *B. vinsonii* subsp. *arupensis* will improve our understanding of the transmission cycle for this emerging pathogen. The success of *Bartonella* spp. in fleas and rodents suggests a high degree of adaptation between these organisms (Gutiérrez et al. 2015). For example, other pathogenic *Bartonella* species include *B. henselae* and *Bartonella quintana*, which are causative agents of cat-scratch disease and trench fever, respectively. The former is transmitted by cat fleas *C. felis* and the latter is typically transmitted by the human body louse *Pediculus humanus humanus*, but *B. quintana* has also been detected in cat fleas, ticks, and head lice (Chang et al. 2001, Rolain et al. 2003, Eremeeva et al. 2019).

In this study, a *R. felis*-like bacterium in *O. leucopus* found on *P. leucopus* was detected. The sequence has an approximately 14-bp difference from a 405-bp segment of the *23S* gene in *R. felis* (GenBank MK693112.1). Additional collection, verification, and sequencing efforts are underway to determine if this represents a sequencing anomaly, a new subspecies of *R. felis*, or a new species altogether. Regardless, this is the first report of infection of *O. leucopus* with a *R. felis*-like bacterium. *Rickettsia felis* causes flea-borne spotted fever and is typically transmitted by the cat flea *C. felis*, but the bacterium has been found in other flea species and arthropod

vectors including ticks. Stevenson et al. (2005) previously reported *R. felis* from a woodrat-associated flea, *Anomiopsyllus nudata*, in western North America (New Mexico). Records of *R. felis* from different ectoparasites provides further evidence that horizontal transmission of pathogens between different arthropods may occur via a vertebrate host. *Rickettsia felis* has been sequenced from rodents, but challenges, such as the absence of a definitive host, impede researchers from fully describing the disease transmission cycle (Pérez-Osorio et al. 2008, Brown and Macaluso 2016, Legendre and Macaluso 2017).

Further research is required to elucidate the transmission cycles of the pathogenic bacteria and parasites that were identified in fleas parasitizing small mammals in this study. Furthermore, measuring reservoir and vector competence would establish key components of the disease transmission cycles. With changing landscapes and the possibility for new or re-emerging disease transmission, it is vital to conduct regular surveillance on potential vectors and hosts to monitor disease dynamics.

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