

## Enzootic Plague Reduces Black-Footed Ferret (*Mustela nigripes*) Survival in Montana

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### Abstract

Black-footed ferrets (*Mustela nigripes*) require extensive prairie dog colonies (*Cynomys* spp.) to provide habitat and prey. Epizootic plague kills both prairie dogs and ferrets and is a major factor limiting recovery of the highly endangered ferret. In addition to epizootics, we hypothesized that enzootic plague, that is, presence of disease-causing *Yersinia pestis* without any noticeable prairie dog die off, may also affect ferret survival. We reduced risk of plague on portions of two ferret reintroduction areas by conducting flea control for 3 years. Beginning in 2004, about half of the ferrets residing on dusted and nondusted colonies were vaccinated against plague with an experimental vaccine (F1-V fusion protein). We evaluated 6-month reencounter rates (percentage of animals observed at the end of an interval that were known alive at the beginning of the interval), an index to survival, for ferrets in four treatment groups involving all combinations of vaccination and flea control. For captive-reared ferrets (115 individuals observed across 156 time intervals), reencounter rates were higher for vaccinates (0.44) than for nonvaccinates (0.23,  $p = 0.044$ ) on colonies without flea control, but vaccination had no detectable effect on colonies with flea control (vaccinates = 0.41, nonvaccinates = 0.42,  $p = 0.754$ ). Flea control resulted in higher reencounter rates for nonvaccinates ( $p = 0.026$ ), but not for vaccinates ( $p = 0.508$ ). The enhancement of survival due to vaccination or flea control supports the hypothesis that enzootic plague reduces ferret survival, even when there was no noticeable decline in prairie dog abundance. The collective effects of vaccination and flea control compel a conclusion that fleas are required for maintenance, and probably transmission, of plague at enzootic levels. Other studies have demonstrated similar effects of flea control on several species of prairie dogs and, when combined with this study, suggest that the effects of enzootic plague are widespread. Finally, we demonstrated that the experimental F1-V fusion protein vaccine provides protection to ferrets in the wild.

**Key Words:** Black-footed ferrets—Black-tailed prairie dogs—*Cynomys ludovicianus*—Deltamethrin—Enzootic—F1-V fusion protein plague vaccine—*Mustela nigripes*—Plague—*Yersinia pestis*.

### Introduction

**B**LACK-FOOTED FERRET (*Mustela nigripes*, hereafter ferret) reintroductions into north-central Montana began in 1994 and continued through 2005 with more than 500 captive-reared animals released in multiple areas. In addition, more than 260 progeny of those released ferrets (wildborn kits) were documented over the years, yet no self-sustaining populations were established. Ferrets require extensive prairie

dog (*Cynomys* spp.) colonies to provide both habitat and prey (Biggins et al. 2006a). Plague is a major obstacle to recovery of endangered ferrets, and epizootics have an easily noticed and dramatic effect, especially on black-tailed prairie dogs (*C. ludovicianus*). An epizootic outbreak will often eliminate entire colonies or complexes of colonies, thereby removing both habitat and prey for ferrets. Moreover, exposure to plague is almost always lethal to ferrets within a few days (Rocke et al. 2004, Godbey et al. 2006).

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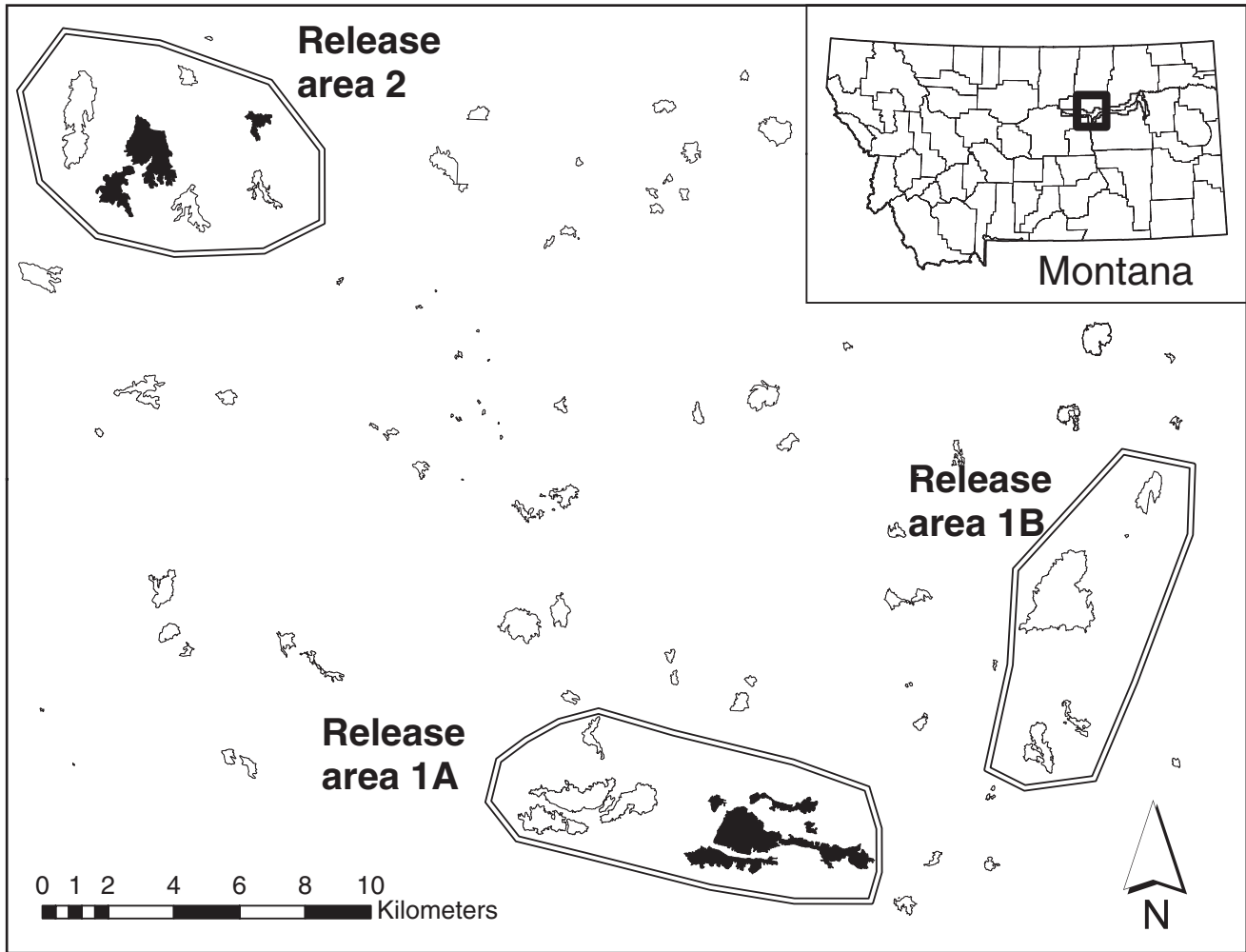
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**FIG. 1.** Location map illustrating study area components. Outlines represent black-tailed prairie dog colony boundaries as mapped in 2004 with solid shaded colonies receiving flea control treatments in 2003, 2004, and 2005.

Epizootic plague was first suspected in Phillips County, MT, during 1992 when routine monitoring revealed that many black-tailed prairie dog colonies had suddenly disappeared. Colonies continued to die-out and nearly 80% of 10,600 hectares (ha) of colonies were eliminated by 1996 (Augustine et al. 2008, Snäll et al. 2008, Matchett, unpublished data). In response, we conducted plague surveillance via carnivore collections (Smith et al. 1984, Williams et al. 1992, Gage et al. 1994) and found high prevalence of positive antibody titers to *Yersinia pestis*, the etiologic agent of plague, detected across all areas sampled in virtually every sampling period. Although the route(s) of exposure to those carnivores is unknown, it seems reasonable that ferrets might be similarly exposed, but not survive.

In addition to epizootics, we hypothesized that enzootic plague, that is, presence of disease-causing *Y. pestis* without any noticeable decrease in prairie dog abundance, could be affecting ferret survival. While the effects of epizootic plague are obvious, the effects of enzootic plague are not well understood and may be very important to better understanding

plague epidemiology that may lead to improved success of wildlife conservation efforts. To evaluate our hypothesis, we manipulated the relative risk of plague on portions of two ferret release areas from 2003 to 2006. We controlled fleas, the primary vector for plague, by applying a pulicide (Seery et al. 2003, Seery 2006) in each burrow on dusted colonies annually for 3 years and compared ferret survival on these areas to ferret survival on nondusted colonies. Beginning in 2004, we vaccinated against plague about half of the ferrets residing on dusted and nondusted colonies using F1-V fusion protein, a bicomponent vaccine antigen developed by the U.S. Army Medical Research Institute of Infectious Diseases (Powell et al. 2005) and shown to protect ferrets from plague in laboratory studies (Rocke et al. 2004, 2006, 2008).

## Materials and Methods

### *Study area and flea control*

Our study took place in southern Phillips County in north-central Montana (47°48'N, 107°54'W) on portions of an ex-

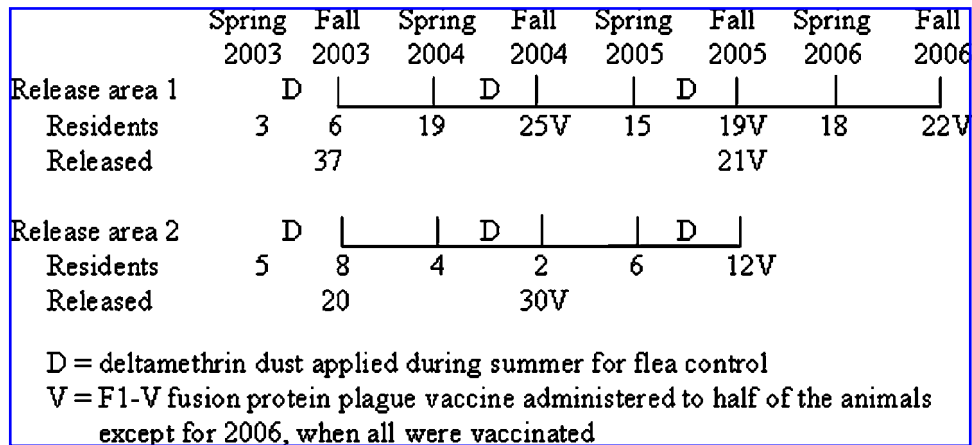


FIG. 2. Black-footed ferret monitoring intervals for the survival analyses and the number of animals present at the beginning of each interval. Wildborn ferrets and those surviving the first interval post-release were considered "Residents."

perimental reintroduction area in which ferrets were first released in 1994 (Fig. 1). Release areas 1A and 1B are on the UL Bend National Wildlife Refuge and managed by the U.S. Fish and Wildlife Service. Until 2007, epizootic plague had never been observed on Release area 1, although it was observed in a prairie dog colony 4 km to the north in 2005. Release area 2 is known as the 40-complex on lands managed by the Bureau of Land Management. Epizootic plague impacted many of these colonies in 1992, but prairie dog populations recovered to levels deemed sufficient to begin ferret releases in 2001. Epizootic plague began affecting one colony (nondusted) on Release area 2 during summer 2005, the last time-interval ferrets were monitored there (Fig. 2).

For flea control, approximately 4 g of DeltaDust® (0.05% deltamethrin; Bayer, Montvale, NJ) was applied into each burrow with Technidusters (Technicide, San Clemente, CA). We treated five colonies encompassing 390 ha in Release area 1A (23,091; 26,911; and 25,620 burrows during 2003, 2004, and 2005, respectively), while four colonies on Release area 1A (350 ha) and four colonies in Release area 1B (450 ha) were not treated to serve as experimental controls (Fig. 1). Similarly, we treated three colonies encompassing 250 ha in Release area 2 (11,656; 15,798; and 15,396 burrows during 2003, 2004, and 2005, respectively), while four colonies (275 ha) were not treated.

*Plague surveillance*

Carnivores, primarily coyotes (*Canis latrans*), have routinely been collected by aerial gunning and ground shooting to assess plague prevalence in ferret reintroduction areas. We tested sera and blood samples on paper strips (Wolff and Hudson 1974) from coyotes and badgers (*Taxidea taxus*) for anti-F1 antibodies, diagnostic for exposure to *Y. pestis*, using passive hemagglutination (World Health Organization 1970) and enzyme-linked immunosorbent assays (ELISA) (Cavanaugh et al. 1979, Willeberg et al. 1979). Our samples were analyzed at the Center for Disease Control (CDC) in Fort Collins, CO, and the Wyoming State Veterinary Laboratory, Laramie, WY.

From 1995 through 2003, we collected blood samples from resident, primarily juvenile wildborn ferrets at Release area 1.

Like for carnivores, these samples were tested for anti-F1 antibodies at CDC and the Wyoming State Veterinary Laboratory. Concurrent with the beginning of our plague vaccine trials during 2004, we attempted to collect blood samples annually from all resident ferrets on Release area 1. These samples were analyzed for antibody to F1 and V antigens at the National Wildlife Health Center using an ELISA as previously described (Rocke et al. 2004, 2006).

We collected fleas from carnivores from 2001 to 2005 and from ferrets from 1996 to 2007. We stored fleas in sterile water or normal saline and tested most of them for plague via mouse inoculation (Poland and Barnes 1979, Quan et al. 1981), but a few were tested with polymerase chain reaction (PCR) (Engelthaler et al. 1999) at CDC for samples collected through 2005. Beginning in 2006, fleas were tested for presence of *Y. pestis* genetic material using a nested-PCR technique (Hanson et al. 2007) at the University of South Dakota.

*Ferret releases and plague vaccinations of captive-reared ferrets*

We released nonvaccinated, captive-reared kits in balanced numbers and sex ratios on dusted and nondusted colonies during fall 2003 on Release areas 1A (*n* = 37) and 2 (*n* = 20) (Fig. 2). Beginning in 2004, captive-reared ferrets before release received a priming dose of F1-V fusion protein vaccine at approximately 60 days of age and a booster at approximately 120 days of age (Rocke et al. 2004, 2008, Powell et al. 2005). We released 15 vaccinated ferrets and 15 nonvaccinates (received placebo injections) on Release area 2 during 2004, split equally among dusted and nondusted colonies. To the extent possible, litters were equally apportioned into vaccine versus placebo groups for both sexes.

In 2005, we released 9 vaccinated and 10 nonvaccinated ferret kits on area 1B, a nondusted area, and 2 female kits, 1 vaccinated and 1 nonvaccinated, on the nondusted portion of Release area 1A. All ferrets involved with this study were marked with passive integrated transponder (PIT) tags that provided for individual identification (Fagerstone and Johns 1987, Biggins et al. 2006b). This research was conducted

in compliance with the Animal Welfare Act and regulations related to animals and experiments involving animals.

#### *Plague vaccinations of wildborn kits and resident ferrets*

During fall 2004 on Release area 1A, we caught and used isoflurane to anesthetize all surviving captive-reared ferrets and all wildborn animals (combined total of 25 ferrets; Fig. 2) for the purpose of implanting PIT tags in wildborn kits, collection of flea, blood and hair samples, and vaccinations against plague and canine distemper virus (Biggins et al. 2006b). We administered the F1-V fusion protein vaccine (a priming dose at first capture followed by a booster at least 30 days later) to approximately half of those 25 resident ferrets, split as equally as possible by litter, sex, and age on both dusted and nondusted areas. We similarly administered placebos to ferrets in the nonvaccinated experimental control group.

We repeated these procedures during fall 2005 for all residents at Release area 1A, with the addition of a single plague vaccine booster (during fall 2005 only) administered to all adults still alive and first vaccinated in 2004. After 2004, we did not administer placebo injections to the animals in the nonvaccinated group; otherwise, handling and processing remained the same. We did not vaccinate any residents at Release area 2 because there were only two ferrets known present before the fall 2004 release, and no wildborn kits were caught there during the duration of monitoring.

#### *Survival monitoring and analysis*

We conducted spotlight surveys (Campbell et al. 1985, Biggins et al. 2006b) to locate as many ferrets as possible each spring and fall throughout each release area. A typical session lasted 1–2 weeks with effort allocated equally across all dusted and nondusted colonies. Sufficient search effort resulted in marking and identifying almost all ferrets present (Biggins et al. 2006b, Matchett, unpublished data). We defined a ferret as “reencountered” if it was individually identified by reading its PIT tag at any time during a session.

We surveyed ferrets across six time intervals, spanning about 6 months each, beginning during fall 2003 and ending during fall 2006 (Fig. 2). Serologic evaluations of ferrets continued through fall 2008. We analyzed data for all ferrets known alive at the beginning of a time interval, along with the covariates age (juvenile vs. adult), area (Release area 1, i.e., both 1A and 1B, or area 2), origin (captive-reared vs. wildborn), sex, vaccination status ( $\pm$ F1-V), and flea control treatment status ( $\pm$ dust). At the end of each interval, we recorded the reencounter status of each ferret. All of those observed during a survey, plus any newly released ferrets, comprised the pool of animals entering the next time interval.

We censored cases from the total data set of 137 individuals (222 ferret-time-intervals) for the following circumstances: residents that received only one plague vaccination dose (7 ferrets across 9 time intervals) and those that had an undetermined or mixed residency status relative to flea control during an interval (5 ferrets across 7 time intervals). Retained in the data set were 3 animals across 4 time intervals that were missed during a survey, but observed during a subsequent survey. Because of their location and behavioral history, we were confident of their location during the missed interval

relative to flea control. Given our overall sample size, this relatively small number of animals missed lends confidence that virtually all animals present during a survey were identified. The resulting data set included 128 individuals observed across 206 ferret-time-intervals.

Our terms “dust” and “vaccine” refer to the binomial variables for ferret residency relative to flea control and plague vaccination history, respectively. To provide an overview of the effect of enzootic plague, we employed a dichotomous variable that categorized plague protection as present (dust, vaccine, or both) or absent (neither dust nor vaccine). We used a simple contingency table for our initial assessment of the effect of any type of protection against plague.

For subsequent multivariate analyses, we used logistic regression (SYSTAT, ver. 12, 2008; Systat Software Inc., Chicago, IL) to assess influences of the primary variables, dust and vaccine, and covariates on reencounter rates. We compared general models to nested submodels using likelihood ratio tests, in an attempt to identify relatively parsimonious models that explained significant variation. We used  $\alpha < 0.10$  for retention of variables in models.

Fully effective flea control or vaccine would be expected to produce a significant statistical interaction between dust and vaccine, whereby ferrets with either treatment would have improved survival compared to ferrets with neither, while effects of these treatments would not be additive (e.g., vaccinated ferrets would survive better than nonvaccinated ferrets on nondusted colonies, but not on dusted colonies). Thus, we initially constructed an omnibus multivariate model, separating vaccine and dust, for evaluating the importance of interactions between these two primary variables and between each of them and all other covariates.

## Results

### *Plague surveillance*

Except for one colony (nondusted) in Release area 2 during the last monitoring interval there, we did not notice any decrease in prairie dog abundance indicative of a plague epizootic from 2003 to 2006. However, antibodies to the F1 antigen were detected in coyotes and badgers in all but 1 year (a sample of only six that year) since we began monitoring in 1993 (Table 1). This suggests common and sustained exposure to plague, both when epizootics were apparent on prairie dog colonies (as expected), and when no epizootics were occurring. The initial collection of carnivores in January 1993 was prompted by the widespread and sudden disappearance of many prairie dog colonies during 1992. This was the first documentation of plague in Phillips County, MT.

We failed to detect *Y. pestis* using mouse inoculation tests of nearly 700 fleas collected from 70 ferrets from 1996 to 2005 at Release area 1. A combination of mouse inoculation and PCR tests of 144 fleas collected from 27 carnivores on Release area 1 from 2001 to 2005 also failed to detect *Y. pestis*. PCR tests at CDC of 25 fleas from 2 ferrets on Release area 1 in 2003 were also negative for presence of *Y. pestis*. In stark contrast, fleas from 3 of 32 ferrets at Release area 1 collected during 2006 and 2007 (total of 373 fleas) tested positive for *Y. pestis* using the Hanson et al. (2007) nested-PCR technique.

From 1995 through 2003, we collected blood samples from 157 individual ferrets on 164 occasions and none tested positive for antibody to the F1 antigen. We collected samples

TABLE 1. PREVALENCE OF PLAGUE AS INDICATED BY EXAMINATION OF CARNIVORE (MOSTLY COYOTES WITH SOME BADGERS) SERA FOR ANTIBODY TITERS TO *YERSINIA PESTIS*

Sampling time	No. of samples	Percent plague-positive	Epizootic plague
January 1993	53	85	Yes
February 1994	34	68	Yes
August 1994	29	86	Yes
February 1995	52	69	Yes
October 1995	49	88	Yes
March 1996	22	91	Yes
August 1996	26	62	Yes
October 1996	34	62	Yes
February 1997	7	43	Yes
October 1997	26	19	No
January 1998	7	43	No
September 1998	17	12	No
December 1999	5	20	No
June 1999	5	40	No
September 1999	16	6	No
September 2000	14	29	No
September 2001	13	23	No
September 2002	9	11	No
September 2003	6	0	No
September 2004	25	8	No
September 2005	10	40	No
September 2006	18	56	No
September 2007	35	23	Yes
Total/mean	512	55	

Samples were collected across southern Phillips County from January 1993 through October 1997. From January 1998 through September 2007, samples were collected from Release area 1, primarily on prairie dog colonies inhabited by black-footed ferrets, on the UL Bend National Wildlife Refuge. Presence of epizootic plague is indicated if prairie dog colonies died-out within the previous 12 months in the vicinity of where carnivores were collected.

from 64 individual ferrets on 85 occasions from 2004 through 2008. All 25 individuals sampled in 2004, before initiating the vaccine trials, tested negative for antibodies to F1 and V antigens, as did all animals that were not vaccinated in subsequent years (except ferret 456 described below). Serology results for vaccinated ferrets are reported below.

*Plague vaccination and serology*

There were 31 vaccinated ferrets among the 115 released individuals and 5 vaccinated individuals among the 20 wildborn ferrets available for study (Table 2). For samples collected at least 1-year postvaccination ( $n=20$ ), we measured positive ( $>1:640$ ) antibody titers to the F1 and V antigens in all but 2 animals (Table 3). One year after receiving a single vaccine dose, ferrets 433 and 461 tested negative via ELISA for F1 and V antigens, forming the basis for censoring animals that received a single dose in our survival analyses.

In contrast, ferret 424 received a single vaccine dose in 2006, yet had the highest F1 titer (655,360) among our observations 1 year later (Table 3). Ferret 456, a wildborn kit in 2007, had an F1 titer of 10,240 and V titer of 640, but had never been vaccinated. The dam for ferret 456 was ferret 445 that received two plague vaccine doses in 2006. Ferret 433 received its two vaccine doses more than 1 year apart and had a minimum

TABLE 2. SAMPLE SIZES (INDIVIDUAL BLACK-FOOTED FERRETS/NUMBER OF TIME INTERVALS) BY REARING ORIGIN

	Vaccinated	Nonvaccinated	Total
Captive-reared			
Dusted	12/17	35/52	47/69
Nondusted	19/27	49/60	68/87
Captive total	31/44	84/112	115/156
Wildborn			
Dusted	3/6	10/28	13/34
Nondusted	2/6	5/10	7 <sup>a</sup> /16
Wildborn total	5/12	15/38	20/50
Dusted	15/23	45/80	60/103
Nondusted	21/33	54/70	75/103
Total	36/56	99/150	135 <sup>b</sup> /206

<sup>a</sup>All seven individuals were male.

<sup>b</sup>This total includes 6 non-vaccinates (1 wildborn, 5 captive-reared) that changed to vaccinates when we began the vaccine portion of the study in 2004 and a single animal (captive-reared) that moved from a non-dusted colony to a dusted colony. Hence, there was actually 128 unique individual ferrets ( $135-7=128$ ).

antibody titer to the F1 antigen of 163,840 and 163,840 to the V antigen 10 months after receiving the last dose.

All four of these animals resided in the portion of Release area 1A that was not dusted and they exhibited the highest observed titers among all of our samples. This area experienced a plague epizootic that became visually apparent during 2007 and continued through 2008, eliminating virtually all prairie dogs on the nondusted portion of Release area 1A. Prairie dog activity and abundance in this area appeared normal during the course of our experiment (2003–2006).

Spotlighting in this prairie dog depauperate portion of Release area 1A during spring and fall 2008 failed to detect any ferrets. In response to this first-ever plague epizootic on Release area 1 (virtually all prairie dogs on Release area 1B also died out during 2007), the remaining portion of Release area 1A, dusted during our experiment from 2003 to 2006 (Fig. 1), was dusted again during early summer 2008. Both prairie dogs and the remaining resident ferrets appeared healthy during September 2008 surveys.

*Reencounter rates and protection from plague*

In our overall assessment of reencounter rates, an index for survival, rates for ferrets with the protection of any combination of dust or vaccine was higher (0.52,  $n=136$ ) than for ferrets having neither protection (0.29,  $n=70$ ,  $\chi^2=10.468$ ,  $p=0.001$ ). Further, the omnibus multivariate model using all 206 records suggested evidence of statistical interactions for vaccine×dust ( $p=0.024$ ) and vaccine×area ( $p=0.027$ ) that prompted construction of submodels.

Sample size and distribution among plague treatments were problematic for wildborn ferrets with only 20 individuals available for study, all at Release area 1 (Table 2). There were only 7 wildborn ferrets (all male) observed over 16 time intervals available to evaluate vaccine effects on nondusted areas. Our analysis using only records for captive-reared ferrets ( $n=156$ ) produced the same evidence of statistical interactions for vaccine×dust ( $p=0.028$ ) and vaccine×area ( $p=0.018$ ). Because of the unbalanced sample distribution related primarily to origin and secondarily to area, and these

TABLE 3. SUMMARY OF SEROLOGY RESULTS FOR BLACK-FOOTED FERRETS VACCINATED AGAINST PLAGUE (EXCEPT FOR FERRET 456) WITH F1-V FUSION PROTEIN VACCINE FROM 2004 TO 2008

Ferret ID <sup>a</sup>	Age	Origin	First plague vaccination date	Second plague vaccination date	Blood draw date	Months since last vaccine dose	ELISA plague	
							F1	V
419	Adult	Wild	8/30/2004	10/20/2004	8/25/2005	10	10240	40960
424 <sup>b</sup>	Adult	Wild	9/2/2006		8/29/2007	12	655360	40960
433	Adult	Wild	8/22/2005		8/30/2006	12	Negative	Negative
433 <sup>b</sup>	Adult	Wild	8/22/2005	10/18/2006	8/30/2007	10	>163840	163840
444	Adult	Wild	8/28/2006	10/15/2006	8/31/2007	11	10240	40960
445 <sup>b</sup>	Adult	Wild	8/28/2006	10/13/2006	8/27/2007	10	>163840	>163840
446	Adult	Wild	8/28/2006	10/13/2006	8/30/2007	11	2560	2560
446	Adult	Wild	8/28/2006	10/13/2006	8/24/2008	22	2560	10240
449	Adult	Wild	8/29/2006	10/13/2006	9/2/2007	11	10240	40960
451	Adult	Wild	9/2/2006	10/13/2006	8/29/2007	11	40960	10240
451 <sup>c</sup>	Adult	Wild	9/2/2006	10/13/2006	8/21/2008	12	163840	40960
456 <sup>b</sup>	Juvenile	Wild			8/29/2007	0	10240	640
459	Adult	Wild	9/9/2007	10/12/2007	8/20/2008	10	10240	40960
461	Adult	Wild	10/16/2007		9/9/2008	11	Negative	Negative
4044	Adult	Captive	8/31/2004	10/18/2004	8/24/2005	10	2560	10240
4125	Adult	Captive	8/30/2004	10/23/2004	8/24/2005	10	2560	10240
4482	Adult	Captive	8/1/2004	10/1/2004	9/8/2005	11	40960	163840
4559	Adult	Captive	8/1/2004	10/1/2004	9/7/2005	11	10240	40960
4960	Adult	Captive	8/18/2005	10/26/2005	9/4/2006	10	40960	40960
4961	Adult	Captive	8/18/2005	10/26/2005	9/2/2006	10	40960	163840

<sup>a</sup>Results are sorted by black-footed ferret ID number.

<sup>b</sup>These four ferrets were the only animals present on the portion of Release area 1A, where epizootic plague erupted during 2007.

<sup>c</sup>Ferret 451 received a third plague vaccination on 8/29/2007.

ELISA, enzyme-linked immunosorbent assays.

statistical interactions, we focused subsequent analyses on captive-reared ferrets within vaccine and dust categories.

Both vaccine ( $p = 0.044$ ) and area ( $p = 0.019$ ) were retained in the model that evaluated vaccine effects on reencounter rates of captive-reared ferrets living on colonies without flea control (Fig. 3). Vaccination did not influence reencounter rates of ferrets living on colonies with flea control ( $p = 0.754$ ), although age ( $p = 0.005$ ) and area ( $p = 0.040$ ) were influential (data not shown).

Nonvaccinates living on colonies with flea control had higher reencounter rates than those living on colonies without flea control (Fig. 4). That model retained the variables dust ( $p = 0.026$ ), age ( $p = 0.016$ ), and area ( $p = 0.001$ ). The only

variable retained in the model that evaluated flea control among vaccinates was age ( $p = 0.023$ ), with dust ( $p = 0.508$ ) having no detectable influence (data not shown).

Nonadjusted point estimates of reencounter rates for captive-reared ferrets were 0.44 for vaccinates on colonies without flea control, 0.41 for vaccinates on colonies with flea control, 0.23 for nonvaccinates on colonies without flea control, and 0.42 for nonvaccinates on colonies with flea control.

## Discussion

Both flea control with deltamethrin and the F1-V fusion protein plague vaccine resulted in greater ferret survival

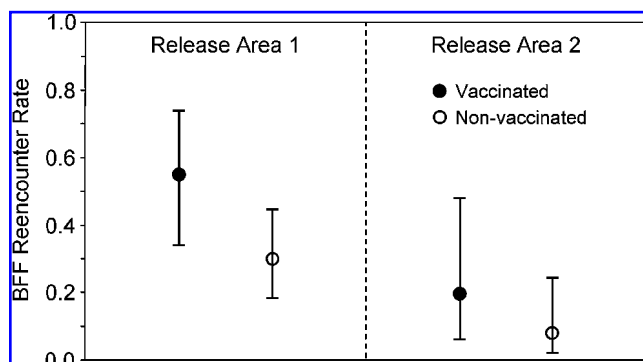


FIG. 3. Effect of vaccine on black-footed ferret survival on colonies without flea control, with area as a covariate (adjusted reencounter rates and 95% confidence intervals).

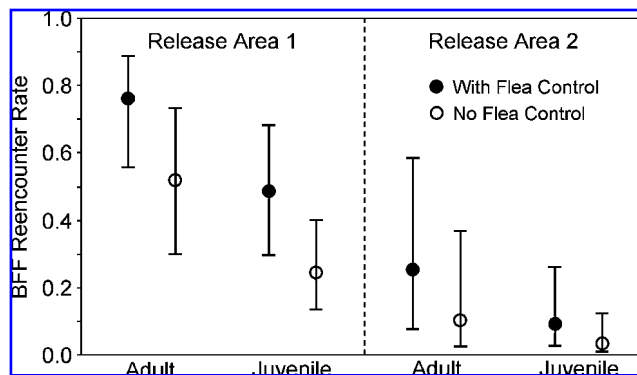


FIG. 4. Effect of flea control on survival of non-vaccinated black-footed ferrets, with area and age as covariates (adjusted reencounter rates and 95% confidence intervals).

compared to ferrets receiving no plague risk-reduction treatments. Our findings demonstrate plague reduced ferret survival when there was no noticeable die off of prairie dogs. We also demonstrated that the F1-V fusion protein vaccine provides protection from plague to ferrets in the wild. Except for ferret 456, we did not observe a single plague-positive titer in nonvaccinated ferrets among the 229 samples we tested from 1995 to 2008. Given the lethality of plague to ferrets, it seems whatever exposure there is at enzootic levels results in death, with few if any survivors.

We observed evidence of a statistical interaction between the two plague treatments, our predicted result if enzootic plague was present and both treatments were effective. We could not detect changes in survival due to vaccine on areas with flea control or due to flea control for vaccinated ferrets. We interpret the different effect of vaccine on ferrets living on colonies with and without flea control to mean that fleas are required for the maintenance, and probably transmission of plague at enzootic levels. This interpretation is consistent with the long-held paradigm that fleas are required for the transmission and maintenance of plague in rodent populations (Pollitzer and Meyer 1961, Bibikova 1977, Lorange et al. 2005). Recently, however, Webb et al. (2006) proposed that classic flea-borne plague transmission (i.e., infectious bite from blocked fleas) does not drive epizootics in prairie dogs. Our finding that vaccine and flea control similarly improved ferret survival, combined with detection of *Y. pestis* genetic material in fleas collected from ferrets, compels a conclusion that fleas played an important role in enzootic plague maintenance and transmission dynamics on our study sites.

Our carnivore serology results indicated common and sustained exposure to *Y. pestis* even when no plague epizootics were observed on prairie dog colonies, yet we could not detect *Y. pestis* in extensive flea samples collected from carnivores and ferrets using common diagnostic methods. Further, Holmes 2003 and Holmes et al. 2006, using standard CDC testing protocols, did not detect *Y. pestis* in fleas collected from burrows, prairie dogs, or small mammals in southern Phillips County, or serological evidence of plague in small mammals. Also in Phillips County, MT, and with similar diagnostic techniques, Biggins et al. (2009) found only a single black-tailed prairie dog that was seropositive for plague and detected no *Y. pestis* in fleas combed from hundreds of prairie dogs in years and at study sites without epizootic plague. In contrast, the nested-PCR technique of Hanson et al. (2007) revealed the presence of *Y. pestis* genetic material in fleas collected from nearly 10% of the ferrets from which we collected fleas in 2006 and 2007. Similarly, Hanson et al. (2007) reported finding evidence of *Y. pestis* in fleas collected on 60% of healthy, normal appearing prairie dog colonies in a study area on the Fort Belknap Indian Reservation, immediately adjacent to our study area.

The greater survival of vaccinated ferrets living on colonies without flea control makes a compelling argument that disease-causing *Y. pestis* was present, but not detected with our sampling and testing before 2006. Differences in assay sensitivity may explain the discrepancy, but those earlier flea samples were not available to re-test to confirm this possibility. To be sure, standard PCR is known to be more sensitive for detection of *Y. pestis* than mouse survival assays (Engelthaler et al. 1999), and nested PCR, a relatively new technique applied to *Y. pestis* diagnostics, is considered even

more sensitive. Clearly, a better assessment of our ability to detect infectious plague is needed along with identifying how/where the pathogen is maintained at enzootic levels.

Ferret 424 showed the highest antibody titer we observed to the F1 antigen 1 year after receiving a single vaccine dose. This observation is important because it suggests an anamnestic response (boosting) from postvaccination exposure to *Y. pestis* in the wild. If true, administration of even a single dose of vaccine may provide a notable degree of protection. In a previous laboratory study of ferrets that received a single dose of vaccine, 25% (2/8) survived an oral plague challenge (Rocke et al. 2008). We suspect that the next two highest F1 titers among our samples (ferrets 433 and 445) may have similarly been elevated as a result of exposure to *Y. pestis* in the wild. These highest F1 titers, plus the single-positive titer from a nonvaccinated kit (Table 3), came from the only four animals living in the area where epizootic plague erupted on Release area 1A during 2007. Ferrets 424, 433, 445, and 456 were resident in this area since being born there in 2004, 2005, 2006, and 2007, respectively.

To our knowledge, ferret 456 is the only nonvaccinated ferret in the wild to have ever shown a positive plague titer. The only other examples of ferret survival to plague exposure occurred in a captive setting where 27 ferrets were likely killed and 2 survived with antibody titers (Godbey et al. 2006). Exposure levels of those animals were likely lower than what might be expected in the wild. Passive immunity may have been conferred to ferret 456 from her vaccinated mother (ferret 445). If so, this example suggests boosting and survival from subsequent exposure to *Y. pestis*. Additional investigations on passive immunity and single-dose vaccinations with subsequent exposure to *Y. pestis* are warranted.

We were not surprised that the covariates age and area were retained in several of the models because similar survival patterns were observed in earlier years. Ferret monitoring before this study in Release area 1 suggested that about 20% of captive-reared kits released in the fall survived until the following summer. Their survival for the following year (after becoming adults) was about 50% (Matchett, unpublished data). A similar pattern relative to age was also observed on Release area 2 before this study, but both rates were lower, not unlike the relationships seen in Figure 4. It is noteworthy that among 17 nonvaccinated juvenile ferrets released on colonies without flea control on Release area 2, not one was observed again.

Although most human risk of plague is associated with epizootic plague (Perry and Fetherston 1997), knowledge presented herein that *Y. pestis* population levels and transmission rates insufficient to cause any noticeable prairie dog die off do in fact affect ferret survival has ramifications for human health, as well as wildlife conservation. Indeed, a recent fatal human case of pneumonic plague acquired from a mountain lion (*Felis concolor*) may be an example of the potential hazard of enzootic plague because no epizootic source could be identified (Wong et al. 2009).

Our observation that enzootic plague negatively effects ferret populations refines the conventional understanding of plague epizootiology as a singular die off wave that progresses through a highly susceptible population after exposure to some reservoir form (Girard et al. 2004, Webb et al. 2006). Further analysis of *Y. pestis* heterogeneity within hyper-variable regions among such enzootic populations may test a

possible role of pathogen adaptation for previously unrecognized disease maintenance between outbreaks. Our findings offer new insights to the current understanding of plague transmission and maintenance between enzootic and epizootic phases. Although many factors affect ferret population establishment, increased ferret mortality from enzootic plague is hindering recovery success. This experiment using ferrets, as well as the additional evidence of broad-scale effects of enzootic plague on survival rates for several species of prairie dogs (Biggins et al. 2009), heightens the growing concern that plague as an invasive disease is in fact disrupting North American ecosystems (Biggins and Kosoy 2001).

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### Disclaimer Statement

The findings and conclusions in this article are those of the authors and do not necessarily represent the views of the U.S. Fish and Wildlife Service. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

### Disclosure Statement

No competing financial interests exist for any of the authors.

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