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ASPECTOS ECOLÓGICOS Y FILOGENÉTICOS DE LA INTERACCIÓN ENTRE BACTERIAS, PULGAS Y CARNÍVOROS SILVESTRES DE LA RESERVA DE LA BIÓSFERA JANOS, CHIHUAHUA, MÉXICO

TESIS

QUE PARA OPTAR POR EL GRADO DE

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Durante el proceso del doctorado se llevaron a cabo las siguientes actividades académicas:

-Participación en cinco proyectos dentro del Programa para Conservación de Especies en Riesgo (PROCER) de la Comisión Nacional de Áreas Naturales Protegidas (CONANP).

-Participación en cuatro Congresos Internacionales y uno Nacional.

-Estancia como investigador invitado en el Centro para el Control y Prevención de Enfermedades (CDC por sus siglas en inglés), en Fort Collins, Colorado, EU.

-Publicación de dos artículos científicos.

RESUMEN

Las enfermedades de transmisión vectorial son de importancia global en la salud pública y veterinaria. En años recientes muchos estudios han reportado un aumento en la distribución y el rango de hospederos y vectores de los patógenos de transmisión vectorial. Esto incluye la emergencia y remergencia de enfermedades como la peste y algunas bartonelosis. Los objetivos de este estudio fueron: (Capítulo 1) evaluar algunos factores que determinan la estructura del ensamble de las especies de pulgas en los carnívoros silvestres de la Reserva de la Biósfera Janos (RBJ), Chihuahua; (Capítulo 2) determinar la prevalencia y la filogenia de *Bartonella* spp. en los carnívoros silvestres y sus pulgas; y (Capítulo 3) analizar la presencia de anticuerpos contra *Yersinia pestis* en carnívoros silvestres. Durante el otoño del 2013 y la primavera 2014 se tomaron muestras de sangre y pulgas de 66 carnívoros silvestres pertenecientes a 8 especies, de los cuales se colectaron 540 pulgas de 7 especies distintas. Las especies dominantes de carnívoros fueron el coyote (*Canis latrans*) y la zorra del desierto (*Vulpes macrotis*), mientras que las pulgas identificadas principalmente fueron *Pulex simulans*, *P. irritans* y *Echidnophaga gallinacea*. La estacionalidad, la especie de los hospederos y la competencia inter-específica fueron los factores estudiados que explican el ensamble de pulgas de los carnívoros. El 10.6% de los carnívoros y el 9.5% de las pulgas procesadas fueron positivas a *Bartonella* spp., siendo *B. rochalimae* y *B. vinsonii berkhoffii* las especies identificadas. Los resultados señalan que las especies de *Bartonella* encontradas en este estudio pueden representar un riesgo incidental para animales domésticos y humanos. A pesar que no pudimos demostrar la presencia de peste (*Y. pestis*) en este estudio, los eventos epizoóticos no pueden ser descartados como una de las posibles causas de la disminución de las poblaciones de perritos de la pradera en el noroeste de México. Por lo tanto, estudios adicionales son requeridos para descartar la presencia de la misma.

ABSTRACT

Vector-borne diseases have grown in importance as global health in humans and animals. In recent years, several studies have shown that some vector-borne parasites have expanded their distribution and the range of vectors and hosts, which in turn have resulted in the emergence and reemergence of diseases such as plague and bartonellosis. The aims of this study were: (Chapter 1) evaluate the drivers that determine the assemblage structure of fleas on wild carnivores from Janos Biosphere Reserve (JBR), Chihuahua; (Chapter 2) determine the prevalence and phylogeny of *Bartonella* species in wild carnivores and their fleas, and; (Chapter 3) asses the presence of antibodies against *Yersinia pestis* in wild carnivores. Sixty-six carnivores belonging to eight species were sampled for blood and 540 fleas belonging to three species were collected during fall 2013 and spring 2014 seasons. Coyotes (*Canis latrans*) and kit foxes (*Vulpes macrotis*) were the predominant host species, while *Pulex simulans*, *P. irritans* and *Echidnophaga gallinacea* were the most abundant fleas in wild carnivore hosts. We found that the abundances and assemblages structure of fleas of wild carnivores were explained by host identity, season and interspecific competition of fleas. We detected *Bartonella* species in 7 carnivores (10.6%) and 27 fleas (9.5%). These positive samples were clustered into *Bartonella rochalimae* and *Bartonella vinsonii* subsp. *berkhoffii*. Our findings support the fact that the presence of *Bartonella* species in wild carnivores raises the issue of their potential risk for humans and domestic animals. Although we could not demonstrate the presence of plague in this study, plague epizootic events cannot be discarded as one of the possible causes of the prairie dog population decline in Northwestern Mexico. Hence, further studies are needed to confirm the presence or absence of plague in JBR.

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host families and black arrows indicate their genotype association (Canidae=IX, XII, XIII and XIV; Mephitidae=IX, X and XI; Mustelidae=X). Species abbreviations are in Table 4. Numbers in parentheses are the number of sequences obtained in blood and fleas from wild carnivores and asterisks are the isolated strains. The dendrogram was constructed with 1000 replications using MEGA 6 software (Tamura et al., 2011).

Figure 3.1. Location of sites where plague surveillance in rodents and carnivores were sampled in Janos Biosphere Reserve, Chihuahua, Mexico.

Figure 3.2. Changes in the distribution areas (solid line) and abundances (dashed line) of black-tailed prairie dog colonies in Janos Biosphere Reserve, Chihuahua, México, in the years 1988 (26), 2000 (58), 2005 (12,30) and 2013 (28). Bold with asterisk indicate the years in which at least one plague surveillance was performed (see table 2).

1. INTRODUCCIÓN

En los últimos años un gran número de enfermedades infecciosas emergentes y remergentes (EIE) han sido reportadas en todo el mundo (1,2). Muchas de ellas al ser de índole zoonótico representan un grave riesgo de salud pública con grandes implicaciones económicas (3). De manera particular, Jones et al. (año) estimaron que el 60% de las enfermedades infecciosas emergentes y remergentes que padece la humanidad son zoonóticas, y que de estás el 71% provienen de la fauna silvestre (2). Los mismos autores señalaron que en la década de los 90´s el 28.8% de los eventos de enfermedades infecciosas emergentes correspondieron a enfermedades vectoriales. Esto coincide con el hecho de que en las últimas décadas muchos parásitos de transmisión vectorial y sus enfermedades han presentado un cambio marcado en el rango de hospederos y en su distribución geográfica (4). Estos saltos taxonómicos y geográficos de los parásitos están determinados por factores ecológicos y evolutivos de la interacción parasito-vector-hospedero, como son el rango geográfico de traslape entre especies de vectores y hospederos y la relación filogenética de los hospederos (5).

Aunado a esto, se sabe que el aumento en la incidencia de enfermedades emergentes y remergentes está asociado al desarrollo de las sociedades modernas, la movilidad de poblaciones humanas, la destrucción de los ecosistemas y la consecuente modificación de los patrones de transmisión de los agentes infecciosos (1,2,6,7). La intensiva destrucción de los hábitats a nivel global ha creado un complejo paisaje caracterizado por cambios en las coberturas (actividades agropecuarias, asentamientos humanos, etc.) que fragmentan los hábitats y la distribución de especies animales y vegetales, dando como resultado la simplificación de los ecosistemas y la composición de las comunidades naturales. Estas alteraciones en las comunidades de especies silvestres pueden afectar la dinámica de las enfermedades, cambiando los patrones de transmisión de patógenos y en algunos casos, desencadenando la emergencia y/o reemergencia de enfermedades infecciosas (8).

A pesar de que el fenómeno del aumento de las EEI está en gran parte explicado por los cambios antropogénicos acelerados, es importante resaltar dos aspectos importantes: 1) detrás de cada EEI de transmisión vectorial existe un sistema parásito-vector-hospedero

particular; 2) las interacciones parásito-vector-hospedero típicamente involucran muchos hospederos, vectores y parásitos (9,10). Tan solo en los carnívoros silvestres, se tiene registro de entre 955 y 980 especies de parásitos encontrados en 159 especies de hospederos, de las cuales 73 especies de parásitos son de transmisión vectorial (11,12). De manera más específica, se reportaron 158 bacterias en 72 especies de hospederos y 147 artrópodos en 61 especies de hospederos (11). Entre las bacterias y los artrópodos reportados en carnívoros silvestres se encuentran *Yersinia pestis* y algunas especies de *Bartonella*, así como especies de pulgas de los géneros *Pulex* y *Oropsylla*. Junto con los carnívoros y otros hospederos, dichas especies forman parte de complejos sistemas parásito-vector-hospedero con implicaciones en la conservación de especies y la salud pública (13).

2. REVISIÓN BIBLIOGRÁFICA

Aspectos epidemiológicos y ecológicos de la peste

Debido a su alta morbilidad y mortalidad, la peste corresponde a una de las enfermedades más devastadoras de la historia humana (14,15). La peste es causada por la bacteria *Yersinia pestis*, un cocobacilo gramnegativo transmitido principalmente por vectores. *Y. pestis* evolucionó a lo largo de los últimos 1,500 a 20,000 años a partir de la divergencia de *Yersina pseudotuberculosis*, un patógeno entérico frecuente en los roedores (15,16).

Se cree que los orígenes de *Y. pestis* provienen de las estepas de Asia Central, desde donde se esparció a través de las rutas comerciales infectando ratas y pulgas (principalmente *Rattus rattus* y *Xenopsylla cheopis*) hasta alcanzar niveles pandémicos en varias ocasiones (17). Se reconocen tres pandemias de peste en la historia de la humanidad, todas ellas causantes de millones de decesos. La primera pandemia o peste de Justiano (541 al 602 D.C.) tuvo sus inicios en Egipto, la segunda pandemia o la Muerte Negra inició en Sicilia en 1347 y se extendió hasta el siglo XVI (17,18). La tercera pandemia, aún vigente, comenzó en la provincia China de Yunnan en 1855 y tras menos de cincuenta años se diseminó por todo el mundo. Solo en la India entre 1898 y 1918 se estima que murieron 12.5 millones de personas a causa de la peste (18,19).

En América, la peste fue introducida en 1899 a través de tres barcos que llegaron a costas de Paraguay, Brasil y Estados Unidos (San Francisco) provenientes de Europa y Asia, desde donde llegó a esparcirse hasta Argentina, Cuba, Trinidad y Tobago, Venezuela, Perú, Ecuador y Bolivia (18). En Norteamérica, siete años después del primer caso reportado en San Francisco en 1900, la peste fue considerada endémica de Estados Unidos y empezó a ampliar su distribución hacia el este. Hasta la década de 1960, la peste se caracterizó por epidemias emergentes que dejaban relativamente pocos decesos (18). Sin embargo, a partir de 1940 el número de casos de peste aumentó década a década, pasando de nueve casos entre 1944 y 1953 en California, Nuevo México y Arizona a 125 casos de 1984 a 1993 reportados en 18 estados situados al Oeste del río Mississippi (20). En lo que respecta a México, solo fueron reportados casos en Mazatlán y Veracruz, en 1903 y 1920 respectivamente, no obstante, no se ha reportado un caso de peste en humanos en los años posteriores (21,22). En

años más recientes, la OMS reportó que entre 2007 y 2010 se han presentado 15 casos de peste en EUA, 52 en Perú, 17 en China y 3,983 concentrados en 6 países de África (OMS 2009-2012).

A pesar de que en el siglo XX y lo que va del XXI la prevalencia de la peste ha disminuido a nivel mundial (14), en algunas partes del mundo se han observado cambios en la ecología y la epidemiología de la enfermedad que señalan que la peste no es una enfermedad erradicada y que continúa siendo riesgo de salud pública. Esta bacteria presenta algunas características evolutivo-ecológicas que, en cierta parte, explican las remergencias de la enfermedad en los humanos y en otras especies. Una de ellas es que presenta factores de virulencia, una capacidad evolutiva que le permiten evitar las defensas de los mamíferos hospederos (14), entre ellos se encuentra la Fracción 1 (F1), un antígeno de la superficie similar a la capsula y el T3SS (por sus siglas en inglés: Type III secretion system), dos factores que inhiben la actividad fagocitaria de los macrófagos y otras células inmunes (23–25).

La otra consiste en la capacidad adaptativa de la bacteria para infectar a nuevas y distintas especies, lo que permite que el rango de la peste se expanda durante brotes locales. Aunque la mayoría de estas expansiones son temporales, ocasionalmente se establecen nuevos focos de infección de *Y. pestis* entre poblaciones inafectadas de roedores silvestres y sus pulgas (17,18). Se sabe que durante la pandemia en curso (tercera pandemia) se han establecido nuevos focos enzoóticos con ciclos activos y estables de la enfermedad en cada uno de los continentes del mundo, con excepción de Australia y el oeste de Asia (14,17,18).

Una vez establecidos, los ciclos enzoóticos de *Y. pestis* pueden mantenerse estables hasta que se presenta un nuevo cambio en la ecología de la enfermedad, resultando en una posible emergencia o reemergencia en la incidencia o prevalencia de la enfermedad en humanos, o bien, continuar su avance por medio de la infección de nuevas especies (26). Un ejemplo de ello fue la reemergencia de peste presentada en la India en 1994, la cual surgió tras más de 15 años sin reporte alguno de la enfermedad después de que ratas con pulgas infectadas fueron atraídas a Bombay por la llegada de grandes cantidades de ayuda en granos y comida ante la necesidad que género el temblor de 1993 (14,18). Otro ejemplo, refiere a la ampliación de la distribución de la peste por Norteamérica, la cual se cree que se difundió rápidamente

desde San Francisco hacia otras regiones al pasar de ratas de ciudad hacia poblaciones de roedores silvestres que habitaban en las afueras de la ciudad, estos a su vez, llevaron la enfermedad a nuevas poblaciones de roedores y pulgas que se distribuían más al este, norte y sur de Norteamérica, lo que resultó en la conformación del área con peste enzoótica más larga del mundo (14,17,27).

Vectores y hospederos silvestres de la peste

La peste se ha registrado en al menos 200 especies y subespecies de mamíferos, incluidos en 73 géneros (14,28). Sólo en EUA se ha identificado la peste en 76 especies de mamíferos (17). Aunque los hospederos más importantes son roedores, como ardillas y perritos de la pradera (17,28), mamíferos carnívoros como el coyote (*Canis latrans*), zorras (*Vulpes macrotis*, *V. velox*), mústelidos (*Martes americana*, *Mustela vison* y *Mustela nigripes*), mapaches (*Procyon lotor*), tejones (*Taxidea taxus*), linces (*Lynx canadensis* y *Lynx rufus*) y pumas (*Puma concolor*) también pueden ser infectados (20,29–32). En México hay indicios de la existencia de un foco enzoótico de peste en roedores en Coahuila (33). Se sabe que en estos focos enzoóticos la enfermedad puede mantenerse latente y logra emerger y remerger en epidemias o epizootias, generalmente, a través de la picadura de pulgas infectada con *Y. pestis*. Existen más de 30 especies de pulgas que pueden transmitir la peste, no obstante, cada una presenta distinta eficacia como vector. Mientras que la pulga de gatos y perros (*Ctenocephalides* spp.), y la de humanos (*Pulex irritans*) son vectores mucho más deficientes que la pulga de la rata oriental (*Xenopsylla cheopis*) y la pulga del perrito de la pradera (*Oropsylla hirsuta*), dos de los vectores competentes para la transmisión de la peste (17,28,34).

Aspectos epidemiológicos y ecológicos de *Bartonella* spp.

Bartonella es un género de bacterias Gram-negativas trasmitidas generalmente por vectores que infectan de manera intracelular a células endoteliales y eritrocitos de hospederos mamíferos (35). Las especies de *Bartonella* son reconocidas como EIE y son de importancia para la medicina veterinaria y humana (36). Dentro del género *Bartonella* se han reconocido entre 30-40 especies y subespecies, de las cuales al menos 13 son consideradas como agentes

zoonóticos (37). Entre ellas se pueden mencionar a la enfermedad del arañazo del gato causada por *B. henselae*, enfermedad de Carrion causada por *B. bacilliformis*, Fiebre de las trincheras causada por *B. quintana* y endocarditis causada por *B. vinsonii* and *B. clarridgeiae* (38).

Aunque entre 1980 y 1992 se identificaron muchas de las especies de *Bartonella* spp. (39), estudios de ADN señalan que este género de bacterias ha infectado a los humanos desde hace aproximadamente 4000 años (40). La infección es de distribución mundial, solo en Estados Unidos se reportan cerca de 22,000 casos al año de enfermedad por arañazo de gato (41), lo que la convierte en la bartonelosis más común. En México, desde que se reportó por primera vez en 1960 (41), solo se han diagnosticado casos esporádicos, sin la presentación de epidemias (42).

Vectores y hospederos silvestres de Bartonella spp.

Debido a la gran cantidad de especies de *Bartonella*, los sistemas parasito-vector-hospedero dentro de este género de bacterias resultan muy diversos. Distintos estudios señalan que existen patrones de co-especiación entre las especies de *Bartonella* y los hospederos (43). Entre las asociaciones bacteria-hospedero están las *B. bacilliformis* y *B. quintana* con el humano como reservorio primario, *B. washoensis* con las ardillas, *B. rochalimae* y *B. vinsonii sub. berkhoffii* con canidos y *B. clarridgeiae* y *B. henselae* con félidos. En términos generales, estudios previos han identificado seis especies y una subespecie de *Bartonella* en al menos 19 especies de carnívoros silvestres, 6 cánidos, 6 félidos, 5 mustélidos, 1 prociónido y 1 vivérrido. Varios estudios han encontrado evidencia serológica de *B. henselae* en felinos silvestres. En California se reportó en pumas (*Puma concolor*), gatos monteses (*Lynx rufus*) y felinos exóticos (*Acynonyx jubatus, Felis* spp. y *Panthera* spp.) (44), mientras que Rotstein et al. (2000) reportaron prevalencias de 18 y 28% en pumas de Florida y Texas, respectivamente (45). En un estudio más amplio que incluyó muestras de gatos monteses y pumas de vida libre y cautiverio en México, Chile y Estados Unidos se encontró una prevalencia de *B. henselae* que fluctuó entre 0 y 33% (46). Un estudio más reciente encontró entre 10 y 40% de prevalencia de *Bartonella* sp. en pumas y gatos monteses capturados en California y Colorado (47).

Los coyotes y otros cánidos por su parte, son sospechosos de ser los principales reservorios de *B. vinsonii* subsp. *berkhoffii* y *B. rochalimae* (48). Lo anterior se apoya en las altas prevalencias de *B. vinsonii* subsp. *berkhoffii* encontradas en coyotes (*Canis latrans*) (26,49,50) y zorra isleña (*Urocyon littoralis*) (51,52). De igual forma se ha podido aislar *B. vinsonii* subsp. *berkhoffii* en coyote (49,53,54), zorra gris (*Urocyon cinereoargenteus*) (53), y la zorra isleña (52); *B. henselae* y *B. koehlerae* en mapache (*Procyon lotor*) (55) y *B. rochalimae* en zorra roja (*Vulpes vulpes*), zorra gris, zorra isleña, mapache y coyote (52,56,57). Adicionalmente, existen algunos reportes de la presencia de *Bartonella* en carnívoros silvestre de Asia, Europa y Australia. En Japón, dos estudios reportaron una nueva especie de *Bartonella* en tejón japonés (*Meles anakuma*) y una cepa cercanamente relacionada con *B. washoensis* en marta japonesa (*Martes melampus*) (58,59). En España se identificaron *B. henselae* in a gato silvestre (*Felis silvestris*), *B. rochalimae* en una zorra roja y un lobo (*Canis lupus*), y *Bartonella* sp. en tejón europeo (*Meles meles*) (60). Finalmente, *B. clarridgeiae* fue detectada en zorra roja en Australia (61).

Hasta este momento los reservorios hospederos de *Bartonella* son mejor conocidos que los vectores predominantes. Al menos un hospedero reservorio es conocido para cada especie de *Bartonella* (35). Mientras que las especies de *Bartonella* suelen ocurrir en interacciones hospedero específicas, se sabe que la riqueza de especies de *Bartonella* es normalmente mayor en sus vectores (62). Existen muy pocos estudios de *Bartonella* en pulgas de carnívoros silvestres (61,63,64). *B. rochalimae* y B.*v.* subsp. *berkhoffii* han sido reportadas en 42 *P. simulans*, una *Ctenocephalides felis* y una *C. inequalis* colectadas en zorras grises de California (63). *B. alsatica* se encontró en *Spilopsyllus cuniculi* obtenida de gato silvestre y una nueva especie de *Bartonella* sp. relacionada filogenéticamente con *B. rochalimae* fue detectada en *Pulex irritans* colectada de zorras (64). Por último, *B. henselae* y *B. clarridgeiae* fueron encontradas en pulgas *C. felis* que infestaban zorras rojas en Australia (61).

Papel de los carnívoros medianos en la ecología y transmisión de las enfermedades

Aunque se ha señalado que *Y. pestis* suele estar mantenida primariamente entre roedores silvestres y sus respectivos vectores (17,65). Algunos carnívoros medianos, como coyotes, zorras y mapaches, pueden ser hospederos reservorios de estos parásitos, lo que sugiere que

pueden ejercer un papel ecológico en la dinámica de enfermedades (66). Los carnívoros medianos, como los coyotes y los mapaches, resultan de importancia en el estudio de la ecología de las enfermedades debido que son especies abundantes y tienen una amplia distribución en Norte América (67,68). Adicionalmente, existen otras características ecológicas que les confiere a los carnívoros un papel importante en la dinámica de enfermedades. Entre ellas se puede nombrar a la interacción depredador-presa que determina el control de especies, como los roedores, que son considerados hospederos reservorios de muchas enfermedades (69); la rápida capacidad de adaptación que presentan algunas especies a áreas perturbadas por los humanos; así como su amplia movilidad (13,66). Los coyotes, por ejemplo, pueden llegar a recorrer largas distancias que van de los 80 hasta 300 y 500 km (70).

Aunque no se ha comprobado, existe la hipótesis de que los coyotes y las zorras puede ser transportadores (vectores) de enfermedades y/o vectores infectados al depredar presas infectadas o al adquirir un vector portador de un agente infeccioso (71–73). McGee et al. (2006) (29) demostraron que tras la epizootia de la peste de perritos de la pradera cola negra (*Cynomys ludovicianus*) ocurrida en Texas en 2004, existían zorras (*Vulpex velox*) y un coyote (*Canis latrans*) con *Oropsylla hirsuta*, una pulga propia de los perritos de la pradera. Esto indica que, al perder su hospedero habitual, *O. hirsuta* puede usar de forma oportunista a canidos silvestres como hospederos transportadores circunstanciales. Consistentemente, se ha señalado que tras una epizootia de peste aumenta el número de pulgas con *Y. pestis* libres en el medio ambiente a medida que la mortalidad de los hospederos aumenta (74). Existen reportes que señalan que algunas especies de pulgas tiene la capacidad de usar un hospedero alternativo si su hospedero primario no está disponible (75). Esto suele ocurrir en el caso de las epizootias, donde aumenta la probabilidad de que las pulgas al abandonar los animales muertos, muerdan a otras especies que no infestan normalmente como a carnívoros y humanos (28).

Aunado a lo anterior, muchos estudios han señalado que algunos carnívoros pueden ser buenos centinelas de algunas enfermedades vectoriales, entre ellas la peste, borreliosis, bartonelosis y anaplasmosis (13,76,77). El papel de los carnívoros como centinelas hace referencia al hecho de que algunas especies poseen un sistema inmune que los protege de patógenos presentando pocos o ningún signo de la enfermedad. De esta manera el sistema

inmune responde generando anticuerpos que además de atacar la infección, pueden ser detectados en pruebas serológicas, sirviendo como indicadores de que el microorganismo está o estuvo presente en el medio ambiente (78). Al respecto, Brown et al. (2011) (79) encontraron una correlación positiva estadísticamente significativa entre el porcentaje de coyotes seropositivos y el número de casos de peste en humano.

Es probable que el acarreo o transporte de la peste por parte de algunos carnívoros silvestres pudiera estar ocurriendo en la RBJ. Esto se apoya en que existen datos publicados de la dispersión y del intercambio faunístico entre E.U. y México (80), y a que la RBJ se encuentra a menos de 100 km de la frontera con Nuevo Mexico, donde se ha reportado la presencia de estas enfermedades en fauna silvestre y en humanos (14,20,81).

Importancia del estudio de enfermedades en la Reserva de la Biósfera Janos y en la conservación de especies

Los cambios en la dinámica de enfermedades no solo resulta importantes para la salud pública, sino que también pueden conllevar a amenazar la conservación de especies silvestres (13). Como se comentó previamente, un cambio en la ecología de una enfermedad puede resultar en la trasmisión del parásito patógeno dentro de poblaciones silvestres que no habían sido expuestas previamente y que por lo tanto no poseen un sistema inmunitario efectivo (82). Ejemplos de esto, son las extinciones locales de perritos de la pradera (*Cynomys ludovicianus*) causadas por la bacteria *Yersinia pestis* (83) y las epizootias de moquillo en distintos carnívoros silvestres de América y África (84).

En el caso particular de los carnívoros silvestres un estudio de revisión encontró que existían al menos 52 parásitos que podían ocasionar distintas enfermedades en 34 especies de grandes carnívoros (85). De estas enfermedades, 31% se debían a enfermedades ocasionadas por bacterias, entre las que se encuentran las enfermedades vectoriales causadas por *Yersinia pestis*, *Francisella tularensis*, *Borrelia burdogferi* y enfermedades rickettsiales. Al respecto, es sabido que los felinos y algunas especies de mustélidos son muy susceptibles a la peste, especies presentes en la RBJ (81,86).

Los perritos de la pradera (*Cynomys* spp.), por su parte, corresponden a uno de los géneros más importantes en el estudio de la ecología y el impacto de *Y. pestis*. Desde los

primeros reportes en 1938 (*C. gunnisoni*) y en los años 40 (*C. ludovicianus*) (87,88), los perritos de la pradera han sufrido epizootias de peste con mortalidades cercanas al 100% (71,89) lo que ha llevado en ocasiones a la extinción local de poblaciones (89). Históricamente, los perritos de la pradera eran especies abundantes, pero en las últimas décadas sus poblaciones han sido diezmadas debido básicamente a tres factores, la pérdida de su hábitat (pastizales templados), a programas de erradicación y a epizootias de peste (90). Sin embargo, además del riesgo de salud pública (entre 5 y 14% de los casos en humanos en EUA se asocian a perritos de la pradera) (14,91), la gran susceptibilidad que presentan los perritos de la pradera a la peste puede tener consecuencias en la conservación de muchas especies (71,89). Esto se explica a que los perritos de la pradera juegan un papel ecológico clave en la inducción de la biodiversidad a través de la creación y mantenimiento del hábitat o como presa de varias especies (92,93). Un ejemplo es el hurón de patas negras (*Mustela nigripes*), una especie en peligro de extinción que enfrenta una doble encrucijada, ya que además de ser susceptible también a *Y. pestis* (94), su dieta se conforma en un 90% de perritos de la pradera (95).

Gracias a la construcción de madrigueras y a la actividad de forrajeo que evita el crecimiento de vegetación arbórea y arbustiva, las numerosas colonias que conforman los perritos de la pradera contribuyen a la conservación y mantenimiento de los pastizales templados (96,97), uno de los hábitats más amenazados del mundo (98). La Reserva de la Biósfera Janos, Chihuahua mantiene 220,000 ha de pastizales nativos, una de las áreas de mayor importancia para la conservación en México y en Norte América (99), donde habita el mayor complejo sobreviviente de colonias de perros de la pradera (100). Debido a los reportes cercanos de *Y. pestis* (Arizona, Nuevo Mexico) (14,20,81) y a la existencia de intercambio y dispersión de fauna silvestre entre la frontera con EUA (por ejemplo, carnívoros medianos), las poblaciones de perritos de la pradera de la RBJ corresponden a un complejo poblacional susceptible a epizootias de peste con impactos en la conservación y la salud pública.

Finalmente, la Reserva de la Biósfera Janos ha sido reconocida como un área prioritaria para la conservación de la biodiversidad en Norte América (101,102). La RBJ alberga varias especies de importancia en la conservación, incluyendo 13 especies de carnívoros, como el

tejón y la zorra del desierto, las cuales están amenazadas en México (103,104). La vegetación de la RBJ está representada por una combinación de pastizal, matorral-pastizal, matorral mezquital y bosques templados (102,105). Sin embargo, la vegetación nativa ha sufrido un rápido y extenso cambio caracterizado por la pérdida de cobertura de pastizal y por la expansión del matorral. Estos cambios se deben básicamente al sobrepastoreo, la agricultura extensiva y la disminución de los perritos de la pradera (105–107). Es bien sabido que los cambios de las coberturas del paisaje debida a la influencia humana, están ligados a cambios en las comunidades y poblaciones de carnívoros silvestres (108). Y como se detalló con anterioridad, adicionalmente estos cambios en el paisaje traen cambios en la ecología y dinámica de las enfermedades. Por lo anterior, resulta de gran importancia el estudio de la ecología de comunidades de los carnívoros silvestres, así como la dinámica de enfermedades transmitidas por vectores.

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CHAPTER 1. **Drivers of abundance and assemblage structure of fleas (Siphonaptera) of sympatric wild carnivores in northwestern Mexico**

INTRODUCTION

Fleas are wingless insects included in the order Siphonaptera, which comprise 2500 species belonging to 15 families (1). They are highly specialized blood-sucking ectoparasites in a wide range of hosts including birds and mammals. In addition to their role as ectoparasites, fleas have medical relevance as vectors of pathogens that infect humans and animals (2). Carnivores are the second most important mammalian hosts of fleas (3), and several studies have suggested that wild carnivores may be potential reservoirs or have a role in the transmission cycles of flea-borne infections such as plague, bartonelloses and rickettsioses. For instance, mephitids and wild canids are suspected to be reservoirs of *Bartonella rochalimae* (4), which have been associated with bacteremia in humans (5). Also, wild canids were identified as a potential carrier of *Yersinia pestis* (the causal agent of plague) among prairie dogs colonies (6), and *Rickettsia felis*, the causative agent of an emergent rickettsiosis, has been reported in fleas collected from beach martens (*Martes foina*) (7). Furthermore, many flea species that are important as vectors of flea-borne diseases (*Pulex* spp., *Oropsylla* spp. and *Ctenocephalides* spp.) have been recorded in various mammalian carnivores such as wild felids, canids, mephitids and mustelids (4,6,8–10).

The drivers of patterns of community structure and composition of fleas have important implications for human and animal health (11). The community structure and composition of ectoparasites are determined mainly by host factors and the off-host environmental conditions. Host factors are included on different ecological levels of organization (individual, species and community), such as host sex and age, body condition, host species, body size, host abundances and diversity (3,12,13). However, many flea species found particularly in carnivores have often accidental associations instead of host-specific and are explained by ecological factors rather than phyletic factors. Fleas could switch between host species due to the exploration or exchange of burrows or could reflect predatory-prey relationships (3,14,15).

On the other hand, the fact that fleas spend most of their life-cycle off-host, including their larvae stage in which rarely are parasites, explain the strong relationship that they have with the environment (16). Environmental factors include habitat and climate, and consequently the season of the year. Several studies have reported a relationship between either development rates of flea larvae or abundances of fleas and temperature and rainfall (17–20). Finally, fleas may be influenced not only by environmental and host factors, but also by the characteristics of interspecific interactions, such as competitive exclusion between sympatric flea species (21–23).

The drivers of patterns of abundances and assemblage structure of fleas have been studied mainly in rodents (12,24–26) and domestic carnivores (17,27), in contrast, these patterns are poorly understood in wild carnivores. To our knowledge, there is only one study on habitat as a driver of flea assemblages in skunks (8).

For these reasons, the aims of this study were to: (1) investigate the flea assemblages in different wild carnivore species, (2) analyze flea abundances change among seasons, habitats and host identity, and (3) examine the interaction between the abundance of different flea species in wild carnivores.

MATERIAL AND METHODS

Study area

Sampling was carried out at five locations (El Cuervo, La Bascula, Monte Verde, Rancho Ojitos and Rancho San Pedro) within the Janos Biosphere Reserve (JBR) in Chihuahua, Mexico (30º 51´50´´N, 108º 30´09´´W) (Figure 1). JBR is in a transition zone between the Sierra Madre Occidental and the Chihuahuan Desert, which comprises a mosaic of grasslands, mesquite shrubland, oak forest and riparian vegetation. The climate is arid-temperate. Mean annual temperatures vary with altitude from a mean annual of 15.7ºC in plains at 1200 m above sea level to a mean annual of 11.8º C at 2700 m above sea level. A rainfall gradient occurs from a mean annual precipitation of 381 mm to 581 mm, with 77% of that precipitation falling from April through August (28).

Hosts sampling, flea collection and identification

Wild carnivores were live-trapped and fleas collected during fall 2013 (October-November) and spring 2014 (May-June) using box traps (Tomahawk Live Trap Inc.WI) and leg-hold traps (Victor Coil Soft Catch^{TM}). Sixteen trapping stations were placed at 0.5-0.8 km intervals along a 10 km transect at each of the five sampling locations. The traps were baited with canned sardine, chicken and commercial lures. Traps were set for nine consecutive days per site and checked at least once a day. Each individual was chemically restrained with an IM injection of ketamine hydrochloride and xylazine hydrochloride, according to the recommended doses for wild carnivores (29). Animals were identified, weighed and sexed. Each carnivore was visually examined and inspected systematically for fleas by combing for 10-15 minutes. Removed fleas were placed in a cryovial containing 70% ethanol and stored in liquid nitrogen. Fleas were placed individually in single petri dishes for examination using a stereo microscope and identified morphologically using published taxonomic keys (30,31). All procedures for handling carnivores were carried out in accordance with the guidelines of the American Society of Mammalogists (32) and were approved by the Ministry of Environment and Natural Resources of Mexico (Permit FAUT-0250).

Habitat classification

The habitat classification was based on 1:250,000 scale landcover GIS database from the National Institute of Statistics and Geography of Mexico (33) and satellite images available in Google Earth (34). Habitat was determined based on the most common land-cover types within a 100-m radius of each trapping sets. A total of four habitat types were distinguished: 1) grassland, 2) shrubland, 3) grassland-forest ecotone and 4) oak forest.

Data analysis

Incidences-based rarefaction and extrapolation were used to evaluate sample efficiency of flea assemblages across wild carnivore species (35). This procedure was based on species richness (q=0) and estimated by sample coverage using iNEXT package (36).

Cumulative relative abundance was plotted to visualize changes in host species rank of the flea assemblage. Relative abundances (RA) were estimated by calculating the abundance of a given species divided by the total abundances of flea assemblages for each wild carnivore host. Morisita-Horn index based on relative abundances were used to compare the similarity coefficient between flea assemblages of each carnivore host species. Similarity coefficient was performed in R software (37), and a dendrogram was created using UPGMA clustering of Morisita-Horn similarity values in Past software (38).

To determine if host and environmental factors had an effect on flea abundances we used generalized linear models (GLM) with quasi-Poisson distribution and log-link function to account for overdispersion in response variables (39). The response variables were the abundance of flea species at a given individual host. The explanatory variables were host identity (carnivore species), season and habitat. We performed the analysis on three flea species (*Pulex simulans*, *Pulex irritans* and *Echidnophaga gallinacea*) which were dominant species at host species.

In order to identify interspecific relationships between flea species in wild carnivores, we conducted two statistical tests. First, Spearman's rank correlations were performed to assess the intensity and direction of the linear relationships between the abundances of *Pulex simulans*, *P. irritans* and *E. gallinacea*. The non-parametric correlation coefficient was used because none of the analyzed datasets are normally distributed. Second, logistic regression models with binomial distribution were used to evaluate if the presence/absence of a given flea species may be explaining the presence/absence of the other species. A P-value < 0.05 was considered statistically significant. Generalized linear models and correlation coefficients were performed using R software (37).

RESULTS

Carnivore and flea assemblage

Sixty-four wild carnivores belonging to eight species of seven genera within five families (Canidae, Felidae, Mephitidae, Mustelidae and Procyonidae) were sampled for fleas. In total, 540 fleas were collected, representing two families (Ceratophyllidae and Pullicidae), six genera and seven species (Table 1.1). The incidence-based and coverage-based

rarefaction procedures suggest that the flea richness was well represented for the eight carnivore species with a sample coverage of over 90% in all cases. The flea assemblage was dominated by three flea species. *Echidnophaga gallinacea* was the predominant species in bobcats (*Lynx rufus*), raccoons (*Procyon lotor*) and badgers (*Taxidea taxus*) with a relative abundance (RA) range between 53.9 and 81.4%. *Pulex simulans* was the most abundant flea on coyotes (*Canis latrans*), gray foxes (*Urocyon cinereoargenteus*) and skunks (*Mephitis* spp.) with a RA range between 61.5 and 81.97%. Both *P. simulans* and *E. gallinacea* were represented in all wild carnivore species. Finally, *P. irritans* was the predominant species and almost strictly associated with kit fox (*Vulpes macrotis*).

The other four species found in this study were less represented in wild carnivore assemblages with only one *Orchopeas sexdentatus* flea collected from a coyote (*Canis latrans*), one *Oropsylla montana* collected from a striped skunk (*Mephitis mephitis*), one *Euhoplopsyllus glacialis* collected from a gray fox (*Urocyon cinereoargenteus*) and two *Thrassis aridis* fleas collected from a hooded skunk (*Mephitis macroura*) and a bobcat (*Lynx rufus*) (Figure 1.1 and Table 1.1).

Flea species composition

Morisita-Horn abundance index of the flea assemblages across carnivore hosts revealed three clusters with a high degree of similarity within each group (>90 %) and a low degree of similarity between groups (<50-10%) (Figure 1.2). These three clusters are consistent with the findings of the flea dominance by host identity mentioned above, being *P. simulans*, *P. irritans* and *E. gallinacea* the species that explain this clustering pattern (Figure 1.1).

Abundance analysis

The GLM shows that the abundances of some flea species are explained by season and host identity (Table 1.2). *P. irritans* and *E. gallinacea* abundances were significantly higher in spring than in fall season (Figure 3). For host identity, the abundance of flea species varied between carnivore host species. Bobcats, raccoons, badgers and kit foxes show statistically higher *E. gallinacea* flea abundances in comparison with coyotes. Although, gray foxes and striped skunks showed the highest abundances of *P. simulans*, only kit fox had significantly

lower abundances compared to the coyote hosts. In contrast, the abundance of *P. irritans* fleas was significantly higher in kit fox than coyotes.

We found a significant negative correlation between flea abundances within wild carnivore hosts. Hosts with high loads of *P. simulans* had low loads of *P. irritans* or *E. gallinacea* fleas (Figure 1.4). Additionally, the logistic regression model showed that the presence of *P. simulans* is more likely to occur in wild carnivore hosts in which *P. irritans* is absent and vice versa (Table 1.3).

DISCUSSION

Our results revealed that the patterns of flea assemblages of wild carnivores in JBR were structured by host identity, season and interspecific interactions. Regarding host identity, we can distinguish some trends of the flea assemblages. Of the seven-flea species collected in carnivore hosts, *Pulex simulans*, *P. irritans* and *Echidnophaga gallinacea* were considered dominant species and *Euhoplopsyllus glacialis, Orchopeas sexdentatus*, *Oropsylla montana* and *Thrassis aridis* were considered rare species. Interestingly, the former group of flea species are classified as generalist and cosmopolitan, whereas the latter are host-specific fleas from rodents and lagomorphs that may be found in wild carnivores as accidental associations.

Several studies coincide with our findings that the fleas belonging to the *Pulex* genus are the most abundant and prevalent on wild canids and mephitids in the United States as well as Europe. This patterns have been described in kit fox (*Vulpes macrotis*) (40) swift fox (*V. velox*) (6,10), Island fox (*Urocyon littoralis*) (41), gray fox (*U. cinereoargenteus*) (42), coyotes (*Canis latrans*) (43), and two species of skunks (*Mephitis mephitis* and *Spilogale gracilis amphiala*) (8,44) from the US and in red foxes (*V. vulpes*) from Spain (9) and Hungary (45). The *Pulex* genus comprises six species (46), but only two of them, *P. simulans* and *P. irritans*, may infest wild carnivore hosts. In concordance to our findings, *P. irritans* is usually the predominant and the most prevalent flea on the *Vulpes* genus, while *P. simulans* is typically the most common flea on gray foxes and coyotes (2,6,10,40,42). As we might expect, we found that *P. simulans* had a broader host distribution than *P. irritans*. While *P. simulans* was found in all carnivore species captured in this study, *P. irritans* was almost restricted to kit foxes. This finding was consistent with a previous study that concluded that

P. simulans is more adaptable to variety of hosts and habitats than *P. irritans* in North America (43). In addition, our results also agree with the hypothesis proposed by the same author that the abundance of *Pulex* species change by latitude distribution, with *P. simulans* usually being more abundant than *P. irritans* on coyotes in the southern part of their distribution.

On the other hand, the sticktight fleas (*Echidnophaga gallinacea*) are cosmopolitan fleas apparently associated with poultry. However, the wide range of hosts indicate that this flea is more likely a generalist flea that utilizes a variety of hosts (47). There are several records of *E. gallinacea* flea in wild carnivores, including all the species captured in this study (48–53). However, in contrast to our findings, the presence and/or dominance of this flea is usually uncommon in flea assemblages of carnivore hosts (2,8,40,54). There are only a few studies that have reported *E. gallinacea* as the dominant and most prevalent flea species on carnivores, including ones involving at least three wild carnivore species from Africa (55–57) and two mephitid species from the US (58).

Although our findings clearly show that host identity is an important driver of flea assemblage structure, the dominance of the different fleas could be additionally explained by interspecific interactions among flea species. It is well known that there are different potential interactions when two or more parasites coexist in a single host and that may result either on facilitation or competition (59,60). The significant negative relationship between the abundances and/or the occurrence between the three most abundant fleas on wild carnivores in JBR suggests competitive exclusion interactions. Specifically, we found that individual hosts with high loads of *P. simulans* usually had low loads of *P. irritans* or *E. gallinacea* fleas and vice versa. In addition, the presence of *P. simulans* explained the absence of *P. irritans*. These results contrast with reports on two species belonging to the genus *Oropsylla*, which showed a positive relationship between the abundances of two flea species in black-tailed prairie dogs (61). The previous authors suggested that the findings regarding the facilitation interactions among fleas might be related to host immune suppression ("top-down" regulation). Also, the apparent competitive exclusion among fleas found in this study may be explained by the direct interaction, through physical or chemical communication, or by indirect interaction, through resource exploitation ("Bottom up" regulation). However,

these hypotheses have been mainly tested in endoparasite systems (60,62,63). Although several studies have described an apparent competitive exclusion among ectoparasites, such as fleas, ticks and lice (21–23,64–66), only two studies were based on experimental approaches and contribute to understanding the effects and mechanisms mediating the interactions among them. The first one found that the removal of dominant tick species impact directly the abundances of chiggers and lice in sengis (*Rhynchocyon* spp.) (64), while the second study found that larvae of different flea species shows competitive interaction which may be driven by resources exploitation (23). Further experimental studies are needed to test the mechanism mediating the apparent competitive exclusion among fleas of wild carnivores in JBR.

As far as we know this is the first evidence of *Orchopeas sexdentatus* collected from coyotes, and *Thrassis aridis* collected from a striped skunk and a bobcat. However, these two-flea species are primarily parasites of woodrats (*Neotoma* spp.) and kangaroo rats (*Dypodomys* spp.), respectively (67,68). In addition, *T. aridis* has been collected from hooded skunks (*Mephitis macroura*) in Mexico (69). *E. glacialis* is a flea that usually infests lagomorphs (70) but has also been collected from gray foxes in Mexico (71) and in the US (40). *Oropsylla montana*, a flea typically associated with ground squirrel hosts, especially *Spermophilus variegatus* (72), has also been commonly found on skunks from north America (8,30,58). All these rodent hosts have been previously recorded in Janos Biosphere Reserve (73,74). Hence, the accidental associations of rodent-specific fleas in wild carnivores may be driven by ecological factors, such as the exchange of burrows by burrow-dwelling hosts or predator-prey relationships (3,14,15).

We found non-significant differences of flea abundances between the different habitats. Various studies have found that the habitat is an important driver for explaining the flea assemblage structure on striped skunks (8) and small mammal hosts (75,76). However, the changes in the patterns of flea assemblages among habitat types within host species are often explained directly by both the habitat selection of the hosts and environmental factors (76). This is consistent with our findings, because the lack of this relationship between flea abundance and habitat type might be explained by the fact that the three distinct assemblage clusters of fleas in wild carnivores were grouped by host identity instead of habitat. The

similarity among all clusters was low and these clusters were composed of different carnivore species found in distinct habitats. Only the cluster characterized by *P. irritans* dominance was specifically associated to kit foxes, a carnivore species which is in turn almost always restricted to grassland habitat. Similarly, previous studies have found that the segregation patterns of flea assemblages in small mammals is better explained by host factors, such as host identity, instead of habitat types (25,77). In addition, similar to other studies (76,77) we found the abundance and assemblage structure of fleas on wild carnivores in JBR to be related to environmental factors explained by seasonal changes. This relationship between fleas and season is often driven by climatic variations, such as temperature and rainfall (17–20). Our results show that the higher abundances of fleas were associated with the rainy season. Although this relationship may vary according to flea species, consistently in most of studies the rainfall was positively correlated with flea abundances (17,24).

Almost all the flea species found in this study have been identified as potential vectors of different flea-borne diseases. Among these fleas are *Echidnophaga gallinacea*, *Euhoplopsyllus glacialis*, *Pulex simulans*, *P. irritans*, *Orchopeas sexdentatus*, and *Oropsylla montana*, all of which have been found naturally infected with *Yersinia pestis*, the causative agent of plague (40,78,79). In addition, *Pulex* flea species and *E. gallinacea* have been reported to be naturally infected with other vector-borne disease agents that are associated with bartonelloses (4,42) and rickettsioses (80,81).

In addition, previous studies have found that the maintenance and transmission of flea-borne diseases is positively related to prevalence and abundance of fleas on hosts (82,83). Hence, our findings may be of relevance regarding flea-borne disease risks. Furthermore, the human settlements and farming activities are increasing in Janos and nearby areas, (84), which in turn may affect the dynamics at the wildlife-livestock-human interface. For instance, a previous study showed that domestic dogs occur at high densities in settlements of JBR and have free roaming activities that may increase encounters with wild carnivores (85). Therefore, the knowledge of flea composition and flea infestation of wild carnivores in this region is important to know not only because identifies the potential flea vectors that may be a risk for the cause health and veterinary concerns but it also provides information

needed to design and implement programs to manage these disease for purposes of human health or wildlife conservation.

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Table 1.1. Flea infestation patterns of wild carnivores from northwestern Chihuahua, Mexico.

Fleas/Hosts (n)	*C. latrans* (17)	*L. rufus* (5)	*M. macroura* (3)	*M. mephitis* (8)	*P. lotor* (4)	*T. taxus* (6)	*U. cinereoargenteus* (7)	*V. macrotis* (14)	Total
Pulex simulans									
% infested hosts	64.7	80	100	75	50	40	71.4	21.4	
Number of fleas	62	7	16	50	5	14	45	8	207
Pulex irritans									
% infested hosts	5.9	-	-	-	-	-	-	92.9	
Number of fleas	2	0	0	0	0	0	0	161	163
Pulex spp.									
% infested hosts	27.8	-	33.3	12.5	25	60	28.6	14.3	
Number of fleas	13	0	6	2	1	4	12	17	55
Echidnophaga gallinacea									
% infested hosts	5.9	80	33.3	50	100	100	14.3	50	
Number of fleas	3	35	3	8	13	21	1	26	110
Euhoplopsyllus glacialis									
% infested hosts	-	-	-	-	-	-	14.3	-	
Number of fleas	0	0	0	0	0	0	1	0	1
Thrassis aridis									
% infested hosts	-	20	33.3	-	-	-	-	-	
Number of fleas	0	1	1	0	0	0	0	0	2
Orchopeas sexdentatus									
% infested hosts	5.9	-	-	-	-	-	-	-	
Number of fleas	1	0	0	0	0	0	0	0	1
Oropsylla montana									
% infested hosts	-	-	-	12.5	-	-	-	-	
Number of fleas	0	0	0	1	0	0	0	0	1
Total	81	43	26	61	19	39	59	212	540

Table 1.2. Results of the generalized linear modelling analyses of the effects of carnivore host identity, habitat type and season on *Pulex simulans*, *P. irritans* and *Echidnophaga gallinacea* flea abundances.

	P. simulans			*P. irritans*			*E. gallinacea*		
Source of variation	Estimate	SE	p	Estimate	SE	p	Estimate	SE	p
Intercept	1.069	0.432	**0.017***	-19.552	4.517e + 03	0.997	-2.183	1.039	**0.041***
L. Rufus	-0.828	0.860	0.340	-17.381	7.531e + 03	0.998	4.079	1.006	**0.000***
M. macroura	0.555	0.702	0.433	-17.243	1.156e + 04	0.999	2.427	1.364	0.081
M. mephitis	0.799	0.491	0.109	-16.183	5.178e + 03	0.998	2.267	1.139	0.052
P. lotor	-1.379	0.978	0.164	-16.737	9.079e + 03	0.999	2.498	1.018	**0.018***
T. taxus	-0.607	0.648	0.354	-18.450	6.786e + 03	0.998	2.824	1.026	**0.008***
U. cinereoargenteus	0.462	0.533	0.390	-1.292	1.001e + 04	1.000	-0.468	1.850	0.801
V. macrotis	-1.687	0.828	**0.047***	3.716	0.920	**0.000***	2.526	1.036	**0.018***
Habitat (Forest)	-0.298	0.556	0.594	0.113	1.023e + 04	1.000	-0.406	1.032	0.696
Habitat (Grassland)	-0.403	0.504	0.428	17.922	4.518e + 03	0.997	-0.556	0.788	0.484
Habitat (Shrubland)	0.050	0.462	0.914	16.492	4.518e + 03	0.997	-0.244	0.699	0.729
Season (Spring)	0.637	0.405	0.122	0.659	0.221	**0.004***	1.140	0.414	**0.008***

*significant p-values are indicated in bold font

Table 1.3. Logistic regression analysis for the relationship between the presence/absence of flea species in wild carnivore hosts

Source of variation	Estimate	SE	p
Intercept	0.974	0.397	**0.014***
Pulex irritans	-2.529	0.826	**0.002***
Echidnophaga gallinacea	-0.523	0.572	0.361

*significant p-values are indicated in bold font

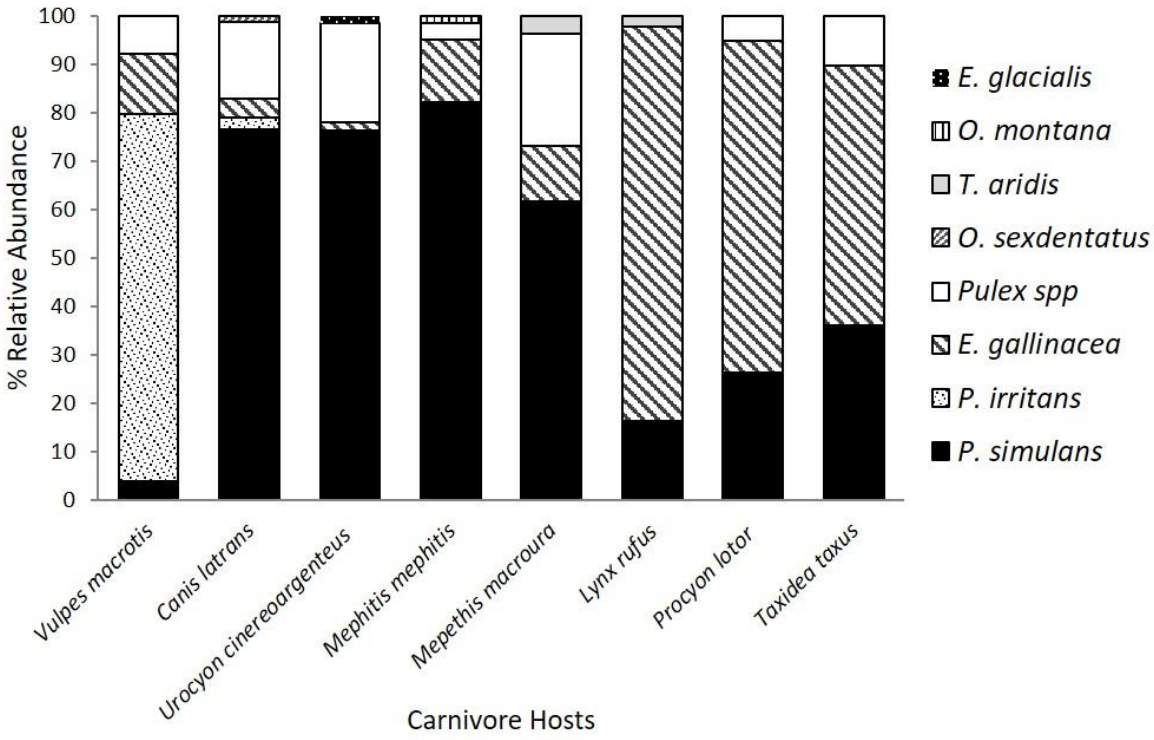

Figure 1.1. Flea-host association in wild carnivores in Janos Chihuahua, Mexico

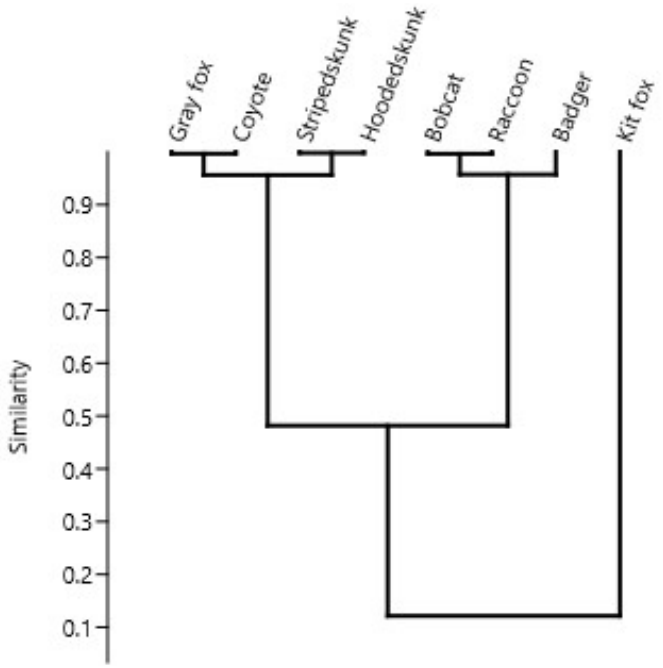

Figure 1.2. Degree of similarity among flea species assemblages in wild carnivores from Janos Biosphere Reserve. Tree was created using UPGMA clustering based on species abundances of Morisita Horn similarity values.

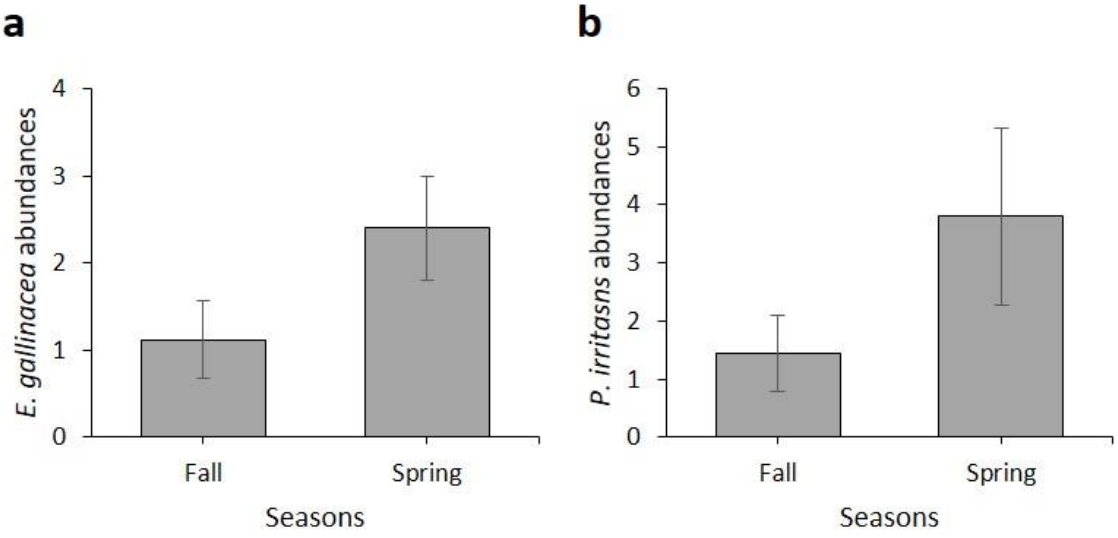

Figure 1.3. Seasonal variation in abundance of *E. gallinacea* (**a**), and *P. irritans* (**b**) in JBR between fall 2013 and spring 2014. The values are means ± s.e.m from sixty-six individuals belonging to eight wild carnivore species.

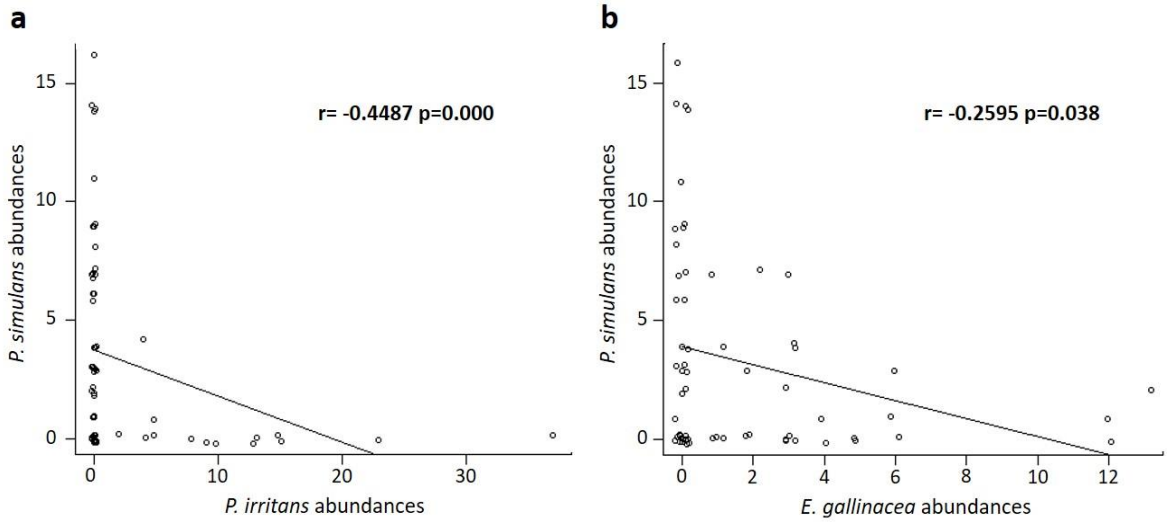

Figure 1.4. Relationship between *P. simulans* abundances and (a) *P. irritans* and (b) *E. gallinacea* abundances across eight wild carnivore species in Janos Biosphere Reserve, northwestern Mexico. Plots show the abundances of flea species in each individual host. Lines represent linear correlation of the relationship between flea dominant species.

CHAPTER 2. **Prevalence and phylogenetic analysis of *Bartonella* species of wild carnivores and their fleas in northwestern Mexico** (see next page)

EcoHealth
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EcoHealth

Original Contribution

Prevalence and Phylogenetic Analysis of *Bartonella* Species of Wild Carnivores and Their Fleas in Northwestern Mexico

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Abstract: The host–parasite–vector relationship of *Bartonella* spp. system in wild carnivores and their fleas from northwestern Mexico was investigated. Sixty-six carnivores belonging to eight species were sampled, and 285 fleas belonging to three species were collected during spring (April–May) and fall (October–November) seasons. We detected *Bartonella* species in 7 carnivores (10.6%) and 27 fleas (9.5%) through either blood culture or PCR. Of the 27 *Bartonella*-positive fleas, twenty-two were *Pulex simulans*, three were *Pulex irritans* and one was *Echidnophaga gallinacea*. The *gltA* gene and ITS region sequences alignment revealed six and eight genetic variants of *Bartonella* spp., respectively. These variants were clustered into *Bartonella rochalimae*, *Bartonella vinsonii* subsp. *berkhoffii* and another genotype, which likely represents a novel species of *Bartonella* spp. Although experimental infection studies are required to prove the vector role of *P. simulans*, our results suggest that this flea may play an important role in the *Bartonella* transmission. The results indicated possible host-specific relationships between *Bartonella* genotypes and the families of the carnivores, but further studies are needed to verify this finding. The presence of zoonotic species of *Bartonella* spp. in wild carnivores raises the issue of their potential risk for humans in fragmented ecosystems.

Keywords: *Bartonella*, prevalence, phylogeny, wild carnivores, fleas, Mexico

Introduction

Bartonella species are vector-borne and gram-negative bacteria that infect the erythrocytes and endothelial cells of mammal hosts (Boulouis et al. 2005; Chomel et al. 2009). They have been recognized as emergent and re-emergent pathogens and are of increasing concern for veterinary and human health (Breitschwerdt et al. 2010; Lantos et al. 2014). To date, about 30–40 species and subspecies of *Bartonella* genus have been described, of which at least 13 are considered zoonotic pathogens (Vayssier-Taussat et al. 2009; Kosoy et al. 2012). Among these, six *Bartonella* species and one subspecies have been reported in two domestic carnivore species and in at least 20 wild carnivore species worldwide. For example, domestic and wild felids are the main reservoirs of *B. henselae*, the causative agent of cat-scratch disease (Chomel et al. 2006). In addition to

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domestic cats, this *Bartonella* species has been found in several free-ranging and captive wild felids such as pumas (*Puma concolor*), bobcats (*Lynx rufus*), cheetahs (*Acinonyx jubatus*) and others (Yamamoto et al. 1998; Chomel et al. 2004; Molia et al. 2004; Bevins et al. 2012). Coyote (*Canis latrans*) and other wild canids are suspected to be the main reservoir of *B. vinsonii* subsp. *berkhoffii* and *B. rochalimae* (Chomel and Kasten 2010), which have been associated with endocarditis and bacteremia, respectively, in humans (Eremeeva et al. 2007; Mogollon-Pasapera et al. 2009). Serological and molecular evidence of *B. v.* subsp. *berkhoffii* infection has been reported in coyote, gray fox (*Urocyon cinereoargenteus*), island fox (*Urocyon litoralis*) and badger (*Taxidea taxus*), while *B. rochalimae* infection was reported in wolf (*Canis lupus*), coyote, gray fox, island fox, red fox (*Vulpes vulpes*) and raccoon (*Procyon lotor*) (Chang et al. 2000; Henn et al. 2007, 2009a; Schaefer et al. 2011; Quinn et al. 2012; Hwang and Gottdenker 2013).

Across the bacteria's range, host specificity of *Bartonella* species is often observed in mammals. However, little is known about predominant vectors or vector transmission to mammalian hosts and/or to people (Chomel et al. 2009). Different arthropods, such as sandflies, fleas, ticks, mites and lice, are considered potential vectors for *Bartonella* spp. infections (Tsai et al. 2011). However, previous studies suggest that fleas are the most important vector involved in the natural cycle of *Bartonella* species in wild carnivores (Sreter-Lancz et al. 2006; Gabriel et al. 2009; Millán et al. 2016). In contrast to the often host-specific relationships observed between *Bartonella* species and mammalian hosts, a variety of *Bartonella* species can infect one or more species of arthropods (Tsai et al. 2011). Although knowledge of the host–vector–parasite interaction is very important to understanding the ecological dynamics of diseases, few studies have specifically examined *Bartonella* species in fleas from wild carnivores. For instance, previous studies on fleas in carnivores reported *B. rochalimae* and *B. v.* subsp. *berkhoffii* in *Pulex simulans*, *Ctenocephalides felis* and *C. inequalis* (Gabriel et al. 2009), *B. alsatica* in *Spilopsyllus cuniculi* from a wildcat and unidentified *Bartonella* genotype in *Pulex irritans* (Marquez et al. 2009), and *B. henselae* and *B. clarridgeiae* in *Ctenocephalides felis* (Kaewmongkol et al. 2011a).

In North America, most studies intended to detect and identify *Bartonella* species in wild carnivores have been conducted in the USA, while in Mexico there was only one study on detection of antibodies against *B. henselae* in captive bobcats and a puma (Chomel et al. 2004).

The objective of this study was to investigate prevalence and genetic diversity of *Bartonella* species in wild carnivores and simultaneously the prevalence and seasonal dynamics of these bacteria in their fleas in northwestern Mexico.

Materials and Methods

Study Area and Sample Collection

We collected blood and fleas from wild carnivores during fall 2013 (October–November) and spring 2014 (April–May) in five locations within the Janos Biosphere Reserve (JBR) in northwestern of Chihuahua, Mexico (30°51′50″N, 108°30′09″W). Sampling sites were categorized as Grasslands (El Cuervo, Ejido San Pedro), Grasslands/Shrublands (Monte Verde), Shrublands (Rancho Ojitos) and Riparian/Oak Forest (Rancho San Pedro).

Sixteen trapping stations were set at 0.5- to 0.8-km intervals along a 10-km transect at each of the five sampling locations. Each station contained one box trap (Tomahawk Live Trap Inc. 30″ × 30″ × 70″ or 60″ × 20″ × 28″) and one leg-hold trap (#1.75 or #3 Victor Coil Soft Catch™) with at least 30 meters of distance between them. The traps were baited with sardine, chicken and commercial lures, such as Coon Digger I, Coyote Urine and Bobcat Gland (Kishel's®). Traps were set for nine consecutive days per site and checked at least once a day. Once captured, each individual was chemically restrained with an IM injection of ketamine hydrochloride (Anesket®) and xylazine hydrochloride (Procin®), according to the recommended doses for wild carnivores (Kreeger et al. 2002). Animals were identified, weighed, sexed and ear-tagged. Blood samples were collected (2–6 ml) from cephalic or femoral veins. Blood was transferred into microtainer tubes with EDTA and stored in liquid nitrogen in the field. It was later transferred to a −70°C freezer until laboratory testing. Each carnivore was visually examined and inspected for fleas by flea combing for 5–10 min. All fleas removed were placed in a cryovial containing 70% ethanol and stored in liquid nitrogen. Fleas were identified morphologically at the Division of Vector-Borne Diseases of the Center for Disease Control and Prevention (CDC) in Fort Collins, Colorado, USA. In order to reduce the chances that fleas might become contaminated with *Bartonella* DNA from other sources, they were removed from respective vials using forceps cleaned with DNA Away® and placed individually in single petri dishes for examination using a sterco

microscope and published taxonomic keys (Hubbard 1968; Furman and Catts 1982).

All procedures for trapping and handling carnivores followed the guidelines of the American Society of Mammalogists (Sikes and Gannon 2011) and were approved by the Animal Care Committee of the Veterinary School (UNAM) and by the Mexican Secretary of Environment and Natural Resources (Permit FAUT-0250).

Bartonella Bacteria Culture from Animals

In order to culture *Bartonella* spp. bacteria, whole blood (0.05 ml) or blood 1:4 diluted in a brain heart infusion medium was plated on brain heart infusion agar plates supplemented with 5% rabbit blood (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD). A supplement of 5% fungizone as a solubilizer was used to reduce fungal contamination. Plates were incubated at 35°C for 7–28 days in an aerobic atmosphere of 5% carbon dioxide. Plates were monitored for bacterial growth at least once a week after initial plating. Bacterial colonies were tentatively identified as *Bartonella* species on the basis of colony morphology (Kosoy et al. 1997). Selected colonies were harvested from subsequent subcultures and stored in 10% Glycerol at −80°C.

DNA Extraction and Polymerase Chain Reaction (PCR)

DNA was extracted from animal blood samples and cultures. For culturing, a heavy suspension of each isolate was placed in a heat block and boiled at 95°C for 15 min. DNA was extracted from blood following the QIAGEN QIAmp Blood and Tissue Kit Protocol (Qiagen, Valencia, CA). Finally, each flea was triturated in sterile tubes containing 100 µl of glycerol 10% and 2.5 mm of sterile glass beads in a mixer mill at 20 beats/s for 5 min. The resulting flea triturate was centrifuged at 14,000 rpm for 1 min. The supernatant was transferred to a clean micro-tube and incubated at 95°C for 10 min. Cultures were confirmed as *Bartonella* spp. by PCR amplification of *Bartonella*-specific sequences of the 16S–23S rRNA intergenic spacer (ITS: 325F–1100R) (Diniz et al. 2007) and citrate synthase gene (*gltA*) primers (781F-1137R) (Norman et al. 1995). PCR was performed on blood and fleas using the same ITS primers used on the cultures, but the *gltA* primers were different (443F-1210R) (Billeter et al. 2011a). The PCR amplifications were performed in a 25 µl reaction mixture containing nuclease free water, 1 µl each of 10 µM forward and reverse primer, 12.5 µl of GoTaqR Green Master Mix (Promega, Madison, WI) and 2.5 µl DNA. The PCR was carried out in a TaKaRa Thermal Cycler Dice TP600 (TaKaRa Bio. Tokyo, Japan), using the following parameters: ITS (325 s, 1100as): a 3-min denaturation at 95°C, followed by 55 cycles of 30 s at 95°C, 30 s at 66°C, and 30 s at 72°C, and ended with 72°C for 7 min; *gltA* (443F-1210R): a 2-min denaturation at 95°C, followed by 38 cycles of 30 s at 95°C, 30 s at 48°C, and 2 min at 72°C, and ended with 72°C for 7 min; *gltA* (781F-1137R): a 3-min denaturation at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 51°C, and 30 s at 72°C, and ended with 72°C for 7 min. All PCR amplification processes were completed by holding the reaction mixture at 72°C for 7 min. To verify the presence of amplicons of appropriate size, PCR products were first electrophoresed in 1.5% ethidium bromide-stained agarose gels and the amplicon bands were then visualized by using a UV light source.

Sequence Analyses

Amplicons of a proper size were purified using a QIAquick PCR Purification Kit (Qiagen, Germantown, MD) and sequenced in both directions using an Applied Biosystems Model 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequencing reactions were carried out in a PTC 200 Peltier thermal cycler (MJ Research, Watertown, MA) using the same primers for PCR assays at a concentration of 3.3 µM. Cycle parameters for the sequencing reactions were 96°C for 1 min, 24 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Sequences were analyzed using MEGA6 (Tamura et al. 2013) to determine the consensus of sequences for the amplified region of the *gltA* and 16 s 23 s ITS. The sequences of this study and the known *Bartonella* species retrieved from the GenBank were aligned using Clustal W. The unweighted pair group method with arithmetic mean by Kimura's 2-parameter distance method and bootstrap calculation was carried out with 1000 replications. A criterion of ≥96% homology to *gltA* was used to define groups. The novel sequences obtained in this study were submitted to GenBank.

Statistical Analysis

Fisher's exact test was used to evaluate *Bartonella* spp. prevalence associations among host species, flea species,

flea sex, season and geographic location for each *Bartonella* species. A P value < 0.05 was considered statistically significant. The analyses were performed in R software (R Core Team 2014).

Results

Bartonella Prevalence in Animals

During the two surveys (fall 2013 and spring 2014), blood samples were collected from 66 wild carnivores representing eight species from five families (Canidae, Felidae, Mephitidae, Mustelidae and Procionidae), including coyotes (*Canis latrans*), kit foxes (*Vulpes macrotis*), gray foxes (*Urocyon cinereoargenteus*), bobcats (*Lynx rufus*), raccoons (*Procyon lotor*), striped skunks (*Mephitis mephitis*), hooded skunks (*Mephitis maroura*) and badgers (*Taxidea taxus*) in JBR located in the northwestern Mexico (Table 1). In total, seven samples were positive for infection with *Bartonella* species through either blood culture or PCR on blood DNA. *Bartonella* isolates were obtained from blood of two striped skunks and one coyote. *Bartonella* DNA was amplified by ITS in four samples (one coyote, two kit foxes and one hooded skunk). Of these, only the hooded skunk was positive by both molecular targets. No *Bartonella* DNA was detected in blood collected from gray foxes, bobcats, raccoons and badgers. Five (7.6%) of these samples were positive for *B. rochalimae*, and the two other (3.0%) were positive for *B. v. berkhoffii* (Table 1).

Difference in the prevalence of *B. rochalimae* in hosts between seasons was marginally significant, having only positive samples during fall. No significant differences were found in prevalence of *B. rochalimae* and *B. v. berkhoffii* in carnivores between host species, sex, season and locations (Table 1).

Detection of *Bartonella* DNA in Fleas

A total of 285 fleas that belonged to three species were collected from the 66 carnivores and tested individually. *Pulex simulans* was the most abundant flea in coyote, gray fox and skunks, while *E. gallinacea* was the dominant species in bobcat, raccoon and badger. Finally, *Pulex irritans* was the dominant species in kit fox, being strictly associated with this host (Table 2).

Bartonella species were detected by ITS and/or *gltA* target in 27 (9.5%) fleas, including 22 (81.5%) *P. simulans*, three *P. irritans* (11.11%), one *Pulex* spp. (3.7%) and one *E. gallinacea* (3.7%). Fifteen (55.6%) of the 27 *Bartonella* PCR-positive fleas were positive for *B. rochalimae*, 11 (40.7%) were positive for *B. v. berkhoffii*, and 1 was positive for *Bartonella* sp. The prevalence of *B. rochalimae* and *B. v. berkhoffii* in fleas varied significantly among the five host species, with the highest prevalences for *B. rochalimae* occurring in fleas from skunks and foxes and for *B. v. berkhoffii* in fleas from coyotes (Table 3). The prevalence of *B. v. berkhoffii* differed noticeably among flea species, with *P. simulans* being the only flea species found to be positive. The prevalence of *B. rochalimae* in fleas varied significantly among locations but at least one flea of each species identified was found to be positive for this *Bartonella* species. No significant differences were found in prevalence of *B. rochalimae* and *B. v. berkhoffii* in fleas between seasons and flea sex (Table 3).

Sequence Analysis

The interactions between hosts, parasites and vectors, along with the specific results obtained for both molecular targets (ITS and *gltA*), are shown in Fig. 1 and in supplementary table (S1). Sequencing analysis of ITS (Fig. 2, variants I–VIII) and *gltA* (Fig. 3, variants IX–XIV) identified eight and six genetic variants, respectively, in blood and fleas from wild carnivores. Six ITS sequences and three *gltA* sequences were novel variants. The new genetic variants were submitted to GenBank with accession numbers KT807800-KT807807, KX169194.

Phylogenetic analysis of ITS showed all variants clustered into three distinct groups (Fig. 2). The first group consisted of four variants (I–IV) of 13 sequences obtained from *E. gallinacea* (1), *P. irritans* (1), and *P. simulans* (6) fleas, and from coyote (1), striped skunk (1), hooded skunk (1) and kit fox (2), all of which clustered with *B. rochalimae* (98.3–99.6% similarity). In this group, one sequence obtained from a *P. simulans* flea collected from a gray fox was identical to a strain previously described in a dog from California (GenBank acc. no. DQ676487) (Henn et al. 2009b). The other three variants were novel and were assigned with GenBank accession numbers KT807800, KT807801 and KX169194. The second group contained one genetic variant (V) with only one sequence obtained from a *P. simulans* collected from a *L. rufus*. It was 99.1% similar to a strain previously isolated from another bobcat from California (GenBank acc. no. KF437497) but distant from all other *Bartonella* genotypes (Fig. 2). The closest species to this presumably novel genotype is *B. henselae* with 91.8%

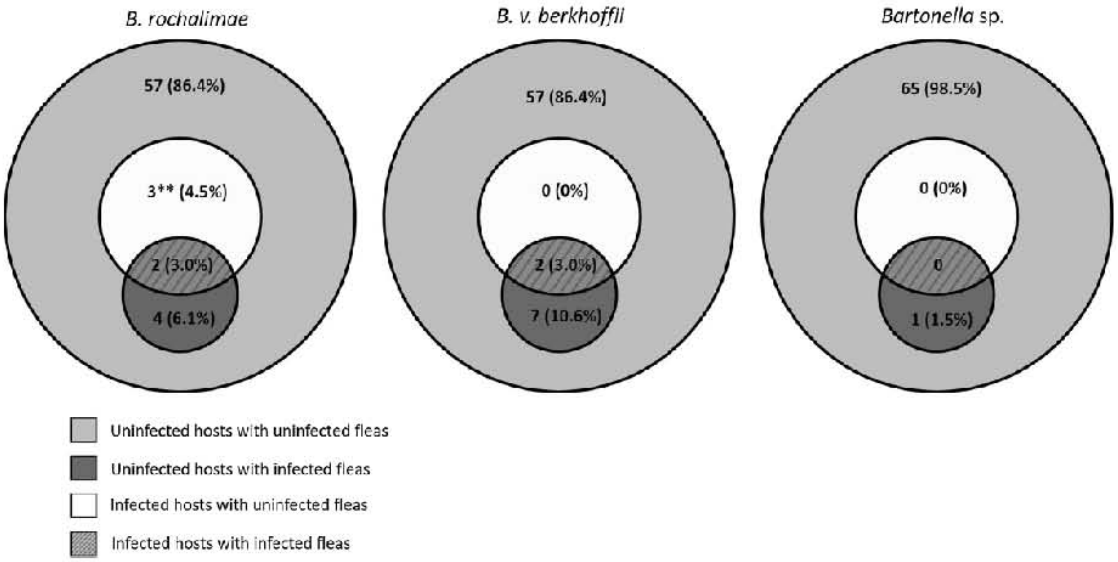

Fig. 1. Venn diagrams of infected/uninfected carnivore hosts ($N = 66$) and their fleas with *B. v. berkhoffii*, *B. rochalimae* and *Bartonella* sp. Both raw numbers of infected individuals and prevalence (%) are indicated. **Fleas were not tested.

similarity, a level of divergence typically considered indicative of a different species (La Scola et al. 2003). The third group included three variants (VI-VIII) of nine sequences obtained from coyote (1), striped skunks (1) and *P. simulans* (7) fleas collected from gray fox and coyotes, and clustered with *B. v.* subsp. *berkhoffii* (97.8-98.7% similarity). In this group, a variant (VIII) represented by one sequence was found in a *P. simulans* from a badger that was identical to a strain obtained in a dog from the USA (GenBank acc. no. DQ059763) (Kordick and Breitschwerdt 1998).

Phylogenetic analysis based on *gltA* showed a total of six variants of 24 sequences clustered into two distinct groups (Fig. 3). The first group clustered with *B. rochalimae* (99.6% similarity) and contained two variants (IX and X) of 12 sequences obtained from striped skunk (1) and hooded skunk (1) carnivores and *P. irritans* (3), *P. simulans* (3) and *Pulex* spp. (1) fleas collected from kit fox, gray fox, badger and striped skunk. These variants were identical to previously described *Bartonella* strains isolated from a human in the USA (GenBank acc. no. DQ683195) (Eremeeva et al. 2007) and from a dog in California (GenBank acc. no. DQ676488) (Henn et al. 2007; 2009b). The second group clustered with *B. v.* subsp. *berkhoffii* (98.5 and 99.6% similarity) (Fig. 3) and included four variants (XI-XIV) of 12 sequences from coyote (1) and striped skunk (1) carnivores, and *P. simulans* (10) fleas. In this group, one variant (XIV) was represented by five sequences obtained from one coyote and five *P. simulans* collected from coyote. These five sequences were identical to a strain previously found in a dog from the USA (GenBank acc. no. CP003124) (Guy et al. 2013) and were very similar (99.6% identity) to the strain detected in a human patient from Europe (GenBank acc. no. AF143445) (Roux et al. 2000). The other three variants were novel and were assigned GenBank accession numbers KT807804-KT807806.

Discussion

This is the first report of the isolation of *B. rochalimae* and *B. vinsonii* subsp. *berkhoffii* from striped skunk, the presence of *B. rochalimae* in kit fox and hooded skunk and their fleas, and the first evidence of *B. rochalimae* and *B. v.* subsp. *berkhoffii* in fleas collected from American badger.

Four carnivore species tested positive (coyote, striped skunk, badger and gray fox) and were infected with two *Bartonella* species, *B. rochalimae* and *B. v.* subsp. *berkhoffii*, while hooded skunk and kit fox were infected only with *B. rochalimae*. The relatively high prevalence of *B. rochalimae* and *B. v.* subsp. *berkhoffii* in skunks and their fleas suggested that skunks are potential reservoirs of both species.

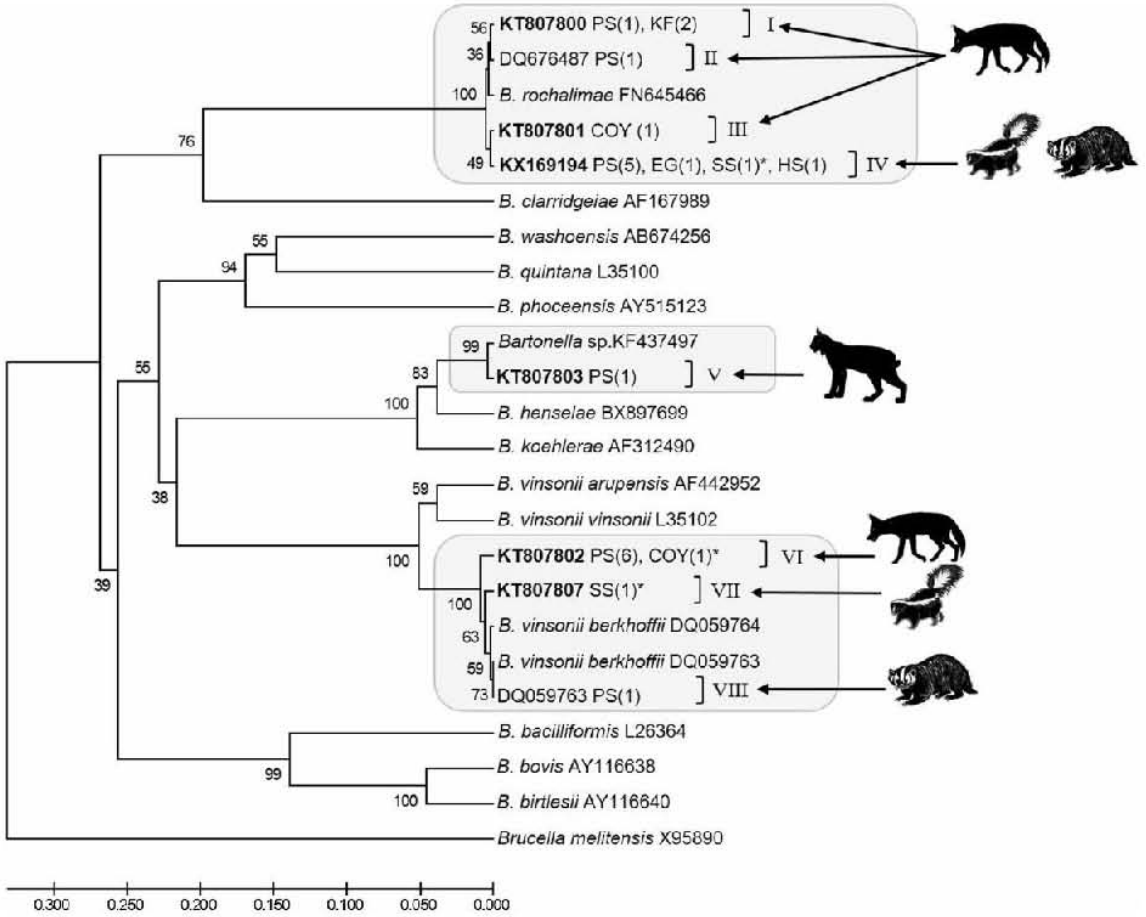

Fig. 2. Unweighted pair group method with arithmetic mean tree based on eight genetic variants (I–VIII) of the 16S–23S rRNA ITS region of three *Bartonella* species in wild carnivore and their fleas from northern Mexico. Each genotype is indicated by *roman numerals* and its GenBank accession number, with novel genetic variants in *boldface*. Drawing depicts the host families, and *black arrows* indicate their genotype association (Canidae = I, II, III and VI; Mephitidae = IV and VII; Mustelidae = IV and VIII; Felidae = V). *Capital letters* refer flea species (PS, *Pulex simulans*; Pspp, *Pulex* spp.; PI, *Pulex irritans*; EG, *Echidnophaga gallinacea*) or carnivore species (AB, American badger; BC, Bobcat; COY, Coyote; GF, Gray fox; HS, Hooded skunk; KF, Kit fox; SS, Striped skunk). *Numbers* in *parentheses* are the number of sequences obtained from blood and fleas from wild carnivores, and *asterisks* are the isolated strains. The dendrogram was constructed with 1000 replications using MEGA 6 software (Tamura et al. 2013).

The low prevalence of *B. rochalimae* in coyotes from Chihuahua was consistent with other studies in California, in which a range of 0–9.5% prevalence was reported (Chang et al. 2000; Henn et al. 2009a). In contrast, the prevalence of *B. v.* subsp. *berkhoffii* in coyotes was very low (5.9%) compared with a previous study in Coastal California that found 28% of coyotes tested were infected with *B. v.* subsp. *berkhoffii* (Chang et al. 2000). Such observation might be related to multiple factors, such as animal ages, seasonal dynamics, and the species of arthropod ectoparasites found on these coyotes.

Several molecular studies have shown that the prevalence of *B. rochalimae* and *B. v.* subsp. *berkhoffii* infection differs between species of foxes. The overall prevalence (13.33%) of *B. rochalimae* in kit foxes from Janos grasslands was higher than the 5.9% (3/51) and 1.6% (1/62) observed in the island fox from Santa Rosa Island and in the red fox from Spain, respectively (Schaefer et al. 2011;

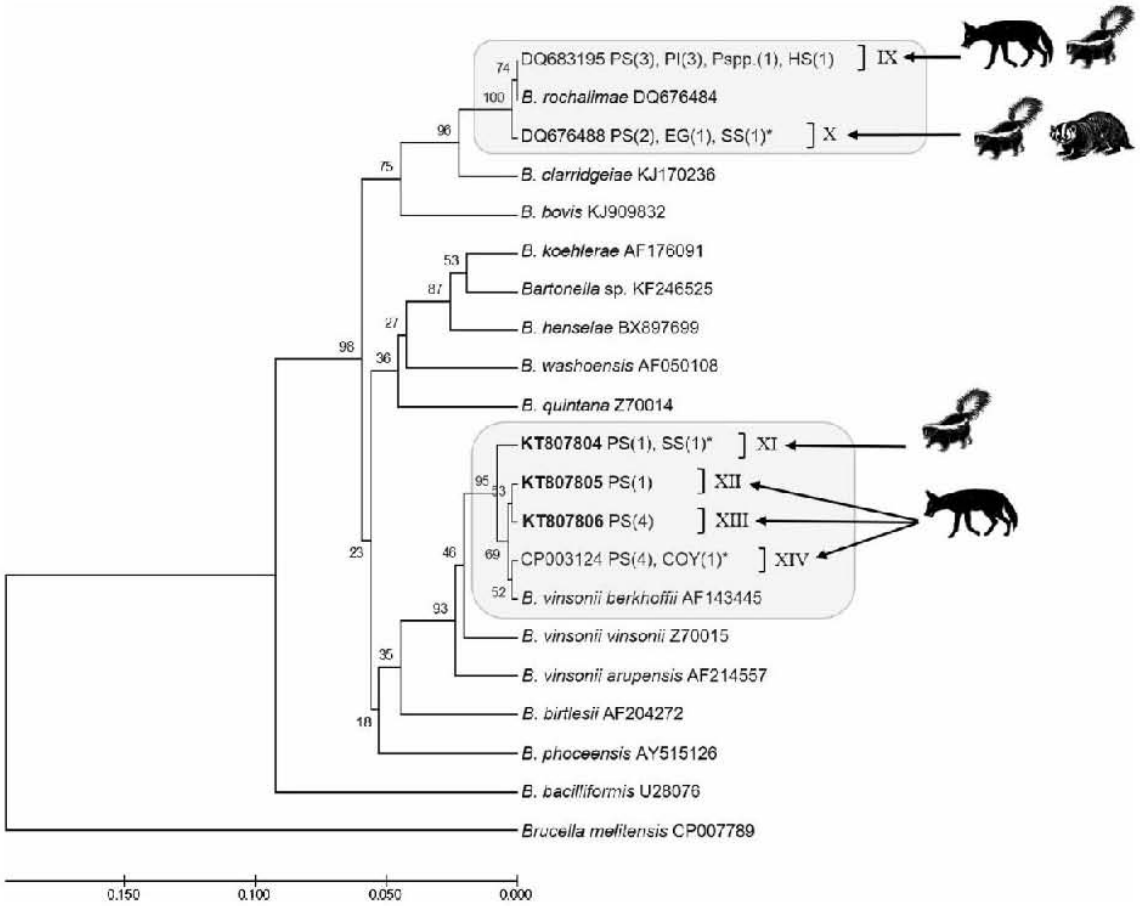

Fig. 3. Unweighted pair group method with arithmetic mean tree based on six genetic variants (IX–XIV) of *gltA* gene fragment of two *Bartonella* species in wild carnivore and their fleas from northern Mexico. Each genotype is indicated by *roman numerals* and its GenBank accession number, with novel genetic variants in *boldface*. Drawing depicts the host families, and *black arrows* indicate their genotype association (Canidae = IX, XII, XIII and XIV; Mephitidae = IX, X and XI; Mustelidae = X). *Capital letters* refer flea species (PS, *Pulex simulans*; Pspp, *Pulex* spp.; PI, *Pulex irritans*; EG, *Echidnophaga gallinacea*) or carnivore species (AB, American badger; BC, Bobcat; COY, Coyote; GF, Gray fox; HS, Hooded skunk; KF, Kit fox; SS, Striped skunk). *Numbers* in *parentheses* are the number of sequences obtained in blood and fleas from wild carnivores, and *asterisks* are the isolated strains. The dendrogram was constructed with 1000 replications using MEGA 6 software (Tamura et al. 2013).

Gerrikagoitia et al. 2012), but less than half of the prevalence reported in gray foxes (42%) from California (Henn et al. 2007).

As far as we know, little research has been done for host–parasite–vector relationship to understand *Bartonella* spp. system in a particular wild carnivore species and its fleas (Gabriel et al. 2009; Kaewmongkol et al. 2011a). These previous studies found distinct *Bartonella* spp. patterns among two fox species and their fleas. Among these, they have reported four distinct patterns: (1) bacteremic host that harbored fleas infected with the same *Bartonella* species; (2,3) bacteremic hosts that carried negative fleas or positive fleas that carried other *Bartonella* species; and (4) non-bacteremic hosts that can harbor *Bartonella*-positive fleas. In bacteremic hosts, we found that four of the seven bacteremic hosts (coyote [1], hooded skunk [1] and striped skunk [2]) harbored at least one *P. simulans* infected with the same *Bartonella* genotype. Furthermore, these same bacteremic carnivore hosts also harbored non-infected *P. simulans* and *E. gallinacea* fleas. These non-infected fleas

Table 1. Carnivore Abundances and Comparison of *Bartonella* Infections in Carnivores Between Host Species, Sexes, Seasons and Locations.

		B. rochalimae		*B. v. berkhoffii*	
	Host abundances	Positive hosts (%)	*P* value[1]	Positive hosts (%)	*P* value[1]
Host species			0.609		0.803
Badger	6	0		0	
Bobcat	5	0		0	
Coyote	18	1 (5.6)		1 (5.6)	
Gray fox	7	0		0	
Hooded skunk	3	1 (33.3)		0	
Kit fox	15	2 (13.3)		0	
Raccoon	4	0		0	
Striped skunk	8	1 (12.5)		1 (12.5)	
Host sex			1.0		0.509
Female	27	2 (7.4)		0	
Male	39	3 (7.7)		2 (5.1)	
Season			0.055[†]		1.0
Fall	35	5 (14.3)		1 (2.9)	
Spring	31	0		1 (3.2)	
Location			0.749		0.743
El Cuervo	11	1 (9.1)		0	
Ojitos	12	1 (8.3)		0	
Monteverde	8	0		0	
San Pedro	23	1 (4.3)		2 (8.7)	
La Báscula	12	2 (16.7)		0	
Total	66	5 (7.6)		2 (3.0)	

[1]*P* value based on Fisher exact test.
[†]Marginally significant.

could be explained by recent host switching from a non-bacteremic host (Gutiérrez et al. 2015) or by poor vector competence. We could not classify the host–parasite–vector relationships of the three other bacteremic hosts, because their fleas were not tested by *Bartonella* PCR. In this study, we did not find any bacteremic host harbored positive fleas with other *Bartonella* species. We did not find evidence for coinfection of different *Bartonella* species in individual animals. These results contrast with some studies that have reported cases of coinfection in rodents and their fleas (Abbot et al. 2007; Birtles et al. 2001; Kosoy et al. 2004; Telfer et al. 2007; Gutiérrez et al. 2014). But our results are consistent with most surveys performed in populations of wild carnivores and/or their fleas that also had no evidence of this patterns (Chang et al. 2000; Kaewmongkol et al. 2011a; Gerrikagoitia et al. 2012; Sato et al. 2012; Bai et al. 2016). As far as we know, there are only three studies that found two different *Bartonella* species or strains in predatory animal or their fleas: one in a coyote (Henn et al. 2009a) and another in *Pulex simulans* fleas collected from gray foxes that appear to be coinfected with *B. rochalimae* and *B. v. berkhoffii* (Gabriel et al. 2009). The third study found two different genotypes of *B. washoensis* in a *Cediopsylla inaequalis* flea collected from a red fox (Brinkerhoff et al. 2010). Two plausible explanations for the lack of finding *Bartonella* coinfection in wild carnivores and/or their fleas could be: (1) the effect of a competitive exclusion between the different *Bartonella* species or genotypes; or (2) the underestimation of the coinfection rate due to the detection assay technique that is usually focused on the predominant genotype instead of low abundant genotypes (Gutiérrez et al. 2014). However, more research is needed to understand the coinfection rates of *Bartonella* and their ecological bases in wild carnivores and their fleas.

Table 2. Flea Abundances and *Bartonella* spp. Infection in Fleas From Eight Wild Carnivore Hosts.

Flea species	Infected fleas/flea abundances							
	Badger	Bobcat	Coyote	Gray fox	Hooded skunk	Kit fox	Raccoon	Striped skunk
P. simulans	2/10	1/6	8/43	4/22	4/12	0/3	0/5	3/27
P. irritans	0/0	0/0	0/0	0/0	0/0	3/54	0/0	0/0
Pulex spp.	0/4	0/0	0/7	0/8	0/0	1/7	0/0	0/3
E. gallinacea	1/20	0/12	0/3	0/1	0/3	0/16	0/12	0/7
Total	3/34	1/18	8/53	4/31	4/15	4/80	0/17	3/37

Finally, 11 non-bacteremic hosts (coyote [3], badger [2], bobcat [1], gray fox [3], kit fox [1] and striped skunk [1]) were carrying *P. simulans* (13), *P. irritans* (3) and *E. gallinacea* (1) positive fleas. Interestingly, we observed two *P. simulans* fleas collected from the same gray fox with two different *Bartonella* species, *B. rochalimae* and *B. v.* subsp. *berkhoffii*.

Despite the fact that *Bartonella* species have been previously reported from analyses of serum and blood from raccoons (Henn et al. 2009a; Sato et al. 2012; Hwang and Gottdenker 2013), American badger (Quinn et al. 2012) and gray foxes (Henn et al. 2007; Schaefer et al. 2012), we did not find *Bartonella* species in these three species. The small sample size may explain these results. However, because we found seven *Bartonella* PCR positives in fleas (*P. simulans* [6] and *E. gallinacea* [1]) taken from three gray foxes and two badgers, some other possible explanations for these observations might be made, including 1) the lack of sufficiently sensitive blood culture and PCR methods for detecting low levels of bacteremia; or 2) a short duration of bacteremia which is possibly associated with non-reservoir hosts (Henn et al. 2007; Breitschwerdt et al. 2010). This second hypothesis would be in contrast with the idea that foxes from the genus *Urocyon* are competent reservoirs for *B. v.* subsp. *berkhoffii* (Schaefer et al. 2011). For this reason, it is likely that the biological explanation lies in intrinsic factors associated with the fleas found infesting these animals. It has been suggested that the *Bartonella* spp. sequences obtained from fleas collected from non-bacteremic hosts may reflect more than just a recent blood meal on a bacteremic host (Gabriel et al. 2009); it could be explained by the reproduction of the bacteria in the flea gut (Higgins et al. 1996; Bouhsira et al. 2013) or by a previous encounter of these infected fleas with another positive host (Gutiérrez et al. 2015). However, further studies will be necessary to prove all these hypotheses.

Similar to our findings, there is a growing number of studies where the inconsistencies between the *Bartonella* phylogenetic trees based on use of different markers were observed. For example, reporting molecular detection of *Bartonella* species in ticks from Peru, Billeter et al. (2011b) found *B. rochalimae* in a tick by ITS and *B. quintana* and *B. elizabethae* from two separate *nuo*G PCRs from the same tick. This can be explained by a coexistence of different *Bartonella* species in one insect, but also by the possibility of recombination events. McKee et al. (2016) argued that the later gene transfer and recombination events could confound patterns of *Bartonella* species cophylogeny. These authors wrote: "the events may not represent invasion by an entirely separate species of Bartonella, but rather the gltA gene that has undergone homologous recombination into a separate genome after coinfection of two species within an individual mammalian or arthropod host." More intensively, this phenomenon has been explored by Buffet et al. (2013) who explained contrasting phylogenies as an evidence of later gene transfer between different *Bartonella* species. On the other hand, the culture-positive, but PCR negative specimens might be related to the cohesion and aggregation factors of *Bartonella* species (Roy et al. 2001).

Significant variations in prevalence of *B. v. berkhoffii* were observed between flea species from wild carnivores. Of the three flea species that were collected in this study, *P. simulans* was most numerous and had the highest *Bartonella* spp. prevalence. Similar findings were reported by Gabriel et al. (2009), who detected 93% of the 42 *Bartonella* PCR-positive fleas as *P. simulans* in gray foxes. These results and the fact that positive hosts harbored *P. simulans* fleas infected with the same *Bartonella* genotype indicate that this flea may play an important role in both, intraspecific and interspecific transmission of *Bartonella* spp. in wild carnivores from North America. However, further investigations are required to elucidate the trans-

Table 3. Frequency of PCR-Positive Reactions for *Bartonella* Species in Fleas From Wild Carnivores by host, Flea Species, Flea Sex, Season and Location.

		B. rochalimae		*B. v. berkhoffii*		*Bartonella* sp.	
	Flea abundances	Positive fleas (%)	*P* value[1]	Positive fleas (%)	*P* value[1]	Positive fleas (%)	*P* value[1]
Host			0.015*		0.005*		0.175
Badger	34	2 (5.9)		1 (2.9)		0	
Bobcat	18	0		0		1 (5.6)	
Coyote	53	0		8 (15.1)		0	
Gray fox	31	3 (9.7)		1 (3.2)		0	
Hooded skunk	15	4 (26.7)		0		0	
Kit fox	80	4 (5.0)		0		0	
Raccoon	17	0		0		0	
Striped skunk	37	2 (5.4)		1 (2.7)		0	
Flea species			0.173		0.003*		1.0
P. simulans	128	10 (7.8)		11 (8.6)		1 (0.8)	
P. irritans	54	3 (5.6)		0		0	
Pulex sp.	29	1 (3.4)		0		0	
E. gallinacea	74	1 (1.4)		0		0	
Flea sex			0.579		1.0		0.3230
Female	191	9 (4.7)		8 (4.2)		0	
Male	94	6 (6.4)		3 (3.2)		1 (1.1)	
Season			1.0		0.209		0.404
Fall	115	6 (5.2)		2 (1.7)		1 (0.9)	
Spring	170	9 (5.3)		9 (5.3)		0	
Location			0.010*		0.085		0.637
El Cuervo	42	6 (14.3)		0		0	
La Báscula	46	0		4 (8.7)		0	
Monteverde	44	0		1 (2.3)		0	
Ojitos	50	4 (8.0)		0		1 (2.0)	
San Pedro	103	5 (4.9)		6 (5.8)		0	
Total	285	15 (5.26)		11 (3.9)		1 (0.4)	

[1]*P* value based on Fisher exact test.
*$P < 0.05$.

mission mechanism(s) of *B. rochalimae* and *B. v.* subsp. *berkhoffii*. On the other hand, the finding of *Bartonella* spp. DNA in only one of seventy-four *E. gallinacea* fleas tested was consistent with several studies from Africa which reported that none of more than three hundred fleas of this species were *Bartonella* spp. positive (Loftis et al. 2006; Sackal et al. 2008; Leulmi et al. 2014). These results may reflect that *E. gallinacea* fleas are less competent and poor vectors for *Bartonella* transmission.

Interestingly, despite the fact that canids, mephitids and mustelids may be infected by *B. rochalimae* and *B. v.* subsp. *berkhoffii*, our results suggest association relationships between host family and genetic variants strains. Variants I, II, III, VI, IX, XII, XIII and XIV were canidae host-specific. Variants IV, VII, VIII, X and XI were identified in mephitids and mustelids. By contrast, variant V was associated with felids. Only one sequence obtained in blood from a hooded skunk was included in variant X, which was mainly associated with canids. Nevertheless, the ITS sequence of this same host was clustered with fleas and blood sequences from mephitids. The finding that *Bartonella* spp. sequences from mustelids were clustered with mephitids may be attributed to the fact that they show a closer phylogenetic relationship than canids and felids (Nyakatura and Bininda-Emonds 2012) and were previously considered to belong to the same family (Mustelidae).

Finally, the low genetic distances among the genotypes of *B. rochalimae* and *B. v.* subsp. *berkhoffii* obtained in this study may reflect a recent split between these *Bartonella* strains. *Bartonella* spp. bacteria may infect a number of mammalian reservoirs and a host specificity of *Bartonella* species is often observed to each host species (Chomel et al. 2009). Previous studies found stronger evidence of host–parasite cospeciation of *Bartonella* with a strong association observed between specific *Bartonella* strains and certain rodent hosts (Ying et al. 2002; Kosoy et al. 2003; Castle et al. 2004). In addition, some authors reported coevolutionary patterns between bat and rodent hosts and *Bartonella* genotypes (Lei and Olival 2014) and a close association between Australian marsupials, their fleas and *Bartonella* species (Kaewmongkol et al. 2011b). One hypothesis previously exposed to explain the host-specificity relationships is the limited host range of their vectors (Vayssier-Taussat et al. 2009). However, all the positives fleas in this study are considered truly generalist (Traub 1985; Eads et al. 2015). For this reason, the most likely explanations for our findings regarding the host-specificity tendency may relate to intrinsic factors of hosts and bacteria, such as immune and virulence factors (Vayssier-Taussat et al. 2009). Finally, it is important to note that because of the modest sample size these findings are not completely conclusive and further studies are needed to confirm this evidence.

The *Bartonella* strains found in this study may be transmitted among different wild carnivores and even incidentally to dogs and humans. We identified two *B. rochalimae* and two *B. v.* subsp. *berkhoffii* genotypes that were identical to strains previously reported in dogs. While variant X was identical to a strain isolated from a damaged aortic valve from a dead dog (Henn et al. 2007, 2009b), variants II, VIII and XIV were identical to different strains previously reported in blood from three healthy dogs (Kordick and Breitschwerdt 1998; Henn et al. 2009b; Guy et al. 2013). In addition, two *Bartonella* genotypes reported in this study were similar to strains previously described in humans. One of them (variant IX) was 100% identical to a strain isolated from a human case of bacteremia, fever and splenomegaly (Eremeeva et al. 2007). Despite the fact that the variant XIV was identical to a *B. v.* subsp. *berkhoffii* strain isolated from a subclinical infection in dogs (Guy et al. 2013), this genotype had 99.62% similarity compared with a strain previously detected from a human case of endocarditis (Roux et al. 2000). These findings suggest that *Bartonella* strains found in wild carnivores and their fleas from JBR are maintained naturally in this region and further studies are needed to identify potential cross-species transmission and possible zoonotic events. In JBR and nearby areas, human settlements and farming activities are increasing (Ceballos et al. 2010); therefore, contacts between wildlife, domestic animals and humans may increase. For instance, in some human settlements at JBR, domestic dogs occur at high densities and have free-roaming activities that may increase encounters with wild carnivores (Almuna 2016). In this case, an example of potential risk of exposure to *Bartonella* infection in humans and domestic dogs may come from *Pulex* spp. that are generalist fleas and are infected in this area with zoonotic *Bartonella* strains. The JBR has suffered extensive and rapid changes in landscape configuration characterized by native grassland loss cover and expansion of shrublands, driven by overgrazing, intensive agriculture and loss of keystone species (Fredrickson et al. 2006; Ceballos et al. 2010; Martinez-Estevez et al. 2013).These changes have affected the distribution, abundance and diversity of wildlife species (Ceballos et al. 2010), which in turn, may modify disease dynamics, affecting disease risk to animals and humans (Daszak et al. 2000; Keesing et al. 2010). In this study, the interactions between kit foxes, *Bartonella* spp. and fleas appear to be particularly sensitive. While *P. irritans* fleas were restricted to kit fox hosts, kit foxes have been mainly associated with grassland, the most threatened habitat in JBR (López-Pérez et al., in preparation). Finally, along with this work, recent studies in this region have reported several zoonotic agents such as *Leptospira interrogans*, hantavirus, and other *Bartonella* species in wildlife (Moreno-Torres et al. 2014; Rubio et al. 2014; Montiel-Arteaga et al. 2015; Rubio et al. 2015; Fernández-González et al. 2016). These findings support the fact that JBR is a study area that needs further investigations on disease dynamics at the interface of wildlife, domestic animals and humans.

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CHAPTER 3. **Can a declining population of black-tailed prairie dogs (*Cynomys ludovicianus*) in the Janos Biosphere Reserve of Mexico be explained by epizootic events of plague? Using wild carnivores as sentinels for plague surveillance**

INTRODUCTION

Sylvatic plague, caused by the gram-negative bacterium *Yersinia pestis*, is a flea borne disease associated with rodents that was introduced to the west coast of North America from Asia in 1899 (1,2). Seven years after the first human case was reported in 1900, plague was considered endemic in the United States and began to expand its distribution, eventually crossing 2,250 km in 40 years to the eastern Great Plains (3). In Mexico, there were reports of human cases in Mazatlan and Veracruz in 1903 and 1920, respectively (4); however, there were no cases reported of human plague in later years (5). Although the mechanisms of the plague expansion in North America are poorly understood, it is known that subsequently new plague foci became established in wild rodent populations in several western states of the United States (3,6). In addition, in the early 1950´s some evidence was found indicating the establishment of plague enzootic foci in rodents from Coahuila, Mexico (7) but the status of these foci is at present uncertain and no human cases or rodent epizootics have been reported from this region in recent decades. It has been speculated that these plague foci are maintained and amplified by two transmission cycles. The enzootic cycle is thought to involve partially resistant hosts (maintenance hosts) characterized by a heterogeneous response to plague infection. Alternatively, the disease may spread from the presumed maintenance hosts supporting this enzootic cycle to an epizootic cycle involving amplifying hosts that are highly susceptible to plague and typically experience high mortality among their populations when exposed to the disease (2,8). However, this hypothesis has been supported by little evidence and consequently has been questioned during the past 10-15 years (2,9). Although, other hypotheses have been proposed to explain the persistence of plague, such as long-term persistence in fleas, hibernating hosts, or soil (8), the spread of plague among rodents and other highly susceptible mammals is well-documented and known to be of importance to both human health and wildlife conservation. Among the mammals this generalist pathogen may infect are prairie dogs (*Cynomys* spp.), a genus of hosts that

appears to be among the most susceptible, as indicated by the high levels of mortality observed in *Y. pestis*-infected colonies of these animals. The black-tailed prairie dog (*Cynomys ludovicianus*) covered over 30 million ha of North America, but their populations have been decimated to less than 2% in the past hundred years (10) due to habitat loss, eradication programs, severe droughts, and plague epizootics (11,12). Since the first reports in 1938 (*C. gunnison*) and the 1940´s (*C. ludovicianus*) (13,14), prairie dogs have suffered plague epizooties with near 100% mortality that has led to the local extinction of some populations (15,16). Plague represents an essential concern for prairie dog conservation efforts, which in turn poses a conservation concern for other species due to its ecological roles these animals play as keystone species and ecosystem engineers (17–20). In these roles prairie dogs are important for creating and maintaining grassland habitats, serving as essential prey for several species of mammalian carnivores and raptor birds, giving refuge in their burrows to many species and increasing regional biodiversity (21–24).

The Janos Biosphere Reserve (JBR), located in the state of Chihuahua, Mexico, consists of 220,000 ha of native grassland and is one of the areas of greatest importance for conservation in Mexico and North America (25). In this Reserve remain the largest complex colonies of prairie dogs (26), and the only significant complex left in the arid grassland system of the American Southwest and northwestern Mexico (27). However, over the last two decades the area that remains actively occupied by black-tailed prairie dog in JBR decreased more than 95% from the 55,258 ha of prairie dog colonies previously reported (28). Although black-tailed prairie dog conservation in JBR faces threats such as habitat loss and natural droughts, the decline of colonies could be a result, at least in part, of epizootic and enzootic plague (29). Consistently, the extinction of some prairie dog colonies in JBR in areas without agricultural activities has been recognized (12,28,30). In addition, there is evidence of enzootic and epizootic plague activity in the border state of New Mexico which has reported a total of 251 human cases during the period from 1965–2012, a figure that comprises 53.6% of human cases for that period in the US (6). Others studies have detected plague positive fleas in Luna County, New Mexico (31), which is located only 100 km away from the closest prairie dog colonies in the JBR.

It is expected, geographically and ecologically, that there is an enzootic cycle of plague in JBR that could explain the decrease of black tailed prairie dogs in the region. In order to detect the presence of *Y. pestis* bacterium in JBR our group has conducted several surveys on different taxa, including molecular and serologic approaches in rodents and their fleas (32–34). Some of these wild hosts and ectoparasites have a key role in the dynamic and ecology of plague. Although not important as hosts for maintaining plague in nature through the infection of feeding fleas, their habit of preying on plague-susceptible rodents means that wild canids often become infected. Once infected they can produce detectable antibody responses that can persist for many months, which makes these animals good sentinel hosts for *Y. pestis* (35,36). For this reason, and to continue assessing the potential presence of this pathogen in the JBR, we initiated a study to detect the presence of antibodies against *Y. pestis* in wild carnivores from this area. The results of this study, which did not find serological evidence of plague in JBR, are described in this manuscript. Additionally, we describe the various plague surveys conducted in JBR and discuss whether the negative results obtained should be considered conclusive regarding the role of the disease on the population ecology of black-tailed prairie dogs in JBR.

MATERIAL AND METHODS

Study sites and trapping

We captured carnivores during fall 2013 (October-November) and spring 2014 (May-June) in five trapping locations (El Cuervo, La Bascula, Monte Verde, Rancho Ojitos and Rancho San Pedro) within the Janos Biosphere Reserve (JBR) in Chihuahua, Mexico (30º 51´50´´N, 108º 30´09´´W) (Figure 1). Sixteen trapping stations were placed at 0.5-0.8 km intervals along a 10 km transect at each of the five sampling locations. Each station contained one box trap (Tomahawk Live Trap Inc.WI) and one leg-hold trap (Victor Coil Soft Catch™) separated by at least 30 meters. Traps were baited with canned sardine, chicken and beef and commercial lures and were open for nine consecutive days per site and checked at least once a day. Each individual was anesthetized with an IM injection of ketamine hydrochloride (Anesket®) and xylazine hydrochloride (Procin® 2%), according to the reported doses for carnivores (37). Animals were identified, weighed, sexed and ear-tagged.

Blood samples were collected via venipuncture of cephalic or femoral and a small volume of blood was applied onto a Nobuto filter paper strip and dried.

All procedures for handling carnivores were carried out in accordance with the guidelines of the American Society of Mammalogists (38) and were approved by the Ministry of Environment and Natural Resources of Mexico (Permit FAUT-0250).

Laboratory Tests

For the extraction of the serum, Nobuto ® strips were placed in a buffered solution of phosphate at pH of 7.2 to 4 ° C for 5 hours. All serum samples were tested for the presence of antibodies to *Y. pestis* Fraction 1 (F1) antigen by the passive hemagglutination (PHA) and inhibition tests (PHI) (39). A titer of $\geq$ 1:16 was considered positive. All samples were processed in the Division of Vector-Borne Diseases of the Center for Disease Control and Prevention (CDC) in Fort Collins, Colorado, United States.

Data analysis

We performed a systematic review of all published literature available to identify studies with data on plague epidemiology, ecology population and distribution of black-tailed prairie dogs from Janos Biosphere Reserve. Data were extracted from a variety of databases-PubMed, and Google scholar using a combination of the search terms: abundances, density, distribution, prairie dogs, Janos, plague, *Yersinia pestis*. We include all reports, theses, conference proceedings and articles that studied abundances and distribution area of the black-tailed prairie dogs inhabit Janos complex and no publication date or publication restrictions were imposed. The search limits were as follows: language ("English and Spanish") and species ("Prairie dogs, *Cynomys ludovicianus*").

RESULTS AND DISCUSSION

We captured sixty-six wild carnivores belonging to eight species of seven genera within five families (Canidae, Felidae, Mephitidae, Mustelidae and Procyonidae) during October-November 2013 and May-June 2014. All serum samples were negative for *Yersina pestis* (Table 1).

In addition to our results, we analyzed the data of three other previous studies on plague ecology and epidemiology (Table 2) and a total of four studies on population ecology of black tailed prairie dog in JBR spanning 25 years (Figure 2).

Although we have not been able to demonstrate the presence of plague in the current or previous studies, the presence of certain factors, such as the lack of a systematical surveys and/or physiological response of the animals could have affected the results which implies that our studies may be inconclusive. Furthermore, the time lapsed since an area's last epizootic, as plague is much more difficult to detect during interepizootic periods when *Y. pestis* is circulating at low levels among enzootic hosts or perhaps in other unknown reservoirs. A total of four studies have attempted to find the presence of *Y. pestis* in JBR. Two of them were based on molecular approaches and focused particularly on fleas from prairie dogs and from small rodents. The other two studies relied on serological tests performed on samples collected from rodents and carnivores. It is well known that these approaches can detect infections over variable windows of time, with the molecular detection of *Y. pestis* DNA in fleas reflecting actual active infections and the detection of plague antibodies in carnivore serum providing evidence of past infections. The values for both molecular detection and antibody titers over these times depend on the immunology of the host species and the diagnostic methods.

Molecular diagnosis methods are based on the ability to detected the presence of bacteria DNA, which is linked to the bacteremic levels in the target host species (40,41). The incubation period for plague is 1 to 4 days in mammals (42), and detectable bacteremia occurred within 24-48 hours after infection in rodents (43) and carnivores (44). Susceptible infected hosts die within 4-20 days (15,44). However, many species of mammals are resistant to plague and fail to develop sufficient levels of bacteremia to infect feeding fleas (8). It is recognized that a flea must feed from an infected host with at least 10^6 cfu/mL bacteremia level to contract the infection (45). In addition, the extrinsic incubation period for plague in fleas (i.e. the time from when the flea first becomes infected until it can actually transmit), including early transmission phase or transmission by blocked fleas, ranges from 1 to 31 days (45,46). Although at least 28 flea species are capable of transmitting *Y. pestis* in North America, the transmission efficiency among these may be highly variable (47) and the

prevalence of infection in fleas can be very low, even in regions with epizootic events (48). For all these reasons the probability of finding fleas positive to plague is relatively low and most likely to occur during or within a few weeks after an epizootic has occurred.

On the other hand, serological tests detect infections over a wider window of time than molecular methods. For this reason, antibodies against *Y. pestis* are preferred for epidemiological researches or for a retrospective confirmation of the disease (49). However, the only previous study that investigated the presence of antibodies against *Y. pestis* in JBR was carried out in black-tailed prairie dogs (32). Because mortality of black-tailed prairie dogs range from 90 to 100% during plague epizootics (15), the probability to detect antibodies in this target host is very low. In order to improve the probability to confirm the presence of plague in JBR system we need to focus on more resistant hosts, such as grasshopper mice (*Onychomys* spp.) or carnivore species. Stapp et al. (9) reported that the emergence of serologic evidence of *Y. pestis* infections in *Onychomys leucogaster* coincided with epizootic die-offs of black-tailed prairie dogs. But as they reported, the detection of antibodies against *Y. pestis* could only be done over a year-long window of time after the epizootic event.

Several studies have found evidence that carnivore species may serve as sentinels for plague. The ability of carnivores to play such a role relies on the fact that although capable of becoming infected, they are mostly resistant to *Y. pestis*-related mortality and display no obvious or only very mild signs of disease despite producing detectable and specific antibody responses (44,50). Carnivores may acquire *Y. pestis* infection by ingesting infected prey or as a result of being bitten by infected fleas (44,51). For this reason, it is hypothesized that sampling a few rodent predators may be equivalent to sampling hundreds of rodents for evidence of plague infection (52). Still, even using carnivores as sentinels, the duration of the antibody response in these animals only persists for several months and the prevalence is often < 5% during inter-epizootic periods (52). Experimental infection studies of plague found that antibody titers persist for at least 219 days in mustelids (53), 330 days in dogs and cats (44), and 100 days (50) and 8 months (51) post-inoculation in other wild carnivores. Despite the fact that serological tests represent the best approach to determine if plague has spread to JBR, we only could detect the infection if it occurred less than one year previously.

Therefore, combining previous results based on the ecology of plague with those obtained using data on the population dynamics of black-tailed prairie dogs in JBR (Figure 2) allows us to understand why all the epidemiology results obtained in JBR are inconclusive. For instance, field-based and modeling approaches have reported epizootic outbreaks with durations of 2 to 10 months (14,54–56). As reported above, considering the antibody response durations of 1-12 months and assuming a contact with bacteria at the end of the epizootic event, it is probable that we have no more than 22 months to detect plague infections in resistant carnivores or rodents once the die-off of prairie dogs starts. Finally, when we compare the window of time of plague detection with the inter-epizootic periods of 1-13 years (2,57) and the black-tailed prairie dog population dynamic in JBR, it is evident that the population data are based on nonrecurring surveys. Those results were obtained from four surveys over 25 years (26,28,30,58) and they are too dispersed in time to know exactly what is the year when the populations of prairie dogs collapse, or how their populations were decreasing. Therefore, the question of whether plague is causing a decrease in the prairie dog population in JBR remains unanswered.

Furthermore, there are important ecology and epidemiology conditions that contribute to the understanding of this system. Natural cycles of plague are often characterized by short epizootic events in susceptible wild rodents, such as prairie dogs, and interspersed by long periods of latent quiescence (2). Although the mechanism to explain interepizootic periods remain unclear, one hypothesis suggests that *Y. pestis* is maintained enzootically between epizootics in resistant rodent hosts (2,8,59). Previous studies have suggested that *Peromyscus maniculatus* and *Onychomys leucogaster* species could be considered enzootic hosts of plague (2,57,60,61). Both species are present in the Janos ecosystem (62,63). In addition, the flea community in rodents and carnivores from JBR is composed of species that include, *Echidnophaga gallinacea*, *Euhoplopsyllus glacialis*, *Oropsylla hirsuta*, *Oropsylla montana*, *Orchopeas leucopus*, *Orchopeas sexdentatus*, *Pleochaetis exilis* and *Pulex* spp. (34) (López-Pérez prep.). All these flea species have been reported to be naturally infected with *Y. pestis* in prairie dog complexes and might play a role in spreading plague (11,64,65). Yet, some evidence suggests that instead of species assemblages, it is the population ecology of some species of rodents that could determine the presence of plague (66). The abundances of

enzootic hosts could be one of the possible explanations of the absence of disease in Janos systems. For instance, previous studies report that *P. maniculatus* and *O. leucogaster* are more abundant in regions where plague has been reported (22,66). In contrast, these two species tend to be rare and less abundant in Janos desert and grasslands (62,63). However, previous studies found that the abundances of *O. leucogaster* are dynamic across areas of plague activity (59,61), being higher during small lapses of time before the epizootic events and decreasing post epizootic.

In conclusion, although we have not detected any evidence of *Y. pestis* in our surveys, there are many hints that still suggest the existence of an enzootic-epizootic cycle of plague and that this disease could provide a viable explanation for the population decrease of black tailed prairie dogs observed in JBR. In order to retest our hypothesis, a systematic multiyear study, such as a long-term ecological research (LTER) project is required, including an integral approach of epidemiological surveillance of plague in resistant hosts and a close and systematic monitoring of prairie dog populations. Prairie dogs are considered good sentinels for plague because their diurnal behavior and large body size make observations easy to detect the presence of plague by the massive die-off (14). We suggest that these monitoring and surveillance efforts must be implemented and conducted as soon as possible in order to better understand the rapid decline of black-tailed prairie dogs in this area of high conservation value. Disease management strategies for prevention and control of plague such as vaccination of prairie dogs (67) and vector control (68) could be applied once there is evidence of *Y. pestis* in the area.

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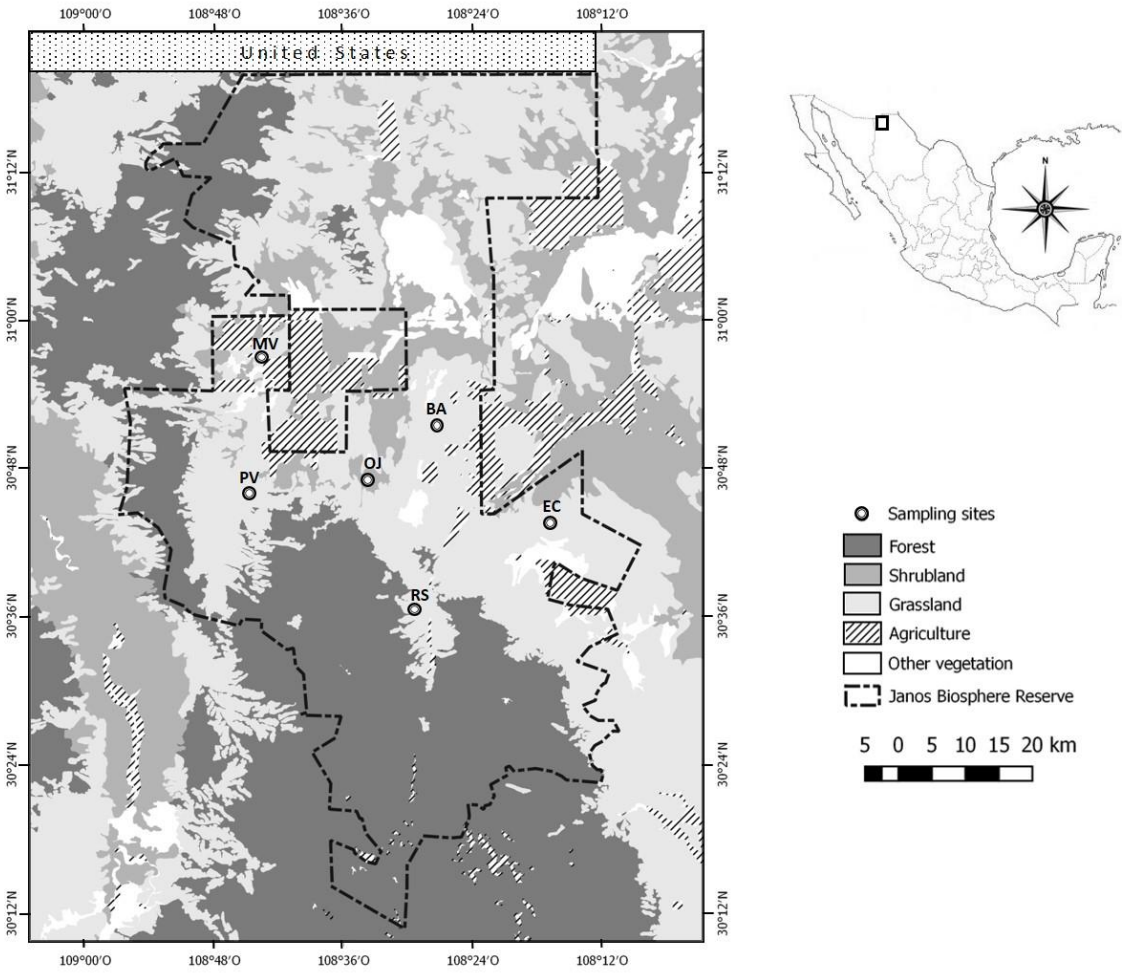

Figure 3.1. Location of sites where plague surveillance in rodents and carnivores were sampled in Janos Biosphere Reserve, Chihuahua, Mexico.

Table 3.1. Wild carnivore sampled in five localities of Janos Biosphere Reserve

Species	Locations					
	Cuervo	Bascula	Ojitos	Rancho San Pedro	Monte Verde	Total
Canis latrans	3	5	2	7	1	18
Lynx rufus	1	0	3	1	0	5
Mephitis macroura	0	0	3	0	0	3
Mephitis mephitis	0	1	1	6	0	8
Procyon lotor	0	0	2	2	0	4
Taxidea taxus	3	1	1	0	1	6
Urocyon cinereoargenteus	0	0	0	7	0	7
Vulpes macrotis	4	5	0	0	6	15
TOTAL	**11**	**12**	**12**	**23**	**8**	**66**

Table 3.2. Studies conducted on plague in Janos Biosphere Reserve, Chihuahua, Mexico.

No.	Collected dates	Target species	Individual tests	Sites[a]	Design methods	Reference
1	2007	Prairie dogs	182	BS, MV, CV, PV, OJ	Extraction of the serum from Nobuto ® strips and PHA serologic test	(32)
2	2009	Fleas from prairie dogs	349	BS, MV, CV, PV, OJ	DNA extraction from fleas and PCR	(33)
3	Mar 2013 Oct 2013	Fleas from rodents	760	OJ, CV	DNA extraction from fleas and PCR	(34)
4	Oct-Nov 2013 May-Jun 2014	Wild carnivores	66	OJ, BS, MV, CV, RS	Extraction of the serum from Nobuto ® strips and PHA serologic test	This study

[a] BS, Bascula; CV, Cuervo; MV, Monte Verde; OJ, Ojitos; PV, Pancho Villa; RS, Rancho San Pedro

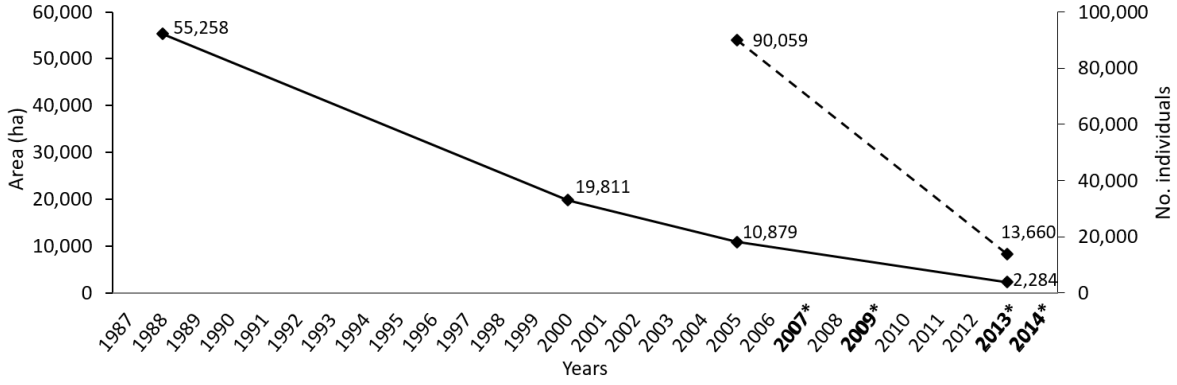

Figure 3.2. Changes in the distribution areas (solid line) and abundances (dashed line) of black-tailed prairie dog colonies in Janos Biosphere Reserve, Chihuahua, México, in the years 1988 (26), 2000 (58), 2005 (12,30) and 2013 (28). Bold with asterisk indicate the years in which at least one plague surveillance was performed (see table 2).

4. CONCLUSIONES GENERALES

En el capítulo 1 se concluye que el ensamble de las pulgas de los carnívoros silvestres de la Reserva de la Biósfera Janos está compuesto por dos grupos de especies caracterizados por aspectos ecológicos, como la abundancia y la amplitud de nicho. El primer grupo fue caracterizado por tres especies dominantes (*Pulex simulans*, *P. irritans* y *Echidnophaga gallinacea*) reconocidas por ser cosmopolitas y generalistas en la selección de hospederos. El segundo grupo correspondió a cuatro especies raras caracterizadas por ser especies especialistas asociadas a roedores y lagomorfos, cuya presencia en carnívoros se explica por la interacción depredador presa. Finalmente, se concluye que la estructura del ensamble de pulgas en los carnívoros silvestres esta explicada por la identidad de los hospederos, la estacionalidad y la competencia intra específica.

A partir de los resultados obtenidos en el capítulo 2 se sugiere que mientras *P. simulans* puede jugar un papel importante en la transmisión de las *Bartonella* sp. entre los carnívoros silvestres, *E. gallinacea* parece ser un vector no competente de dichas bacterias. Adicionalmente, se observó una relación entre los distintos genotipos encontrados de *Bartonella* sp. y las familias de los carnívoros hospederos. La presencia de especies de *Bartonella* sp. previamente reportadas como zoonóticas indica un riesgo potencial para la salud pública en la zona de estudio.

Por último, los resultados del capítulo 3 concluyen que a pesar de los resultados negativos obtenidos en cuatro aproximaciones distintas durante 2009 y 2014, incluyendo el presente estudio, estos no son concluyentes con respecto a la ausencia de *Yersinia pestis* en la Reserva de la Biósfera Janos. Por lo tanto, no se descarta que la disminución de las poblaciones de perritos de la pradera de cola negra (*Cynomys ludovicianus*) pueda deberse a eventos epizoóticos de peste. Para dar seguimiento a este estudio se recomienda un monitoreo ecológico a largo plazo que incluya el estudio sistemático de la dinámica de la población de los perritos de la pradera, así como el estudio epidemiológico de *Yersinia pestis*.