

Evidence for the involvement of an alternate rodent host in the dynamics of introduced plague in prairie dogs

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Summary

1. The introduction of plague to North America is a significant threat to colonies of prairie dogs (*Cynomys ludovicianus*), a species of conservation concern in the Great Plains. Other small rodents are exposed to the causative agent, *Yersinia pestis*, during or after epizootics; yet, its effect on these rodents is not known, and their role in transmitting and maintaining plague in the absence of prairie dogs remains unclear.

2. We live-trapped small rodents and collected their fleas on 11 colonies before, during and after plague epizootics in Colorado, USA, from 2004 to 2006. Molecular genetic (polymerase chain reaction) assays were used to identify *Y. pestis* in fleas.

3. Abundance of northern grasshopper mice (*Onychomys leucogaster*) was low on sites following epizootics in 2004, and declined markedly following plague onset on other colonies in 2005. These changes coincided with exposure of grasshopper mice to plague, and with periods when mice became infested with large numbers of prairie dog fleas (*Oropsylla hirsuta*), including some that were infected with *Y. pestis*. Additionally, several *Pleochaetis exilis*, fleas restricted to grasshopper mice and never found on prairie dogs on our site, were polymerase chain reaction-positive for *Y. pestis*, indicating that grasshopper mice can infect their own fleas. No changes in abundance of other rodent species could be attributed to plague, and no other rodents hosted *O. hirsuta* during epizootics, or harboured *Y. pestis*-infected fleas.

4. In spring 2004, grasshopper mice were most numerous in colonies that suffered plague the following year, and the pattern of colony extinctions over a 12-year period mirrored patterns of grasshopper mouse abundance in our study area, suggesting that colonies with high densities of grasshopper mice may be more susceptible to outbreaks. We speculate that grasshopper mice help spread *Y. pestis* during epizootics through their ability to survive infection, harbour prairie dog fleas and, during their wide-ranging movements, transport infected fleas among burrows, which functionally connects prairie dog coterries that would otherwise be socially distinct.

Key-words: ecology of vector-borne diseases, epizootic-enzootic cycles, host-parasite relationships, invasive diseases, multi-host pathogens

Forecasting and controlling the spread of zoonotic diseases requires a thorough understanding of the ecology of the pathogen, its hosts and vectors in the field (Keesing, Holt & Ostfeld 2006). For multi-host pathogens, i.e. pathogens

capable of infecting multiple host species, hosts may take any number of different roles, including asymptomatic carriers, amplifying hosts, hosts supporting vector populations that do not contribute directly to disease spread, and spillover hosts (Fenton & Pedersen 2005), making such diseases particularly difficult to study. In addition, some multi-host pathogens, such as West Nile virus, the Lyme disease bacterium and the avian malaria parasite, pose serious threats to wildlife as well as human populations. When such pathogens

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are introduced beyond their native range, they can have devastating effects on naïve host populations (e.g. Woodworth *et al.* 2005).

Plague, a disease caused by the bacterium *Yersinia pestis* (Lehmann and Neumann) van Loghem, was introduced to the USA in ~1900 and spread to the Great Plains by the 1940s (Barnes 1993). The pathogen infects a broad range of mammals, but ground squirrels such as prairie dogs (*Cynomys*) are particularly susceptible to mortality. Populations of these colonial animals, which may cover 100s of hectares of grasslands, are wiped out by plague, often within months after an epizootic starts (Cully & Williams 2001). Indeed, plague represents the greatest threat to the long-term persistence of large colonies of black-tailed prairie dogs (*Cynomys ludovicianus* Ord), a species that plays an important role in grassland ecosystems by providing habitat and serving as prey for several species of conservation concern, including the black-footed ferret (*Mustela nigripes* Audubon and Bachman; United States Fish & Wildlife Service 2004). The introduction of *Y. pestis* into prairie dog populations thus has important implications for the conservation of grassland biodiversity beyond the threat to prairie dogs themselves.

Plague-induced die-offs, or epizootics, of prairie dogs occur sporadically, and are interspersed with prolonged periods of latent quiescence (enzootic or maintenance phase); however, the mechanisms of spread and possible persistence of the disease on the landscape remain poorly understood (Gage & Kosoy 2005). Under natural conditions, transmission of *Y. pestis* can occur in a variety of ways, including flea bites, direct contact with infected hosts, or consumption of infected host tissues. Because mortality of prairie dogs is so high during epizootics (90–100%; Cully & Williams 2001; Pauli *et al.* 2006), it has been argued that *Y. pestis* must persist between epizootics in different rodent hosts, so that die-offs of black-tailed prairie dogs are the result of spillover from enzootic hosts. In the Great Plains, deer mice (*Peromyscus maniculatus* Wagner) and thirteen-lined ground squirrels (*Spermophilus tridecemlineatus* Mitchell) have been hypothesized to be enzootic hosts, largely based on retrospective serological surveys (Lechleitner *et al.* 1968; Fitzgerald 1970), or from the isolation of *Y. pestis* in fleas collected from or associated with these rodents (Anderson and Williams 1997; Cully *et al.* 1997; Cully & Williams 2001). However, there is little direct evidence that other small mammals act as such hosts (e.g. Salkeld & Stapp 2008a), in part because of the difficulties in detecting plague before epizootics are well underway, and because most research has focused on prairie dogs. An alternative explanation is that plague persists solely in populations of prairie dogs and their specific fleas, and that die-offs occur as a result of increased transmission rates associated with periods of high population densities within colonies (Stapp, Antolin & Ball 2004; Gage & Kosoy 2005). In this scenario, *Y. pestis* may be maintained at a landscape scale through the spatial isolation of colonies and time-lags between the timing of initial infection, epizootic events and opportunities for inter-colony movements of infected hosts or vectors.

We studied populations of small mammals and their fleas in black-tailed prairie dog colonies in northern Colorado,

USA, to determine their possible involvement in epizootic and enzootic cycles of plague in prairie dog colonies. Although deer mice and thirteen-lined ground squirrels are found in prairie dog colonies in our study area, our efforts focused on the northern grasshopper mouse (*Onychomys leucogaster* Wied-Neuwied), for several reasons. Grasshopper mice are the most abundant small rodents in prairie dog colonies in shortgrass steppe (Stapp 2007). Some individuals have been shown to be resistant to plague mortality in the laboratory (Holdenreid & Quan 1956, Thomas *et al.* 1988), and they carry fleas that are competent vectors of *Y. pestis* (Thomas 1988). In addition, they regularly use burrows of other animals (Stapp 1997), exposing them to many species of fleas, and in the laboratory may be infected with *Y. pestis* by consuming infected rodent prey (Thomas *et al.* 1989). Moreover, recent serological tests showed that grasshopper mice are infected with *Y. pestis* during epizootics, whereas deer mice and ground squirrels apparently are only exposed the year afterward, suggesting different modes of infection, e.g. consumption of infected carcasses below-ground rather than by bites of prairie dog fleas, and little or no involvement in prairie dog epizootics (Stapp *et al.* 2008b).

Here, we report changes in relative abundance of small rodents before, during and after epizootics on a total of 11 colonies over a 3-year period (2004–2006). We also describe patterns of flea infestation of rodents during epizootics, and evidence of the infection of fleas with *Y. pestis*, to determine the extent to which small rodents exchange fleas with prairie dogs, which might indicate involvement in the transmission of the disease. As such, our study documents changes in populations of small mammal hosts and flea vectors on colonies throughout epizootic events in prairie dogs, which provides new insights into the potential role of alternative small mammal hosts in the dynamics of plague.

Materials and methods

STUDY AREA AND FIELD SAMPLING

Our study area was the Pawnee National Grasslands (PNG; Fig. 1), a mix of federal, state and private lands administered by the US Department of Agriculture Forest Service. The climate is semi-arid and vegetation is shortgrass steppe (Lauenroth & Milchunas 1991).

To estimate relative abundance, in 2004 small rodents were live trapped on active prairie dog colonies that ranged in size from 0.8 to 175.7 ha the previous year (the active area of each colony was estimated in late summer each year by walking or driving its perimeter and mapping locations of active burrows using a global positioning system unit; see Stapp *et al.* 2004). Rodents were also trapped on four colonies (hereafter, 'PL04' colonies) where plague extirpated prairie dogs in approximately February (PNG74, PNG3), May (PNG41), and June (PNG62) 2004 (Table 1; Fig. 1). Sites were trapped once in May and again in early August. In an attempt to increase the number of captures, three trapping grids were established on two plague colonies (PNG3, PNG62) and three active colonies (PNG17, PNG35, PNG78) in late August 2004. These five colonies were trapped again in late August and September 2004 (Table 1).

In 2005, the four PL04 colonies and four active colonies (PNG17, PNG35, PNG78, PNG84) were trapped in May and early June.

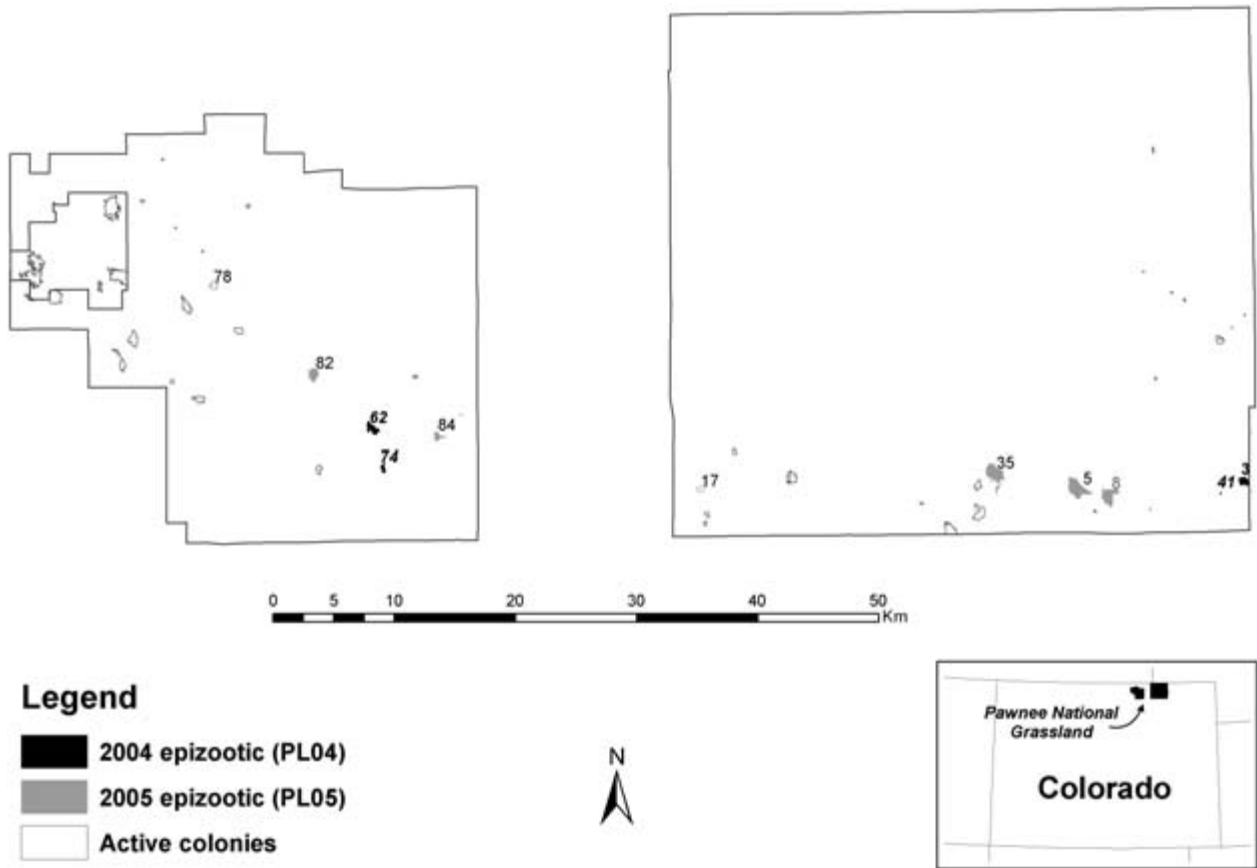


Fig. 1. Map of Pawnee National Grasslands, Colorado, USA, showing the location of black-tailed prairie dog colonies that suffered plague in 2004 (PL04; black fill) and in 2005 (PL05; grey fill), and those that remained active throughout the study period (no fill). The size and shape of polygons reflects the estimated active area in 2003. Numbers correspond to colony numbers assigned by USDA Forest Service.

Grids were established on two colonies (PNG8, PNG5) where we suspected plague (hereafter, 'PL05' colonies); these were trapped in late June 2005. Grids were also established on a third colony (PNG82) that was extirpated by plague in July–August 2005. For the remainder of summer 2005, as many active and plague colonies were sampled as possible (Table 1). Finally, evidence of plague was detected in September and October 2005, after trapping ceased, on two colonies (PNG35, PNG84) that had been used as controls, i.e. active colonies. Prairie dogs were present on some areas of PNG84 until June 2006. All 11 focal colonies were trapped once more from late May to early July 2006. In total, we sampled four to six times on each of four PL04 colonies, five PL05 colonies, and two colonies that remained active throughout the study period (Table 1).

All grids were 2.25 ha and consisted of 100 large Sherman live traps spaced 15 m apart, except for sampling in May–July 2004, when grids were 1.35 ha (60 traps). Except for May–July 2004 and three smaller colonies (PNG41, PNG74, PNG8), which always had one grid, all study colonies had three 2.25-ha grids, spaced ≥ 100 m apart, that were trapped concurrently. Trapping sessions consisted of four consecutive nights. Traps were set at dusk, checked at dawn, and then re-set for an additional 4–5 h to capture diurnal ground squirrels. Traps were shaded with PVC pipe to reduce heat-related mortality. All animals captured were weighed, identified by sex, age and reproductive status, and marked with a uniquely numbered aluminum ear tag. Fleas were combed from anesthetized animals, stored in cryotubes in a mixture of Tween-80 detergent and saline

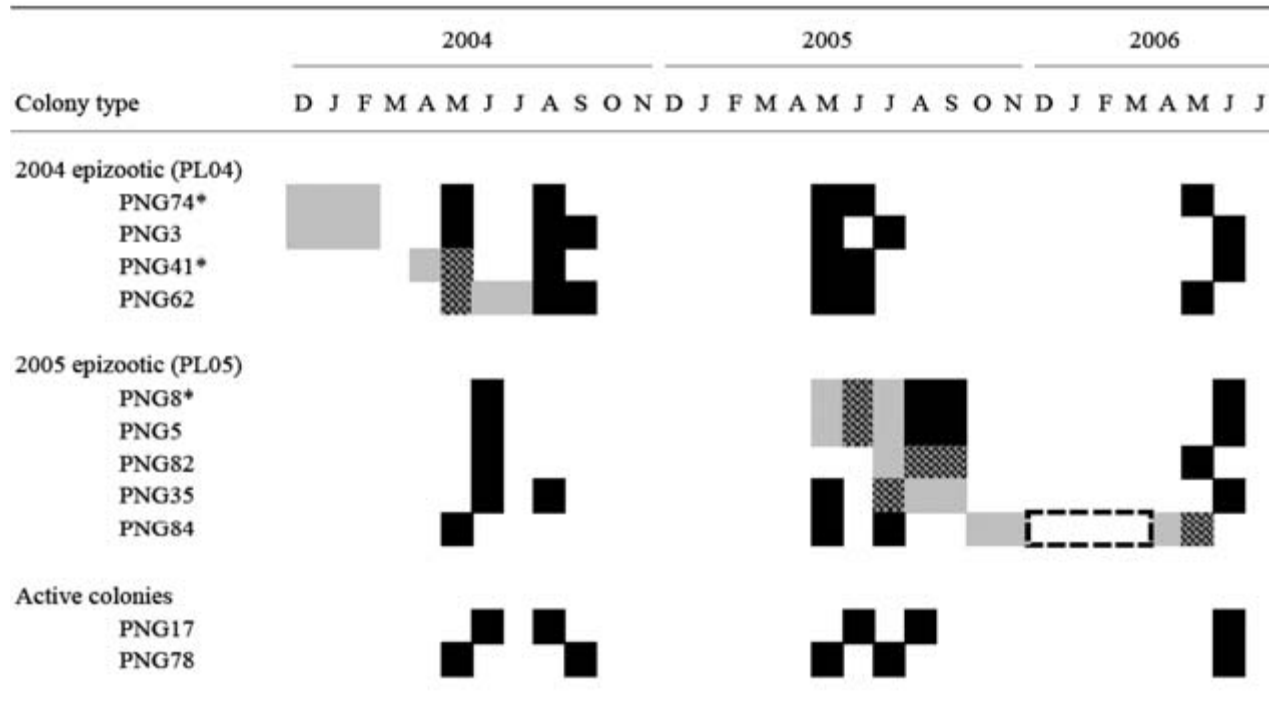
solution, and then frozen at -80 °C. Blood and ear tissue samples were also collected before animals were released. Animal handling procedures were approved by the Institutional Animal Care and Use Committee at California State University Fullerton, following American Society of Mammalogists (1998) guidelines.

Data on long-term patterns of abundance of rodents on the study area were available because the PNG is one of the primary locations of the Shortgrass Steppe Long-Term Ecological Research project (SGS-LTER), funded by the US National Science Foundation. Between 1994 and 2006, population densities of small mammals were determined by live trapping in May and September each year on three 3.14-ha trapping webs in saltbush [*Atriplex canescens* (Pursh) Nutt.]-dominated vegetation, which represent high-quality habitat for grasshopper mice (Stapp, Van Horne & Lindquist 2008a). Although long-term webs were in areas without prairie dogs and were several kilometres from our focal colonies, they provided the best available information on temporal changes in rodent populations.

MOLECULAR ASSAYS FOR *Y. PESTIS* IN FLEAS

Fleas were thawed, identified to species and scored as fed or not fed based on the presence of blood in the gut. To determine the presence of *Y. pestis*, fleas were triturated with a MM301 mixer mill (Retsch, Newtown, PA USA) in 100 μ L brain heart infusion (BHI; Difco) broth with three 3-mm sterile Pyrex glass beads at 20 beats/s for 2–4 min. Triturated flea material was centrifuged at $15\,600 \times G$ for 10 s

Table 1. Black-tailed prairie dog colonies in northern Colorado that were sampled intensively for small rodents from 2004–06. Gray bars denote our best estimate of the timing of most prairie dog mortality, based on surveys of colony activity. Solid bars indicate sampling periods outside of epizootics; hatched bars indicate periods when sampling coincided with epizootics. The dashed box for PNG84 indicates that the epizootic that began in autumn 2005 likely continued over the winter. The presence of plague was ultimately confirmed in each colony by detection via fluorescent antibody stains of tissue from prairie dog carcasses; serological analyses of rodent blood; or polymerase chain reaction analyses to detect *Yersinia pestis* in fleas (Stapp *et al.* 2008b)



*only one grid was trapped on these colonies.

and heated to 95 °C for 10 min, and then centrifuged for an additional minute to pellet the debris. An aliquot of the supernatant containing the DNA (1.25 µL) was used for the polymerase chain reaction (PCR) template.

The PCR targeted the *pla* gene, as described previously (Hinnebusch and Schwann 1993), with minor modifications. Each 25 µL PCR contained 1× PCR buffer with 1.5 mM MgCl₂, 200 µM deoxynucleotide triphosphates (dNTPs), 0.5 U *Taq*-polymerase (Promega, Madison, WI, USA), 1.25 µL of flea triturate, and 10 µM each of primers Yp1 (5'-ATCTTACTTTCCGTGAGAAG-3') and Yp2 (5'-CTTGAT-GTTGAGCTTCCTA-3'; Stevenson *et al.* 2003). Negative controls (master mix reagents only) and two positive controls [0.6 ng of *Y. pestis* DNA extracted from a wild type isolate (CO963188) from the CDC, and CO963188 diluted to 10–100 cfu/100 µL in HIB (Heart Infusion Broth) and heat lysed] were included in each 96-well plate of reactions. Reactions were amplified in a PTC-200 thermocycler (MJ Research, Watertown, MA, USA), with an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 1 min at 95 °C, 1 min at 55 °C, 1 min at 72 °C, and a final extension step of 10 min at 72 °C. Amplification with the *pla* primers produced product lengths of 478 bp. Products were visualized by separating 5 µL of the PCR mixture on 2% agarose gels containing 1% ethidium bromide. Previous studies have shown the sensitivity of the *pla* PCR to be 10–100 cfu (Engelthaler *et al.* 1999).

DATA ANALYSIS

Because population densities of rodents in shortgrass steppe are relatively low ($\leq 4 \text{ ha}^{-1}$ – Stapp *et al.* 2008a), the number of individuals

per 100 trap-nights (TN) was used as an index of relative abundance. Our grids were relatively large (2.25 ha) and we chose to trap on multiple colonies and replicate grids on large colonies rather than sample single large grids, which still might not have yielded sufficient captures for more precise modelling of population density. The total number of TN was adjusted by subtracting 0.5 TN for every trap that was sprung (tripped and empty). For colonies with multiple grids, the number of individuals captured of each species on all grids was summed to estimate abundance during each trapping session. Logistic regression was used to examine the effects of rodent abundance and the active area of colonies on the probability that colonies were extirpated by plague. Abundance estimates and colony area were square-root transformed before analysis.

Our analyses of patterns of flea infestation were restricted to first-time captures of rodents within a trapping session. To assess the ability of different rodent hosts to harbour multiple flea species independent of plague, species accumulation curves were generated using counts of fleas combed from rodents captured in the two active colonies in 2005–06 (PNG17, PNG78), as well as an additional 15 active colonies, i.e. without plague, that were sampled in 2004. Accumulation curves were generated for each rodent species separately in PRIMER 6.0 (version 6.1.5; Clarke & Gorley 2006) to show the total number of flea species observed as a function of sample size (individual hosts). Comparisons of curves among host species allowed us to assess the relative ability of each host species to support multiple flea species. Flea counts were $\log(x + 1)$ -transformed before analysis. We used the UGE option in PRIMER, based on Ugland, Gray & Ellingsen (2003), to generate an average species accumulation curve based on 999 permutations, with samples entered in random order.

Because we were primarily concerned here with the ability of other rodents to exchange fleas with prairie dogs, our analysis of flea infestation was focused on *O. hirsuta* Baker, which make up 76% of fleas on prairie dogs during the months we sampled (D. Tripp, M. Antolin, in litt.). The load of *O. hirsuta* on a colony was calculated as the mean number of *O. hirsuta* on combed individuals of a species during a sampling period.

Results

EFFECTS OF PLAGUE ON SMALL MAMMAL POPULATIONS

Grasshopper mice, thirteen-lined ground squirrels and deer mice were the most common species captured during the study, constituting 35.6%, 29.2% and 18.8%, respectively, of the 1803 individuals captured over ~44 395 trap-nights (44 044 trap-days for squirrels). Ord's kangaroo rats (*Dipodomys ordii* Woodhouse; 13.0%) and silky pocket mice (*Perognathus flavus* Baird; 2.8%) were also present on some colonies. Other species (hispid pocket mouse, *Chaetodipus hispidus* Baird; western harvest mouse, *Reithrodontomys megalotis* Baird; plains harvest mouse, *R. montanus* Baird; prairie vole *Microtus ochrogaster* Wagner; house mouse, *Mus musculus* Linnaeus) represented $\leq 0.3\%$ of captures. On average, we captured 15.2 individuals (SD = 12.9) and 3.0 species (SD = 1.1) per grid during a 4-night trapping session over the 3-year study period ($n = 119$ grid-sessions).

Grasshopper mice were very abundant in the five PL05 colonies in May–June 2004, but declined dramatically after plague extirpated prairie dogs on these sites (Fig. 2). Including abundance estimates from nine additional active colonies that were trapped in May–June 2004 (see Stapp 2007), the abundance of grasshopper mice in 2004, combined with colony active area, were significant predictors of whether a colony suffered plague in 2005 (logistic regression, colony area $X^2_{1,15} = 9.86$, $P = 0.002$, grasshopper mouse abundance $X^2_{1,15} = 4.30$, $P = 0.038$, $n = 16$ colonies; neither deer mice nor ground squirrel abundance was significant predictors in separate models, $P > 0.05$). On PL05 colonies, abundance of grasshopper mice decreased significantly, by an average of 69.4% (SD = 10.2), between the trapping session preceding plague onset in 2005 and the subsequent trapping session (paired t -test, $t_4 = 5.68$, $P = 0.005$). There were no significant changes in abundance of ground squirrels (paired t -test, $t_4 = 0.57$, $P = 0.600$ or deer mice ($t_4 = 0.66$, $P = 0.554$) over the same period. Grasshopper mouse populations appeared to recover in PL05 sites by 2006, except on the two colonies (PNG35, PNG84) that suffered plague in late 2005 and early 2006 (Fig. 2).

In May–June 2004, grasshopper mice were significantly more abundant in PL05 than PL04 colonies, with the two active colonies intermediate and not significantly different than either plague group (ANOVA, $F_{2,8} = 5.19$, $P = 0.036$). If PL05 colonies were considered to be 'active' in May–June 2004 and pooled with the two active colonies, grasshopper mice were significantly less abundant in PL04 colonies than in

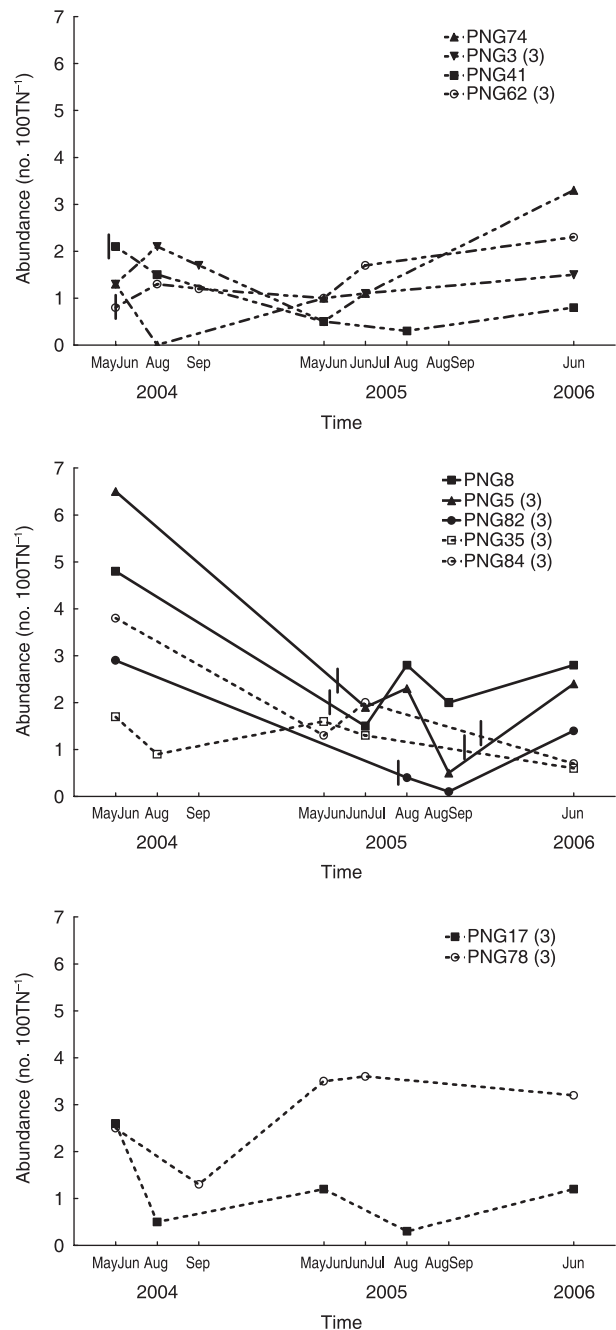


Fig. 2. Relative abundance (number of individuals per 100 trap-nights, TN) of northern grasshopper mice (*Onychomys leucogaster*) on prairie dog colonies that suffered plague in 2004 (top) and 2005 (middle), compared to that in colonies that remained active throughout the study period (bottom). Except for May/June 2004 and for colonies PNG64, PNG3 and PNG8, upon which only a single grid was trapped, relative abundance was calculated by summing all unique individuals captured across three grids (in parentheses). Vertical lines represent approximate timing of prairie dog mortality on each colony; prairie dogs were extirpated on PNG74 and PNG3 during winter 2004, before sampling.

active ones ($F_{1,9} = 8.83$, $P = 0.016$). There were no significant differences between PL04, PL05 and active colonies in May–June abundance of grasshopper mice in 2005 ($F_{2,8} = 1.76$, $P = 0.233$) or 2006 ($F_{2,8} = 0.30$, $P = 0.749$). Comparing

May–June estimates of abundance between PL04, PL05 and active colonies, there were no significant differences in abundance of squirrels or deer mice between plague and active colonies in any year (separate ANOVA tests by year, range of $F_{2,8} = 0.01 - 1.25$, $P = 0.338 - 0.969$). Abundance of these species was extremely variable among colonies and over time (see Figs S1 and S2), reflecting, in part, the reduced activity of squirrels as they enter hibernation in late summer (Flake 1974). None of these patterns, however, could be obviously attributed to plague.

Differences in the number of grids and variation in the number and timing of trapping sessions on different sites relative to plague epizootics precluded formal analysis of survival. All colonies except PNG82 were trapped twice between May–June and July–August 2005. On average, in July–August 2005, 16.4% (SE = 5.9, $n = 4$) and 25.2% (SE = 2.9, $n = 4$) of grasshopper mice in PL04 and PL05 colonies, respectively, were previously marked, compared to 36.9% (SE = 3.6, $n = 2$) in active colonies (ANOVA, $F_{2,7} = 3.68$, $P = 0.081$). At the same time, 44.4% (SE = 29.4, $n = 3$) and 48.4% (SE = 13.4, $n = 3$) of deer mice in PL04 and PL05 colonies, respectively, were marked, whereas 31.0% (SE = 2.4, $n = 2$) of deer mice in active colonies were recaptures ($F_{2,5} = 0.15$, $P = 0.862$). No comparisons were made for squirrels, which frequently had damaged ears and lost tags, making it difficult to determine recaptures, and which showed more seasonal variation in above-ground activity than the two mouse species. Too few individuals were recaptured in May–June 2006 for meaningful analysis of plague effects.

RATES OF INFESTATION AND *Y. PESTIS* INFECTION OF FLEAS

On active colonies, grasshopper mice consistently hosted the greatest diversity of fleas, with the most rapid increase in richness occurring over the first 30 hosts (Fig. 3). Eight species were collected from grasshopper mice ($n = 223$ hosts; flea species, in order of abundance: *Pleochaetis exilis* Jordan, *Thrassis fatus* Jordan, *Foxella ignota* Baker, *Meringes parkeri* Jordan, *O. hirsuta*, *Aetheca wagneri* Baker, *Orchopeas leucopus* Baker, *Epitedia wenmanni* Rothschild), whereas deer mice had a maximum of five species ($n = 77$; *A. wagneri*, *O. leucopus*, *T. fatus*, *P. exilis*, *O. hirsuta*) and ground squirrels had two species ($n = 158$; *T. fatus*, *P. exilis*). For a sample size of 77 individuals, the sample size for the least common species (deer mouse), grasshopper mice were infested with an average of 6.7 species of flea, whereas deer mice and ground squirrels harbored 5.0 and 1.7 species, respectively (Fig. 3).

O. hirsuta, the primary flea of prairie dogs, was occasionally found in low numbers on grasshopper mice in active colonies (Fig. 4) and, in one instance, several kilometres away from the nearest active colony. Infestation by *O. hirsuta* increased markedly during epizootics (Fig. 4), presumably as a result of fleas abandoning dead prairie dogs for remaining live hosts, or recently emerged fleas seeking blood meals in the absence of prairie dogs. During these periods, grasshopper mice infested with *O. hirsuta* had an average of 3.8 (± 0.5) of

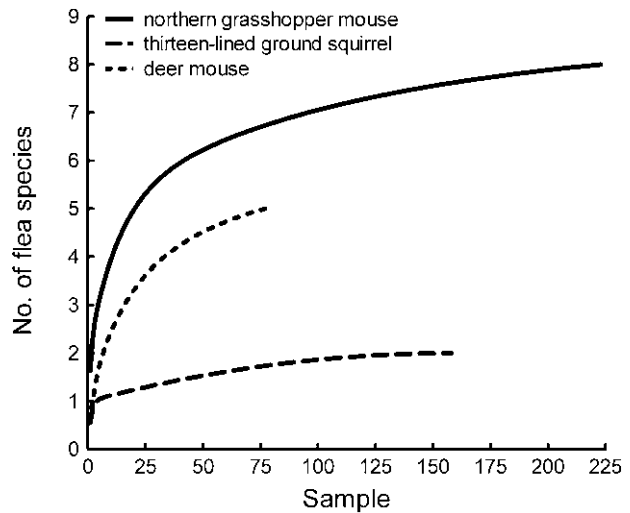


Fig. 3. Species accumulation curves for fleas on northern grasshopper mice, deer mice and thirteen-lined ground squirrels captured in 17 active black-tailed prairie dog colonies between May 2004 and July 2006. Curves are the means of curves generated in PRIMER from 999 permutations in which samples (individual hosts) were entered in random order.

these fleas (range: 1–35, $n = 114$ mice, 436 fleas), compared to 1.6 ± 0.4 during non-epizootic periods (range: 1–7; $n = 17$ mice, 27 fleas). During the peak of the summer 2005 epizootics, 81% of grasshopper mice were infested with *O. hirsuta*; yet, *O. hirsuta* was not found on any other small rodent during epizootics and was extremely rare on other rodents otherwise. None of the 543 ground squirrels examined (910 fleas) were infested with *O. hirsuta*, and of the 252 fleas collected from deer mice (322 individuals), only three were *O. hirsuta*: one from PNG62 in May 2005, and one each on PNG84 and PNG78 in July 2005. One *O. hirsuta* was found on a kangaroo rat on PNG35 in June 2006 (69 fleas examined, 227 individuals). Interestingly, relatively large numbers of *O. hirsuta* were collected on grasshopper mice early in July 2005 on two colonies (PNG35, PNG84) for which there was no sign of prairie dog mortality until at least September 2005 (Fig. 4).

Pleochaetis exilis was the most common flea on grasshopper mice, infesting 66.3% of 688 individuals and making up 59.6% of all fleas ($n = 3485$) combed from this species. *Pleochaetis exilis* was rarely found on other rodents (10 *P. exilis*, all singletons, of 1231 fleas from 1092 deer mice, ground squirrels and kangaroo rats combined), and to our knowledge, has never been collected from prairie dogs on the PNG (~17 345 fleas; D. Tripp, M. Antolin, *in litt*). Using *pla*-PCR assays, *Y. pestis* was detected in three of 289 *P. exilis* (1.0%) and eight of 247 *O. hirsuta* (3.2%) combed from grasshopper mice on PNG5 and PNG8 in July and August 2005. One *O. hirsuta* combed from a grasshopper mouse on PNG84 in June 2006, after the epizootic had swept through the prairie dogs, was also PCR positive, as were four *P. exilis* from one grasshopper mouse on a colony that suffered plague in May and June 2006 (8.9% of 45 *P. exilis* tested from colony CPER127). We do not

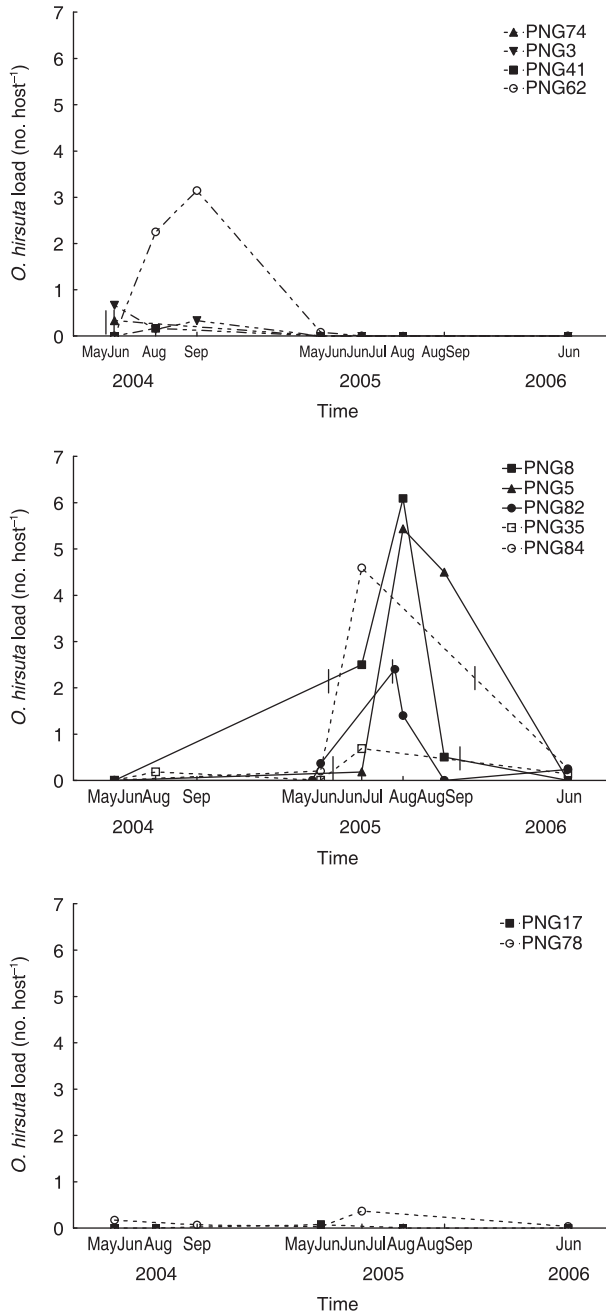


Fig. 4. Loads of prairie dog fleas (*Oropsylla hirsuta*) on northern grasshopper mice (*Onychomys leucogaster*) on prairie dog colonies that suffered plague in 2004 (top) and 2005 (middle), compared to that in colonies that remained active throughout the study period (bottom). Vertical lines represent approximate timing of prairie dog mortality on each colony; prairie dogs were extirpated on PNG74 and PNG3 during winter 2004, before sampling.

know if these *O. hirsuta* were infected by grasshopper mice or prairie dogs, but given the absence of *P. exilis* on prairie dogs, their scarcity on other rodents, and the lack of evidence of *Y. pestis* infection of other rodents during epizootics (Stapp *et al.* 2008b), *P. exilis* fleas were almost certainly infected with *Y. pestis* by grasshopper mice.

Discussion

In shortgrass steppe of northern Colorado, the northern grasshopper mouse is the small rodent that is most likely to be involved in the transmission and maintenance of plague. Grasshopper mice were infected by *Y. pestis* during prairie dog epizootics (Stapp *et al.* 2008b), which may in part explain why plague colonies had fewer grasshopper mice. Abundance of grasshopper mice on PL04 sites was low in late spring and summer 2004, following epizootics that had mostly wiped out these colonies earlier in the winter and spring. Grasshopper mice reached highest densities in May–June 2004 on colonies that suffered plague the following year, and then declined dramatically during and immediately after the periods of highest prairie dog mortality above-ground (Fig. 2). Although the apparent changes in grasshopper mouse abundance were significant (a 69.4% decrease), it is important to note that grasshopper mice continued to be captured on prairie dog colonies at all stages of prairie dog epizootics, i.e. there were no massive die-offs of grasshopper mice of the magnitude usually seen in prairie dogs. Between 11.1–23.1% of grasshopper mice on plague colonies were seropositive for antibody to *Y. pestis* (Stapp *et al.* 2008b), indicating that some individuals survived infection and therefore were resistant to mortality, confirming earlier studies (Holdenreid & Quan 1956; Thomas *et al.* 1988). No other rodent species were seropositive for plague during epizootics (Stapp *et al.* 2008b), or showed any population changes suggesting plague-related mortality, despite the fact that we sampled multiple colonies of each type.

During epizootics, grasshopper mice were infested with large numbers of prairie dog fleas (*O. hirsuta*), some of which were infected with *Y. pestis*, suggesting that grasshopper mice were infected with plague as a result of bites of prairie dog fleas or, perhaps, by consuming carcasses. No other species shared fleas with prairie dogs to any significant extent, perhaps because grasshopper mice are more likely than other species to encounter *O. hirsuta* through their widespread use of prairie dog burrows (Kraft 2009), or because grasshopper mice are more suitable hosts. We suspect that these fleas had abandoned dead or dying prairie dogs because the numbers of *O. hirsuta* in burrows increases dramatically during epizootics (Salkeld & Stapp 2008b). To our knowledge, such a rapid switch in hosts in response to plague mortality has not been reported, although such behaviour has been expected to occur (Poland & Barnes 1979). High burdens of *O. hirsuta* were observed on grasshopper mice on two colonies 2 months or more before there was any evidence of prairie dog mortality, suggesting that plague epizootics may go undetected for long periods, with much mortality occurring below-ground, and that infestation of grasshopper mice by large numbers of *O. hirsuta* may signal an incipient outbreak.

We also found evidence of *Y. pestis* infection of *P. exilis*, a flea species that was largely restricted to grasshopper mice and was never found on prairie dogs during our field studies. This species has been found naturally infected with *Y. pestis* (Miles, Wilcomb & Irons 1952; Gage, Montenieri & Thomas 1994) and can transmit *Y. pestis* in the laboratory (Kartman

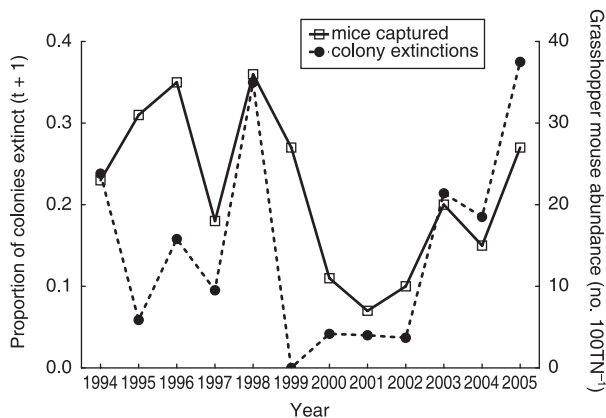


Fig. 5. Extinctions of prairie dog colonies tend to occur in years following periods of high regional abundance of grasshopper mice. Values on the right y-axis are the total numbers of grasshopper mice captured on three 3.14-ha saltbush trapping webs (124 traps each) during 4-night trapping sessions conducted each September as part of the SGS-LTER project (Stapp *et al.* 2008a). Values on the left y-axis are the proportion of active prairie dog colonies on the Pawnee National Grasslands, Colorado, that went extinct in the subsequent year, based on field surveys conducted in late summer and early fall from 1995–2006 (see Stapp *et al.* 2004).

& Prince 1956). Our results strongly suggest that grasshopper mice can infect their own fleas, which would be necessary for them to maintain the pathogen enzootically, i.e. in the absence of prairie dogs. It is also possible that grasshopper mice were the source of infection for the *Y. pestis*-positive prairie dog fleas that were combed from them, suggesting a mechanism by which grasshopper mice might eventually infect susceptible prairie dogs that re-colonized an area.

Although our study implicates grasshopper mice in dynamics of plague in prairie dog colonies in shortgrass steppe, their exact role is not clear. One of our most interesting results was that colonies that suffered plague in 2005 tended to have high numbers of grasshopper mice the previous year (Fig. 2). Moreover, when we compared long-term patterns of grasshopper mouse abundance (SGS-LTER data) to the long-term record of extinctions of colonies on the PNG, we found that epizootics tended to occur more frequently following years when grasshopper mice are abundant (Fig. 5). Although it is possible that the temporal dynamics of grasshopper mouse populations off colonies and plague epizootics in prairie dogs are both driven by some common but unknown environmental factor, the degree of concordance between these otherwise independent variables is noteworthy.

We hypothesize that, all other things being equal, colonies with high densities of grasshopper mice may be prone to plague because the wide-ranging behaviour of grasshopper mice (Stapp 1997, 1999), combined with their frequent use of burrows (Stapp 1997; Kraft 2009) and ability to act as at least temporary hosts for flea vectors of *Y. pestis* (Thomas 1988; Fig. 3), allows them to spread infected fleas across the territorial boundaries of prairie dog social units (coterie). In a network of prairie dog colonies in which a source of *Y. pestis*

is regionally present, invasion of a colony by a *Y. pestis*-infected rodent and/or flea, e.g. transported by a mammalian carnivore (Salkeld *et al.* 2007), might only result in the death of members of a single coterie, which would go unnoticed and not lead to an epizootic (e.g., Cross *et al.* 2005). However, by transporting fleas among coterie, or by becoming infected themselves by scavenging carcasses, grasshopper mice may connect infected and uninfected parts of a colony, functionally reducing the threshold of plague invasion in the prairie dog population and ultimately sparking a widespread epizootic. In laboratory challenges, grasshopper mice that die typically do so 4–9 days after exposure to *Y. pestis* (Thomas *et al.* 1988), which would give ample time for infected mice to infect and spread fleas among burrows, even if these mice eventually succumbed to disease [laboratory studies, e.g. Burroughs (1947), Engelthaler *et al.* (2000), suggest that rodents must have fatally high bacteremias to infect feeding fleas]. Individual grasshopper mice that are resistant to mortality also may maintain infections of *Y. pestis* bacilli in their tissues for 25 d or more post-exposure (Holdenreid & Quan 1956; Thomas *et al.* 1988).

Such a role for grasshopper mice in epizootics could help resolve a key question that has emerged from our studies of prairie dog plague in shortgrass steppe: why large colonies are more susceptible to plague extinction. Stapp *et al.* (2004) found that large colonies (>15 ha) were nearly as likely to go extinct as small ones during epizootic periods associated with El Niño Southern Oscillation events, when relatively mild, wet winters were expected to increase forage production for, and ultimately, population densities of prairie dogs. Stapp *et al.* (2004) argued that rates of disease transmission might be higher during such periods because of elevated host and flea population densities and increased encounter rates. Although above-ground plant biomass is positively related to winter precipitation ($r = 0.49$, $P = 0.030$, unpublished SGS-LTER data, 1982–2002; D. Milchunas, *in litt.*), colony-active area actually *decreases* with increasing winter precipitation ($r = -0.31$, $P = 0.005$, $n = 24$ years, 1982–2005), i.e., colonies grow larger during drier years. A likely explanation is that colony perimeter expands during dry years as animals are forced to widen their search for nutritious forage (Crosby & Graham 1986), so that population densities may be similar or lower in large-sized colonies than in smaller ones. Using mark-resight methodology, Magle *et al.* (2007) reported lower population density of prairie dogs on a large PNG colony than on a small one.

If prairie dog densities are not markedly higher in large colonies, then some other mechanism, perhaps related to colony area, is needed to explain the higher probability of epizootics in large colonies. In 2004, the study year during which the most colonies were active, grasshopper mouse abundance was positively correlated with both colony active area ($r = 0.52$, $P = 0.037$) and the density of prairie dog burrows ($r = 0.66$, $P = 0.005$, $n = 16$ colonies), suggesting that prairie dog colonies are high-quality habitat for grasshopper mice (Stapp 2007). Use of prairie dog burrows by grasshopper mice also increases with grasshopper mouse abundance (Kraft 2009). Therefore,

if large colonies tend to support more grasshopper mice, the susceptibility of colonies to plague epizootics may be determined by the abundance and behaviour of grasshopper mice, in addition to the density of prairie dogs (Lloyd-Smith *et al.* 2005). Although labour-intensive, regular monitoring of abundance and flea loads of grasshopper mice, especially on colonies of special conservation importance, may help identify sites that are at highest risk of epizootics, which could then be targeted for control of flea vectors (e.g. Beard *et al.* 1992; Seery *et al.* 2003) or, possibly, prairie dog vaccination efforts (Mencher *et al.* 2004).

The above scenario assumes a consistent, regional source of *Y. pestis* to infect and re-infect colonies in a prairie dog metapopulation. It is not known whether grasshopper mice can serve as enzootic hosts or be the source of a new epizootic. In many ways, grasshopper mice exhibit characteristics suggested by others to be associated with enzootic hosts (Poland & Barnes 1979). First, their populations are heterogeneous in their responses to *Y. pestis* infection, with some individuals dying after a few days of illness and others recovering from their infections (Thomas *et al.* 1988). The fact that some grasshopper mice harbour *Y. pestis*-infected fleas and are susceptible and die following *Y. pestis* infection suggests that these animals will develop the high bacteremias necessary for them to infect their own fleas (*P. exilis*) and perhaps other fleas, such as *O. hirsuta*, for which they could serve as temporary hosts. As noted previously, grasshopper mice also harbour an extraordinarily high number of fleas normally associated with other rodents, which should increase their likelihood of acquiring *Y. pestis* or carrying *Y. pestis*-infected fleas from one location to another. Also, at least a fraction of the grasshopper mouse population appears resistant to plague mortality (Thomas *et al.* 1988; Stapp *et al.* 2008b), and could contribute to the survival of grasshopper mouse populations in plague-affected areas. These same resistant mice also could serve as hosts for fleas that first became infected with *Y. pestis* after feeding on other, more susceptible grasshopper mice or other rodents, thus contributing to at least a short-term reservoir of plague and the movement of *Y. pestis*-infected fleas. To date, however, we have found no evidence of persistent, circulating infections by *Y. pestis* in grasshopper mice, and have only indirect evidence of a possible route of re-transmission of the bacterium back to prairie dogs. Thus, while it appears that grasshopper mice are involved in epizootic spread of plague in shortgrass steppe, more evidence is required to establish their involvement in the maintenance of *Y. pestis* between epizootics.

More broadly, our results provide general insights into the ecology of vector-borne diseases, especially those that involve multiple hosts and vectors. Even if they are not found to have a significant enzootic role, wide-ranging, continuously distributed secondary hosts such as grasshopper mice may alter spread of disease at different spatial scales, e.g., by amplifying the rate of spread during epizootics; by homogenizing local heterogeneity in vector densities across burrows and among social units of syntopic hosts through their movement behaviour; and possibly, by connecting isolated subpopulations in a

broader metapopulation (Gog, Woodroffe & Swinton 2002). Moreover, species such as grasshopper mice that are capable of supporting many different vector species and interact more with other potential hosts provide possible alternate avenues for persistence of the pathogen in different habitats. Finally, in our system, the role of other rodent species as disease reservoirs and sources of re-infection remains unresolved, despite our intensive field and laboratory efforts, which underscores the difficulties in determining the significance of interspecific transmission as a mechanism of disease persistence in multi-host pathogen systems (Swinton *et al.* 2002). The fact that the ecology of plague in prairie dogs seems to differ markedly from that in other areas of western North America, where the disease is presumed to persist enzootically in a number of different hosts (Davis *et al.* 2002; Foley *et al.* 2007), suggests that the widely held model of distinct epizootic-enzootic plague cycles may not apply in all systems, and highlights the importance of understanding the interactions between a wide range of hosts and vectors across a disease's geographical range, including areas where the pathogen has been introduced.

Acknowledgements

Our research was funded by a grant from the National Science Foundation (EID-0327052) to M. Antolin, K. Gage, P. Stapp and C. Webb. Additional support was provided by the Shortgrass Steppe Long-Term Ecological Research project (DEB-0217631) and the Department of Biological Science at California State University, Fullerton. We thank D. Kite, J. Holm, C. Cannon, A. Benson, H. Houghton, C. Knox, C. Wermager, E. Humphrey, and M. Lindquist for their assistance. B. Flynn prepared the map of our study area. Comments from two anonymous reviewers improved the manuscript.

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Received 4 September 2008; accepted 11 February 2009

Handling Editor: Mike Boots

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Relative abundance (number of individuals per 100 trap-nights, TN) of thirteen-lined ground squirrels

(*Spermophilus tridecemlineatus*) on prairie dog colonies that suffered plague in 2004 (top) and 2005 (middle), compared to that in colonies that remained active throughout the study period (bottom).

Fig. S2. Relative abundance (number of individuals per 100 trap-nights, TN) of deer mice (*Peromyscus maniculatus*) on prairie dog colonies that suffered plague in 2004 (top) and

2005 (middle), compared to that in colonies that remained active throughout the study period (bottom).

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