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Management and Conservation Article

Changes in Kit Fox Defecation Patterns During the Reproductive Season: Implications for Noninvasive Surveys

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ABSTRACT Noninvasive survey methods based on analyzing DNA extracted from feces can be useful for carnivores that are difficult to study by other methods. Changes in fecal deposition patterns associated with reproduction in kit foxes (*Vulpes macrotis*) might affect results of such surveys. We used a trained dog to collect fresh scats on 2-km transects in the home ranges of 11 radiocollared female kit foxes in January, February, and March 2008 and determined sex of the individual that deposited the scats by amplifying the zinc finger protein gene. Female foxes give birth in mid-February to mid-March. We found a similar number of scats each month. In January, the sex ratio of the scats was not different from the expected 1:1. However, in February there were almost 2 male scats for every female scat and in March there were >8 male scats for every female scat. Comparing March to January, there were more male scats on all 11 transects and fewer female scats on 10 of 11 transects. Around the time pups are born, both sexes appear to show changes in fecal deposition patterns that make it easier to find male scats and harder to find female scats. Effects of these changes on survey results will vary depending on the purpose and design of the survey. Surveys to determine distribution and relative abundance would probably not be negatively affected by these changes. However, if surveys to estimate abundance are conducted during the reproductive season, they could result in an underestimate of population size unless the increased heterogeneity in scat detectability is taken into account.

KEY WORDS capture heterogeneity, feces, kit fox, noninvasive surveys, scat, sex ratio, *Vulpes macrotis*, zinc finger protein gene.

Many carnivore species are difficult to study by direct observation or capture–recapture methods. Population densities may be low, home ranges large, and the animals nocturnal and secretive. Because of these problems, there is increasing interest in developing more effective noninvasive survey methods for carnivores (Long et al. 2008). Noninvasive survey methods include recent approaches based on isolating and analyzing DNA extracted from hairs or feces (scats; Schwartz and Monfort 2008). Molecular genetic analyses of DNA extracted from feces can be used to document distribution and abundance (Smith et al. 2006*a*), determine sex ratios (Smith et al. 2006*b*), or estimate population size (Frantz et al. 2003, Bellemain et al. 2005, Prugh et al. 2005, Ruell et al. 2009, Cubaynes et al. 2010).

Studies of the accuracy of noninvasive surveys based on fecal DNA have focused on genotyping errors (Taberlet et al. 1996, Mills et al. 2000, Miller et al. 2002, McKelvey and Schwartz 2004, Smith et al. 2006*b*). Differences in fecal deposition patterns related to age, sex, social, or reproductive status might also lead to errors in analyses based on fecal DNA genotypes (Kohn et al. 1999, Cubaynes et al. 2010). However, for most species little is known about the details of such differences in fecal deposition patterns or how they might affect results of noninvasive surveys based on fecal DNA.

Differences in the number of scats found per kilometer searched have provided information on distribution and relative abundance of San Joaquin kit foxes (*Vulpes macrotis mutica*; Smith et al. 2006*a*). Because these surveys must cover a broad area and funding to survey different areas becomes available at different times, different areas have been surveyed at different times of year. The kit fox is a seasonally breeding species in which females give birth annually in mid-February to mid-March (Egoscue 1956, Zoellick et al. 1987). We speculated that scats of females might become more difficult to find during the pupping season. If so, surveys at this time of year could give a misleading impression of kit fox distribution and relative abundance. To evaluate this hypothesis, we collected kit fox scats during the last 2 weeks of each month in January, February, and March 2008 and determined sex of the individual that deposited the scats by amplifying a short fragment of the zinc finger protein genes that is a fast and reliable method of determining sex from feces in kit foxes (Ortega et al. 2004).

Kit foxes are socially monogamous and mated pairs remain together throughout the year (Ralls et al. 2007); thus the expected sex ratio for live animals is 1:1. Numerous field studies (summarized in Moehrenschlager et al. 2004), as

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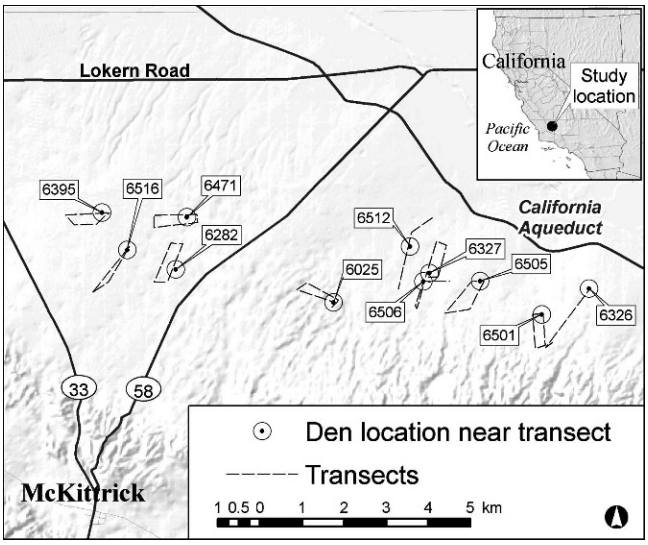

Figure 1. Study area in Lokern Natural Area, California, USA, showing one of the dens used by each of the 11 individual female kit foxes and the scat-collection transects we established in January 2008.

well as molecular sexing of scats collected in July (Smith et al. 2006*b*), confirmed this expectation. Thus, we expected to find approximately equal numbers of male and female scats each month if the scats of males and females were equally likely to be found.

STUDY AREA

We conducted fieldwork in the southern San Joaquin Valley, California, USA, at the Lokern Natural Area. Lokern was in western Kern County, approximately 40 km west of Bakersfield (Fig. 1). The Mediterranean climate was characterized by hot, dry summers and cool, wet winters. Annual precipitation averaged about 14 cm but was highly variable (Spiegel 1996). February was the wettest month during our study; monthly rainfall totals for Bakersfield in 2008 were 1.68 cm in January, 2.08 cm in February, and a trace in March (National Oceanic and Atmospheric Administration 2009). Vegetation consisted primarily of nonnative annual grassland with some areas of arid-land shrubs. Detailed descriptions of the area are given in Spiegel (1996) and Nelson et al. (2007).

METHODS

Most pups have dispersed from their natal territories by January, so the population on the study area consisted mainly of resident adult pairs. Eleven radiocollared adult females (Fig. 1) were captured previously as part of a different study using wire-mesh live traps (model 109; Tomahawk Equipment Company, Tomahawk, WI) and fitted with an approximately 40-g very high frequency (VHF) radiocollar (model 1930; Advanced Telemetry Systems, Isanti, MN). We were authorized to study San Joaquin kit foxes under Endangered Species Act permit TE-825573-3 from the United States Fish and Wildlife Service and a Memorandum of Understanding with the California Department of Fish and Game. We followed standard methods designed to minimize risk of stress or injury (Cypher et al. 2000), approved by both agencies, for trapping, handling, and radiocollaring kit foxes. We tracked each radiocollared female to her den once per week.

Previous studies indicated that trained dogs find more scats than do human observers (Smith et al. 2003), so we used a professional detection dog and handler team (Working Dogs for Conservation, Three Forks, MT) to search for scats. The dog and handler had extensive experience collecting kit fox scats. We used only one dog in our study. In an earlier study in a nearby area (Smith et al. 2003), the mitochondrial DNA extracted from scats found by this dog indicated that it was consistently accurate at finding only kit fox scats (and ignoring those of other species such as coyotes [*Canis latrans*]) in this area.

To ensure that we collected scats from multiple fox home ranges, we established a 2-km survey route within an area we judged to be part of each radiocollared female's home range based on locations of dens. We gave the investigator that established these transects information on the general area used by each female but not on the locations of her dens. Most transects were looped or continuous routes to eliminate backtracking. We included unpaved roads and vegetated areas as available, because dogs found numerous scats along both of these substrates in other studies (Smith et al. 2003). Previous work indicated that dogs found about 25 scats/km in the Lokern area (Smith et al. 2006*a*), so we expected to find about 50 scats per transect or 550 scats each month. Although dogs locate many old scats that contain DNA that is too degraded for successful polymerase chain reaction (PCR) amplification of the zinc finger protein genes used for determining sex (Ortega et al. 2004), we expected to find about 150 fresh, useable scats per month. Chi-square simulations indicated that sexing as few as 125 scats per month would give us a high degree of statistical power to distinguish between a sex ratio of 60 male to 40 female scats and the expected 50:50 ratio.

We conducted scat searches in January, February, and March 2008 for 6 to 7 days during the last 2 weeks of each month. When the dog alerted its handler to presence of a kit fox scat, the location of the scat was geo-referenced with a Global Positioning System (GPS). We collected and used for DNA analysis only scats deposited by adult foxes (adult scats are much larger than scats produced by young pups) with the physical characteristics of fox scats <8 days old as determined by our freshness rating method (see Smith et al. 2003). We stored scats in plastic bags containing 1 teaspoon of silica gel for desiccation (Fisher Scientific, Pittsburgh, PA) and shipped samples within 7 days of collection to the genetics laboratory at the National Zoological Park for storage at -20° C.

Laboratory Procedures

Shortly after the samples arrived at the genetics laboratory, we extracted DNA from every scat sample using the QIAGEN DNeasy™ extraction kit (Qiagen Inc., Valencia, CA) and following the modified protocols outlined in Eggert et al. (2005). We conducted DNA extractions in a separate facility away from the genetics laboratory to avoid

Table 1. Number of fresh kit fox scats we found and sexed in the Lokern Natural Area of California, USA, in 2008, number of male scats, number of female scats, sex ratio, polymerase chain reaction amplification success, and allelic dropout rate each month.

Month	No. found	No. sexed	M	F	Sex ratio	% amplified	Dropout
Jan	171	130	58	72	0.8:1	77.3	4.7
Feb	202	148	96	52	1.8:1	73.3	3.1
Mar	174	154	138	16	8.6:1	75.4	14.5

contamination from PCR products. We isolated each sample ≥2 times and typed it ≥3 times to test for errors and ambiguities in sex identification. Negative controls (no scat material added to the extraction), used to check for contamination, accompanied each set of extractions. We also included positive controls from tissue samples of kit foxes of known sexes in each set of PCR amplifications.

We determined the sex of the animal that deposited each scat using a modification to an earlier protocol developed in our laboratory by Ortega et al. (2004). Both methods use primers that amplify a short (195 base pair [bp]) fragment of the zinc finger protein genes (Zfx and Zfy). In kit foxes, this fragment contains a Taq$^{\alpha}$I digestion site unique to the Zfy gene. In an agarose gel, successful PCR products show a double band for males and a single band for females (Ortega et al. 2004). However, degraded samples with low quantities of DNA produced PCR products that were difficult to detect in agarose gels. To increase efficiency of screening many fecal samples and to improve our ability to detect fragments, we modified the original protocol by adding a carboxyfluorescein label to the forward primer (ZFKF 203L) designed by Ortega et al. (2004) and by running the digested PCR fragments directly onto an ABI PRISM* 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA).

In our modified protocol, we performed PCR amplifications in an MJ Research PTC-100 thermocycler (MJ Research, Waltham, MA) in 10-μL reaction volumes and fluorescently labeled the forward primer (ZFKF 203L) with 6-FAM (Operon Technologies Inc., Alameda, CA). The final reaction conditions were as follows: 0.5 U Ampli Taq Gold DNA Polymerase (Applied Biosystems, Inc.), 1× AmpliTaq Buffer II, 0.4 μM fluorescently labeled forward primer, 0.4 μM unlabeled reverse primer, 2 mM $MgCl_2$, 0.2 mM each deoxyribonucleotide triphosphate, 10× bovine serum albumin (New England Biolabs, Ipswich, MA), and 2–3 μL of the DNA extract. Polymerase chain reaction conditions and protocols for restriction enzyme digestion of PCR products were the same as described in Ortega et al. (2004); however, in our study, we added 1 μL of the digested PCR product to 9 μL of formamide/ROX solution (Applied Biosystems), electrophoresed and detected on an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). We performed fragment size analysis using the Genotyper® 2.5 or GeneMapper® software (Applied Biosystems). We determined samples to be males when ≥1 of 3 PCR amplifications yielded 2 fragments (representing the 2 genes: the 154-bp fragment [*Zfx*] and the 195-bp fragment [*Zfy*]) and females when we consistently detected one fragment (representing the *Zfx* gene) in ≥3 independent amplifications.

Our modified protocol was an effective way of processing and detecting PCR products of Zfy and Zfx. With this protocol, we loaded samples onto a 96-well plate and ran them directly on an ABI PRISM 3100 Genetic Analyser for 2.5 hours. Although fluorescently labeled primers are more expensive than unlabeled primers, peak detection of PCR products with fluorescently labeled primers was more sensitive and accurate than detection of bands and determination of fragment size in an agarose gel using ethidium bromide and ultraviolet fluorescence to visualize the fragments.

RESULTS

We collected 547 fresh scats and successfully amplified DNA from 432 of them (Table 1). Because we amplified DNA samples ≥3 for each scat, we could estimate amplification success and rates of allelic dropout when one of the 2 fragments failed to amplify in a particular reaction. Average amplification success was 75.3% (range 73.3–77.3%) and we found low rates of allelic dropout in January and February (Table 1). We detected an increase in rates of allelic dropout in the March samples, when 57 out of the 394 successful PCR amplifications failed to amplify one of the 2 fragments. Only 19 of the 57 reactions showed dropout for the 154-bp fragment (Zfy) so most of the genotyping error (38/57 cases) was due to dropout of the 195-bp fragment (Zfx). However, if allelic dropout had introduced a bias against females in our study, we should have observed a lower amplification success in the month that had the most female scats (Jan) but this was not the case.

We found similar numbers of scats each month but the sex ratio varied across months (Table 1). In January, we found 58 male and 72 female scats, which was not different from the expected 50:50 ratio ($\chi^2 = 1.66$, $P = 0.280$). In February, we found almost 2 male scats for every female scat, which was different than expected ($\chi^2 = 8.88$, $P = 0.003$), and in March we found >8 male scats for every female scat, which again was different than expected ($\chi^2 = 62.7$, $P = 0$).

Tabulating the number of male and female scats found on each transect per month showed a high degree of consistency in the trends over time across the 11 transects (see Table S2, available at <http://dx.doi.org/10.2193/2009-401.s1>). We found more male scats on all 11 transects in March than in January ($n = 11$, $P < 0.001$, sign test) and we found fewer female scats on 10 of the 11 transects ($n = 10$, $P < 0.001$, sign test).

DISCUSSION

We found and sexed approximately the same number of scats each month, but the sex ratio changed markedly from

month to month, reflecting the expected equal sex ratio in January but becoming increasingly male-biased in February and March. As expected, the number of female scats we found decreased each month, from 72 in January to 16 in March. Unexpectedly, however, the number of male scats we found increased each month, from 58 in January to 138 in March. Because we collected only scats deposited by adults, these results suggest that adults of both sexes showed changes in fecal deposition patterns associated with the birth of pups in late February or early March.

Male kit foxes bring food to females with young pups (Moehrenschlager et al. 2004), and males likely spend more time hunting when feeding their mates as well as themselves. Males may deposit fewer scats near their den and more scats in other areas of their home range, making male scats more evenly dispersed and easier to find. In contrast, females with young pups must change their defecation behavior so that their scats are harder to find. However, our suspicion that females might defecate near the den during this period was incorrect. We continued to monitor dens of the radiocollared females during the 2009 pupping season and found that there were no scats present at dens when pups were still below ground. However, once pups began to emerge aboveground at about 1 month of age (as documented by direct observations or motion-triggered cameras near dens), we found both pup and adult scats near the den. Females with young pups may defecate in a latrine chamber underground or in an aboveground latrine away from the immediate vicinity of the den (Ralls and Smith 2004).

It is unlikely that our results can be explained by female foxes depositing fewer scats during early lactation because of fasting at this time. The use of maternal reserves as a lactation strategy is only feasible in large mammals such as bears, seals, and whales (Oftedal 2000). Small mammals such as foxes expend energy at a much higher rate relative to body mass than do large mammals; thus small mammals are physically unable to store sufficient energy to cover the energy expenditures associated with lactation for any length of time (Oftedal 2000). In carnivores with litters, such as foxes, lactation poses a large nutrient demand on mothers and thus food intake increases with the onset of lactation (Oftedal and Gittleman 1989); thus, one might expect that more rather than fewer feces would be produced by lactating females.

The effect of changes in fecal deposition patterns that we observed on the results of surveys using fecal genotyping would depend on the purpose of the survey. Contrary to our initial hypothesis, surveys to determine distribution and relative abundance of kit foxes, such as those by Smith et al. (2006*a*) would not be affected, because the number of fresh scats found per kilometer surveyed was similar across months, ranging from 5.9 (130 scats/22 km) in January to 7 (154 scats/22 km) in March. However, detectability of female scats in March was extremely low. Even though we searched in areas known to be used by individual female foxes, we found no scats in the home range of 3 of the 11 females and only one scat in the range of 4 others (see Table S2, available at <http://dx.doi.org/10.2193/2009-401.s1>). Furthermore, the Lokern area has a high density of kit foxes; Smith et al. (2006*a*) found about 25 scats/km (counting both old and fresh scats) there during previous surveys. We suspect that if we had conducted surveys in March in an area with a low density of kit foxes, such as the Ciervo-Panoche area where Smith et al. (2006*a*) found <2 kit fox scats per kilometer in previous surveys, without knowledge of the general areas used by individual females, we would have failed to detect many females.

Although no noninvasive mark–recapture surveys for kit foxes based on scats have been undertaken to date, such surveys would be possible because kit fox scats can be identified to the individual level based on microsatellite and sex genotype (Smith et al. 2006*b*). Capture heterogeneity, that is, differences in probability of finding scats of different individuals, is an important consideration in mark–recapture studies because it can lead to an underestimate of population size (Otis et al. 1978). Substantial individual heterogeneity in scat detection probability has been documented in bobcats (*Lynx rufus*; Ruell et al. 2009) and wolves (*Canis lupus*; Cubaynes et al. 2010). Capture heterogeneity can result from individual differences in defecation rates, road use, or territory marking, or variation in the quantity of DNA found in scats of different individuals (Bellemain et al. 2005, Eggert et al. 2005, Lukacs and Burnham 2005, Cubaynes et al. 2010). Our results indicate that changes in individual fecal deposition patterns associated with reproduction can be an additional source of capture heterogeneity that should be considered in the design of noninvasive surveys based on scats. Even though detection heterogeneity can be modeled if the survey design includes >2 sampling events, it is always advisable to minimize it (Long and Zielinski 2008). Detection heterogeneity associated with kit fox reproduction could be avoided by scheduling sampling events for mark–recapture surveys outside of the reproductive season. If surveys are conducted during the reproductive season, mark–recapture models that explicitly account for individual detection heterogeneity, such as those developed by Cubaynes et al. (2010), should be used to avoid underestimating population size. In noninvasive surveys for wolves, models that ignored individual detection heterogeneity underestimated population size by an average of 27% (Cubaynes et al. 2010).

Changes in male and female defecation patterns during the reproductive season may occur in other small canids, such as swift foxes (*Vulpes velox*), in which males help care for pups (Allardyce and Sovada 2003). Researchers collecting scats of island foxes (*Urocyon littoralis*) on San Nicolas Island, California, for diet studies found that scats were much harder to find during the reproductive season, with many fewer scats along roads than at other times of year, which suggests that scats of male as well as female island foxes are more difficult to find during the reproductive season (C. Cory, The Nature Conservancy, personal communication; F. Ferrara, Institute for Wildlife Studies, personal communication). Changes may also occur in canid species that live in larger social groups, because detection

probability of wolf scats was higher before than after the breeding period (Cubaynes et al. 2010) and in females of carnivore species that rear their young without male help. For example, female mountain lions (*Felis concolor*) bury their feces when they have young kittens (Seidensticker et al. 1973). More detailed studies of variation in defecation patterns of other carnivore species during the reproductive season may help improve accuracy of noninvasive surveys based on feces.

MANAGEMENT IMPLICATIONS

Noninvasive survey methods are increasingly used to determine animal presence and assess abundance. For methods involving scats, heterogeneity in scat detection rates among sex, age, or social classes could adversely affect survey results. Around the time kit fox pups are born, both parents appear to show changes in fecal deposition patterns that make it easier to find male scats and harder to find female scats. Effects of these changes on survey results will vary depending on the purpose and design of the survey. Surveys to determine distribution and relative abundance would not be affected. However, if surveys to estimate abundance are conducted during the reproductive season, they could result in an underestimate of population size unless the increased heterogeneity in scat detectability is taken into account.

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