Journal of Wildlife Management 74(5):1050–1058; 2010; DOI: 10.2193/2009-305

Management and Conservation Article

Efficient, Noninvasive Genetic Sampling for Monitoring Reintroduced Wolves

JENNIFER L. STENGLEIN, *University of Idaho, Department of Fish and Wildlife Resources, Moscow, ID 83844, USA*
LISETTE P. WAITS,[1] *University of Idaho, Department of Fish and Wildlife Resources, P.O. Box 441136, Moscow, ID 83844, USA*
DAVID E. AUSBAND, *Montana Cooperative Wildlife Research Unit, 205 Natural Sciences Building, University of Montana, Missoula, MT 59812, USA*
PETER ZAGER, *Idaho Department of Fish and Game, 3316 16th Street, Lewiston, ID 83501, USA*
CURT M. MACK, *Nez Perce Tribe, Gray Wolf Recovery Project, P.O. Box 1922, McCall, ID 83638, USA*

ABSTRACT Traditional methods of monitoring gray wolves (*Canis lupus*) are expensive and invasive and require extensive efforts to capture individual animals. Noninvasive genetic sampling (NGS) is an alternative method that can provide data to answer management questions and complement already-existing methods. In a 2-year study, we tested this approach for Idaho gray wolves in areas of known high and low wolf density. To focus sampling efforts across a large study area and increase our chances of detecting reproductive packs, we visited 964 areas with landscape characteristics similar to known wolf rendezvous sites. We collected scat or hair samples from 20% of sites and identified 122 wolves, using 8–9 microsatellite loci. We used the minimum count of wolves to accurately detect known differences in wolf density. Maximum likelihood and Bayesian single-session population estimators performed similarly and accurately estimated the population size, compared with a radiotelemetry population estimate, in both years, and an average of 1.7 captures per individual were necessary for achieving accurate population estimates. Subsampling scenarios revealed that both scat and hair samples were important for achieving accurate population estimates, but visiting 75% and 50% of the sites still gave reasonable estimates and reduced costs. Our research provides managers with an efficient and accurate method for monitoring high-density and low-density wolf populations in remote areas.

KEY WORDS *Canis lupus*, noninvasive genetic monitoring, population density, population estimation, probability of capture, wolf.

Since their reintroduction into the Northern Rocky Mountain (NRM) ecosystem, gray wolves (*Canis lupus*) have been carefully monitored, primarily using radiotelemetry. Radiotelemetry is a preferred method for monitoring small populations of wolves because it is reliable for obtaining territory size, calculating density, determining dispersal distance, and documenting pack size and breeding status (Fritts 1983, Fuller and Snow 1988, Ballard et al. 1998, Mitchell et al. 2008). Although telemetry has many advantages, it is labor intensive and requires trapping and handling of animals, and it can typically be maintained only in a small subset of the population or at a smaller spatial scale (Kunkel et al. 2005). Also, radiotelemetry largely depends on visual detection from the air for pack counts (Fuller and Snow 1988), so it is potentially less effective in areas with dense tree cover. As the NRM wolf population grows and stabilizes, it will be difficult to maintain radiocollars in a high percentage of the packs, but the requirements for monitoring will persist. For long-term management of NRM wolves, it is imperative to develop a cost-effective and efficient method for monitoring the population.

Noninvasive monitoring techniques, such as howling surveys, winter tracking, hair collection, camera trapping, and scat surveys have been useful for monitoring carnivores (Harrington and Mech 1982*a*, Paquet 1991, Lucchini et al. 2002, Clevenger and Waltho 2005, Long et al. 2008). Even though they exist in low densities, wolves are well-suited to noninvasive monitoring because they are territorial and often leave sign in prominent places along roads, trails, or junctions (Barja et al. 2004, MacKay et al. 2008). Of these methods, scat surveys are an established wildlife management technique that has been shown to be useful in detecting the presence of a target species, but it has limited utility in monitoring population trends because individuals cannot be identified and field identification of sign is difficult (Reynolds and Aebischer 1991, Davison et al. 2002). With genetic analysis, however, individuals can be identified from scat samples, providing a method for monitoring wolf populations (Waits and Paetkau 2005).

Long-term wildlife monitoring programs require methods that can efficiently produce reliable data annually, and genetic monitoring has been proven a useful tool (Schwartz et al. 2006). A monitoring method using genetic data collected noninvasively can produce minimum counts and home ranges, pack counts, accurate population estimates, and territory sizes and can document breeding status of individual packs (Taberlet et al. 1997, Lucchini et al. 2002, Lukacs and Burnham 2005, Adams 2006, Ausband et al. 2010). Over time, a genetic monitoring program could assess connectivity between recovery areas, track population trends, detect hybridization events, and assess genetic impacts from harvest (Adams et al. 2003, Prugh et al. 2005, Williams et al. 2009). To assess whether noninvasive genetic sampling (NGS) could effectively and efficiently contribute to long-term gray wolf monitoring, we conducted a 2-year study in Idaho, USA. We surveyed areas with a high probability of having reproductive wolf packs. Our objectives were to 1) estimate the minimum number of

[1] *E-mail: lwaits@uidaho.edu*

wolves and determine whether we could distinguish high and low wolf density areas with NGS minimum counts, 2) compare population estimates from single-session models using noninvasive genetic data with estimates derived from radiotelemetry, and 3) determine the most efficient and cost-effective NGS protocol by assessing how reduced sampling affected the minimum count, the population estimate, and the probability of capturing known, radio-collared wolves.

STUDY AREA

We conducted NGS surveys for wolves from 15 June to 20 August during the summers of 2007 and 2008 in an 11,335-km^2 portion of central Idaho, USA, comprising 6 game management units (GMUs; Fig. 1). Using a wolf occupancy-probability map for habitat selection and interagency reports tracking wolf packs, we chose 2 areas of low wolf density (1–3 wolf packs; GMU 43 and GMU 24) and 2 areas of high wolf density (4–7 wolf packs; GMUs 33, 34, and 35 and GMU 28) for sampling (Oakleaf et al. 2006, Nadeau et al. 2007, Ausband et al. 2010). The 4 study areas were in mountainous regions of primarily United States Forest Service lands (84.8%) and private lands (9.1%), and the main land uses were forest (88.0%), rangeland (8.9%), and irrigated agriculture (6.5%). The climate was characterized by average summer temperatures of 16.7° C and yearly temperatures ranging from about 44.4° C to 43.3° C with 23–82 cm of average annual total precipitation.

METHODS

Survey and Detection

We used resource-selection maps based on vegetation, meadow size, topography, elevation, and slope to identify places within the study areas with the highest probability of being wolf rendezvous sites (Ausband et al. 2010). Targeting probable rendezvous sites reduced sampling effort while increasing our chances of finding wolf scat samples and reproductive packs. We divided predicted rendezvous sites (>70% suitability) into 1,500-m^2 areas for ease of sampling.

Field survey crews traveled to predicted rendezvous sites and surveyed during dawn and dusk when wolves were most active (Harrington and Mech 1982*b*). At each site, technicians howled as described by Harrington and Mech (1982*a*). If pups howled back, or if there was other evidence that the site was occupied, we made an extensive effort to locate the activity center. When we found an occupied rendezvous site in 2007, we surveyed for the freshest scat and hair samples. In 2008, a 6-person crew comprehensively sampled occupied sites for 3–5 hours collecting all hair (including daybeds) and scat samples (marked with a toothpick to avoid double sampling) in the immediate vicinity and on trails leading from the site.

If there was no response to howling, 2 technicians spent ≥30 minutes traveling ≥1.5 km surveying, collecting, and recording wolf sign. We collected scat samples that appeared to be from large canids. If scat was ≥2.5 cm in diameter, we considered it adult wolf scat, and if scat was <2.5 cm, we considered it wolf pup scat (Weaver and Fritts 1979). We did not collect canid scats outside of predicted rendezvous sites in 2007. In 2008, we collected putative canid scats (>2.5 cm in diam) outside of predicted rendezvous sites if we detected them while traveling to or from predicted sites, and we labeled them as incidentals.

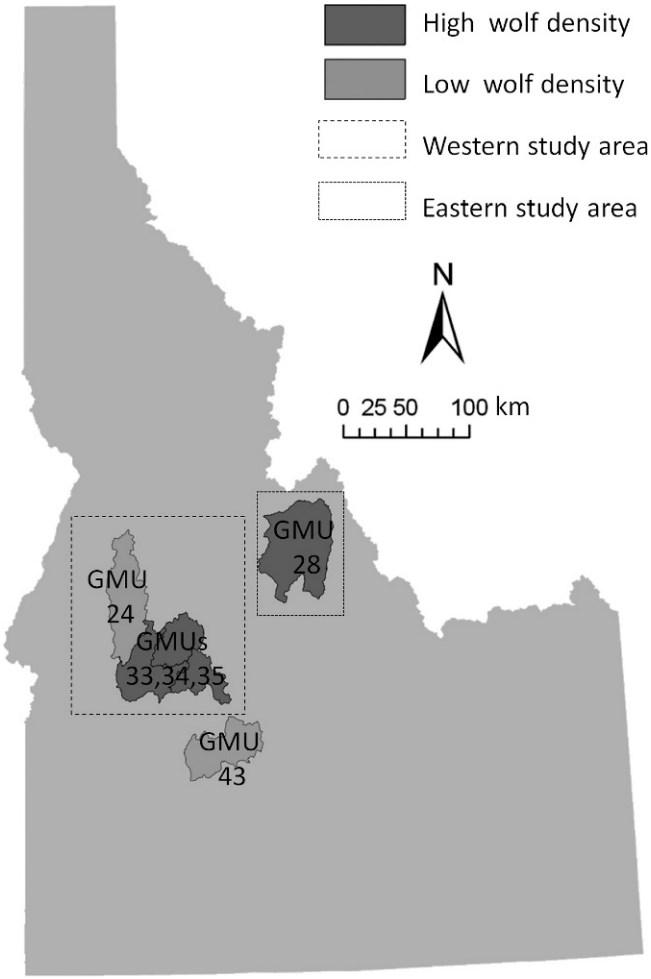

Figure 1. Location of our study area in Idaho, USA, with high and low gray wolf density game management units (GMUs) shown. For population analyses, we identify the western study area and the eastern study area that we sampled in the summers of 2007 and 2008.

When we found wolf scat samples, we collected a small sample of the side of the scat with sterilized forceps and placed it in DMSO/EDTA/Tris/salt solution buffer (Frantzen et al. 1998, Stenglein et al. 2010). In 2007, we did not collect scats appearing old (typically brittle and white), but in 2008, we collected all scats and noted them as fresh or old based on appearance. We collected hair samples from bedding sites and den sites using sterilized forceps; we distinguished samples collected from daybeds (hereafter, daybed hairs) in 2008 from hair samples found in clumps on the ground and clinging to bushes and trees (hereafter, other hairs). To minimize mixed samples, we placed each distinct clump of hair in an individual envelope.

In 2007, 13 radiocollared wolves, representing 8 packs, were in our study area at the time of sampling, and 24 radiocollared wolves, representing 13 packs, were present in 2008 (total radiocollared individuals = 26). We obtained

genetic samples for all these individuals from the United States Fish and Wildlife Service Forensics Laboratory (Ashland, OR). We calculated the proportion of radio-collared wolves (probability of capture [$\hat{P}$]) captured with a scat or hair sample. Because pack membership of collared wolves was known, we calculated the proportion of known packs detected with NGS.

Genetic Analysis

We extracted DNA in a laboratory designed specifically for genetic analysis of noninvasive genetic samples using the QIAamp DNA stool mini kit (Qiagen Inc., Valencia, CA) for scat samples and the DNeasy tissue kit (Qiagen) for hair samples. We included one extraction negative in each group of extractions to check for contamination. We extracted all follicles (≤ 10) and underfur from each hair sample in 2007, but in 2008, we only extracted hair samples with ≥ 3 guard hairs or underfur because preliminary analyses demonstrated poor amplification success (15%) for samples with only 1–2 guard hairs. In 2007, we conducted microsatellite analysis for 8 loci using the primers and conditions outlined in Stenglein et al. (2010). Because of decreased success and increased error rates with locus Pez15, we replaced this locus with Cxx.119 and C09.173 in 2008, and we reran all unique 2007 genotypes for these 2 loci (Holmes et al. 1994, Neff et al. 1999, Breen et al. 2001). In 2008, we conducted a mitochondrial DNA control region species-identification test before microsatellite analysis to cull all coyote (*Canis latrans*) samples and low-quality DNA samples (i.e., samples that failed to amplify; Onorato et al. 2006).

In 2008, we combined 9 microsatellite loci with a polymerase chain reaction (PCR) product size of <250 base pairs (C09.173, Cxx.119, FH2001, FH2054, FH2088, FH2137, FH2611, FH2670, FH3725) into one PCR multiplex (Breen et al. 2001, Guyon et al. 2003). The 7-μL PCR mix consisted of 0.05 μM FH2054, FH2137, and C09.173; 0.1 μM FH2001, FH2088, FH2611, and FH3725; 0.2 μM FH2670; 0.3 μM Cxx.119 primer pairs; 1× concentrated Qiagen Master Mix; 0.5× concentrated Qiagen Q Solution; and 1 μL DNA extract. The PCR profile had an initial denaturation step of 15 minutes at 94° C, followed by a touchdown with 13 cycles of 30 seconds at 94° C, 90 seconds at 62° C with a decrease in annealing temperature by 0.4° C each cycle, and 1 minute at 72° C, followed by 28 cycles of 30 seconds at 94° C, 90 seconds at 57° C, and 1 minute at 72° C. We included a PCR negative in each group of reactions to test for contamination.

We sized alleles using a 3130 × 1 ABI capillary machine (Applied Biosystems Inc., Foster City, CA) and viewed them with GeneMapper 3.7 (Applied Biosystems) software. Initially, we amplified and ran each wolf sample twice using the 9-locus multiplex. For samples with successful amplification at 5–9 loci, we performed 1–3 additional PCRs to finalize the consensus genotype, and we discarded samples with <5 loci working. We did not accept an allele in a heterozygous consensus genotype until we saw it at least twice, and we required ≥ 3 independent PCR replicates to accept a homozygous consensus genotype. After we confirmed 8–9 loci for a sample, we assessed the genotype using GIMLET (version 1.3.3, <pbil.univ-lyon1.fr/software/Gimlet/gimlet.htm>, accessed 12 Jan 2008; Valiere 2002) with all the consensus genotypes to check for matches. We estimated the probability that siblings ($P_{(\mathrm{ID})\mathrm{sibs}}$) would have identical genotypes in GIMLET as 1.6×10^{-4} for 8 loci and 3.8×10^{-4} for 9 loci (Waits et al. 2001). We ran genotypes observed in only one sample through RELIOTYPE (<www.webpages.uidaho.edu/~joyce/Lab%20Page/reliotype.html>, accessed 24 Jan 2008; Miller et al. 2002) to determine whether further repetitions were needed to obtain 95% certainty of accuracy for the genotype. We conducted sex identification for all consensus genotypes using the primers and protocol of Seddon (2005). We initially ran the sex-identification PCR 3 times for each sample, and we needed to see the X chromosome alone 3 times to confirm a female and the Y chromosome 2 times to confirm a male.

To assess DNA quality, we evaluated PCR amplification success rates and error rates that were due to allelic dropout and false alleles for all 2008 samples for the categories of scat and hair. We calculated PCR amplification success as the number of successful PCRs out of the initial 2 PCRs for each sample. We calculated genotyping errors separately for allelic dropout and false alleles and based them on the 2 initial PCRs (Broquet and Petit 2004).

Minimum Count, Density, and Population Estimates

We determined the minimum count of wolves as the number of unique genotypes in each year. To determine whether NGS could be used to detect differences in wolf density better than using sign survey alone, we compared density estimates from 3 scenarios with increasing levels of genetic analysis and assessed which scenario best matched the known high- and low-density GMU designations. The scenarios were 1) sign survey alone, calculated as the number of sites where we collected a scat or hair sample; 2) genetic species identification, calculated as the number of sites where we obtained positive wolf identification; and 3) genetic individual identification, calculated as the minimum number of wolves. We standardized the statistics by GMU area so we could make comparisons among GMUs.

We estimated population sizes for the western portion of the study area (GMUs 24, 33, 34, and 35) and the eastern portion of the study area (GMU 28; Fig. 1) separately because the regions were geographically separate and we did not detect any wolves moving between regions. We left GMU 43 out of the population estimates because it was disjunct from GMUs 24 and 33, 34, and 35, and we detected just 5 wolves there over both years. For each rendezvous site, we coded the data so that we could detect an individual once with scat and once with hair. Therefore, multiple detections only occurred between sites and between data types (i.e., scat and hair; Eggert et al. 2003, Gervasi et al. 2008). Condensing multiple recaptures of the same data type by site is a way to achieve an even capture probability, which is an important assumption for most single-session models (Lukacs and Burnham 2005). Because of our

rigorous genetic error-checking protocol, we assumed all genotypes were correct. All sampling in each study area occurred within 1 month, when reproductive wolf packs were localized to rendezvous sites and individual wolves were not readily dispersing, thus increasing our probability of meeting closure assumptions (Otis et al. 1978, Mech and Boitani 2003).

We used 2 single-session closed population estimators developed for NGS studies. We used the maximum-likelihood CAPWIRE population estimator (<www.webpages.uidaho.edu/~joyce/Lab%20Page/capwire.html>, accessed 22 Sep 2007; Miller et al. 2005) and implemented the likelihood ratio test to choose between the null even-capturability model (ECM) and the model accounting for 2 unequal capture probabilities (the 2 innate rates model [TIRM]) at $P = 0.1$ with the default parameters. The likelihood ratio test for selection between the models has been criticized for failing to reject the ECM when sample size is low and when there is heterogeneity in the data that does not conform to the TIRM (Miller et al. 2005, Puechmaille and Petit 2006). An indication of incorrect ECM selection would be an underestimate of the population size and with a low P value close to the threshold (Miller et al. 2005). Because of the difficulty of rejecting the ECM, we carefully evaluated the results of the likelihood ratio test; if the P value was close to the cutoff criterion and the estimate was downwardly biased compared with the telemetry estimate, we considered the TIRM as the correct model. Additionally, we implemented the single-session, sequential Bayesian method that assumes equal capture probability with a maximum population size of 200 for each study area (Gazey and Staley 1986, Petit and Valiere 2006). We calculated 95% confidence intervals or 95% credibility intervals (95% CrIs) on all estimates.

Because ≥ 1 individual in each pack was collared, we had accurate data for the number of wolves in each pack derived from summer ground searches and observations during flights every 2 weeks. For each study area, we summed the number of wolves in each pack and added 9% to account for lone wolves to obtain a population estimate that served as a comparison for the estimates from NGS techniques (M. Lucid, Idaho Department of Fish and Game, unpublished data).

Developing a Cost-Effective NGS Protocol

To provide sampling recommendations for future NGS studies, we compared results from our extensive sampling in 2008 to those from 2 subsampling scenarios. The first subsampling scenario focused on the type of samples we collected or analyzed and included 1) removing incidental scats, 2) removing daybed hair samples, 3) removing all hair samples, and 4) removing all old scat samples. The second subsampling scenario focused on reducing field effort, and we randomly subsampled 75%, 50%, and 25% of sites, respectively. For each subsampled data set, we recalculated the minimum count; the probability of capturing known, radiocollared wolves ($\hat{P}$; see Survey and Detection section above); and the population size, and then we compared results to the full data set and telemetry estimates, when appropriate. Also, we assessed the average number of observations per individual for the population estimates for each scenario.

We calculated the cost of analyzing the samples for each year and divided it into the cost associated with laboratory supplies (collection supplies and DNA extraction, PCR, and ABI capillary machine supplies and fees) and laboratory labor (US$20/hr). Our supply cost estimate should be interpreted as raw supply cost only and does not include the costs of purchasing or maintaining laboratory equipment. Laboratory labor included hours associated with laboratory work and analysis time of obtaining consensus genotypes. We recalculated total cost, cost per wolf, and cost per genotype for all subsampling scenarios. We did not include the large costs associated with collecting samples in the field in our calculation of the costs of analyzing samples.

RESULTS

Survey and Detection

We visited 476 and 488 predicted sites in 2007 and 2008, respectively (79% of predicted sites across both yr). We collected 248 samples in 2007 from 12% of visited sites and 1,495 samples in 2008 from 27% of visited sites. We collected scats from 12% (2007) and 27% (2008) of visited sites and hair samples from only 1% (2007) and 4% (2008) of visited sites, making scats the most prevalent sample type in both years. In 2008, we collected 58 scat samples (7%) as incidentals (i.e., not within predicted rendezvous sites).

We detected 8 ($\hat{P} = 0.62$) radiocollared wolves in 2007 and 15 ($\hat{P} = 0.63$) radiocollared wolves in 2008 (total radiocollared wolves = 18). We collected scat or hair samples from 75% and 69% of packs in 2007 and 2008, respectively. We always detected radiocollared wolves with NGS when we located their pack's rendezvous sites.

Genetic Analysis

In 2007, we identified 9% of adult scat samples as coyote, and in 2008, 14% of adult scat samples and 2% of pup scat samples were coyote (Table 1). The increase in coyote scat samples in 2008 may have been due to collecting incidental scat samples because 48% of incidental samples were coyote, whereas only 6% of samples for predicted sites were from coyotes. Our 2008 hair and daybed samples were reduced from 637 to 480 after removing samples with just 1–2 guard hairs. Overall PCR amplification success for the species-identification test in 2008 was 86% for scat and 92% for hair samples. We determined 36 sites (8%) in 2007 and 88 sites (18%) in 2008 to be occupied by wolves based solely on the species-identification test.

Polymerase chain reaction amplification success for microsatellite analysis (across all loci) was 81% for scat and 72% for hair samples. Error rates averaged per locus that were due to allelic dropout and false alleles were 13% and 3%, respectively, for scat samples, and 20% and 5%, respectively, for hair samples. Microsatellite analysis resulted in a consensus genotype for 126 samples (51%) in 2007 and 694 samples (52%) in 2008. Pup scat samples had the

Table 1. Total number of gray wolf hair and fecal samples collected and analyzed in 2007 and 2008 from central Idaho, USA. We removed coyote and mixed (>1 individual) samples from further analysis. Genotyped indicates the number of samples that produced a consensus genotype.

Yr	Sample categories	Ad scat	Pup scat	Hair	Daybed hair[a]
2007	Total	110	75	62	
	Coyote	10	0	0	
	Mixed	0	0	5	
	Genotyped	57	42	27	
2008	Total	493	365	328	152
	Coyote	69	8	0	0
	Mixed	1	4	32	32
	Genotyped	263	234	138	59

[a] We did not distinguish daybed hair from other hair samples in 2007.

highest percentage of samples producing a genotype, whereas hair samples had the lowest (Table 1). Mixed samples (i.e., multiple individuals/sample) also occurred at a higher rate with hair samples and were highest for daybed samples in 2008 (21%; Table 1). We removed all mixed samples from further analysis.

Minimum Count, Density, and Population Estimates

We detected 59 wolves in 2007 with individual wolves identified 1–9 times. We detected 45 wolves with scat alone, 5 wolves with hair alone, and 9 wolves with both scat and hair. We detected 98 wolves in 2008, with individuals identified 1–45 times. We detected 55 wolves with scat alone, 1 wolf with hair alone, and 42 wolves with both scat and hair. We detected 35 wolves (59%) identified in 2007 again in 2008. Across both years, we detected 122 wolves (63 M, 59 F).

We collected samples from fewer sites, standardized by area, in 2007 in the low-density wolf areas (Table 2). However, in 2008, we collected samples from the most sites per area in a low-density GMU (Table 2). After species identification of samples to genetically confirm wolf occupancy at a site, number of sites per area with positive genetic wolf identification was highest for one high-density GMU and one low-density GMU in both years (Table 2). We detected between 1 and 27 wolves per GMU study area in 2007 and between 4 and 45 wolves in 2008. There were >2.5 times more wolves per area based on DNA minimum counts in the high-density GMUs compared with the low-density units in both years (Table 2).

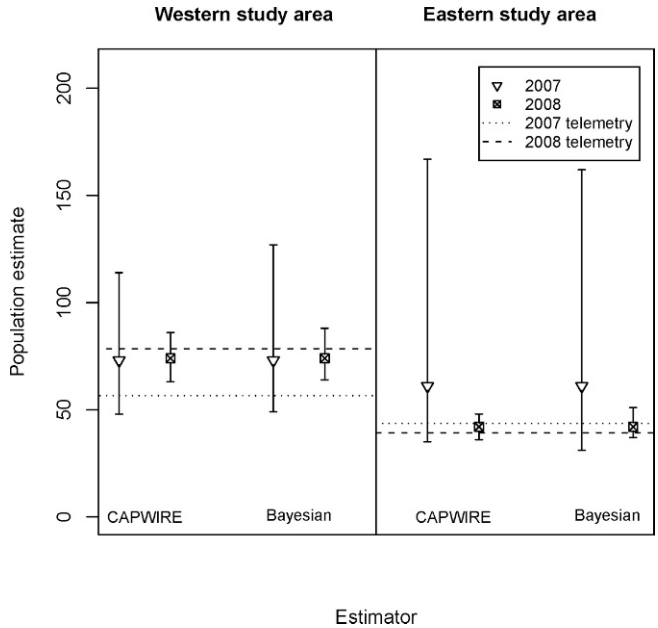

Figure 2. Population estimates of gray wolves in 2 areas (western and eastern study areas) of central Idaho, USA, in 2007 and 2008. The CAPWIRE and Bayesian, single-session estimates and the 95% confidence intervals and 95% credibility intervals are shown. The horizontal, dotted and dashed lines depict the population estimate from radiotelemetry in each year.

We did not detect heterogeneity in capture probability for either study area in either year ($P > 0.47$), and, therefore, we used the ECM population estimate. In 2007, when we sampled less comprehensively, both methods overestimated population size by >25%, and 95% CIs and 95% CrIs were large but overlapped the telemetry estimate (Fig. 2). In 2008, both population estimates were <6 wolves different from the telemetry estimate and the width of the 95% confidence intervals and CrIs were 9–35% smaller than the confidence intervals and CrIs in 2007 (Fig. 2).

Developing a Cost-Effective NGS Protocol

Removing incidental scats from the data set reduced the minimum count by 5 wolves, but $\hat{P}$ of radiocollared wolves inhabiting the study areas was unaffected (Table 3). When

Table 2. Number of sites with samples, number of sites with gray wolf samples (wolf ID), and estimated wolf population density, standardized by area, for selected game management units (GMU) in central Idaho, USA, 2007–2008.

Yr	GMU	Density	Area (km^2)	Rendezvous sites visited	Sites with sample/ 1,000 km^2	Sites with wolf ID/ 1,000 km^2	Wolves/ 1,000 km^2
2007	43	Low	1,813	44	3.3	0.6	0.6
	24	Low	2,274	112	3.5	4.0	4.0
	33, 34, 35	High	3,861	210	7.0	4.7	7.0
	28	High	3,388	110	4.7	3.5	6.5
	Total		11,335	476	5.0[a]	3.2[a]	5.2[a]
2008	43	Low	1,813	47	5.5	3.3	2.2
	24	Low	2,274	120	16.7	8.8	5.7
	33, 34, 35	High	3,861	173	15.3	11.7	11.7
	28	High	3,388	148	7.1	5.0	10.6
	Total		11,335	488	11.6[a]	7.8[a]	8.6[a]

[a] Value is an average.

Table 3. Reduced sampling scenarios from a complete 2008 data set of scat and hair samples collected from predicted gray wolf rendezvous sites in central Idaho, USA. We estimated the effect of subsampling on number of samples collected and number of wolves identified, and we estimated associated costs under different subsampling scenarios.

Type of sampling	Scats	Hairs	$\hat{P}$[a]	Wolves (n)	Laboratory supply cost (US$)	Total lab cost (US$)	Cost/wolf (US$)	Cost/genotype (US$)
2007	185	62	0.62	59	3,469	9,569	162	76
2008	858	480	0.63	98	11,446	21,766	222	31
No incidental scats	800	480	0.63	93	10,950	20,822	224	31
No daybed hairs	858	328	0.63	98	10,145	19,292	197	30
No hair samples	858	0	0.63	97	7,339	13,957	144	28
No old scats	480	480	0.54	74	8,212	15,616	211	36
75% of sites	587	280	0.46	81	7,416	14,103	174	34
50% of sites	539	269	0.46	78	6,911	13,144	169	33
25% of sites	294	130	0.29	53	3,627	6,897	130	33

[a] Probability of capture of known radiocollared wolves.

we removed daybed hair samples and all hair samples, $\hat{P}$ was unaffected; removing all hair samples only reduced the minimum count by one wolf. When we removed old scats, the minimum count dropped by 24 wolves, and $\hat{P}$ was reduced by 9% (Table 3). The minimum count and $\hat{P}$ were reduced when we simulated visitation to 75%, 50%, and 25% of sites, with only 29% of known wolves detected with NGS when we visited 25% of sites (Table 3).

Population estimates were least affected when we removed incidental scat samples and daybed hair samples from the data set in the western study area (Fig. 3). In both areas and for both methods, population estimates were biased high and had 95% confidence intervals and CrIs >2 times the width of the confidence intervals and CrIs for the data set when we removed all hair samples (Fig. 3). Removing old scat samples resulted in larger changes in the western study area, where all methods showed increased bias, and the CAPWIRE method had 95% confidence intervals that did not overlap the telemetry estimate (Fig. 3). Reducing sampling intensity to visiting 75%, 50%, and 25% of sites increased 95% confidence intervals and CrIs >2 times the original width of the 95% confidence intervals and CrIs for both methods (Fig. 3). The Bayesian method failed to overlap the telemetry estimate twice. The methods performed similarly, but CAPWIRE failed to overlap the telemetry estimate in 3 scenarios.

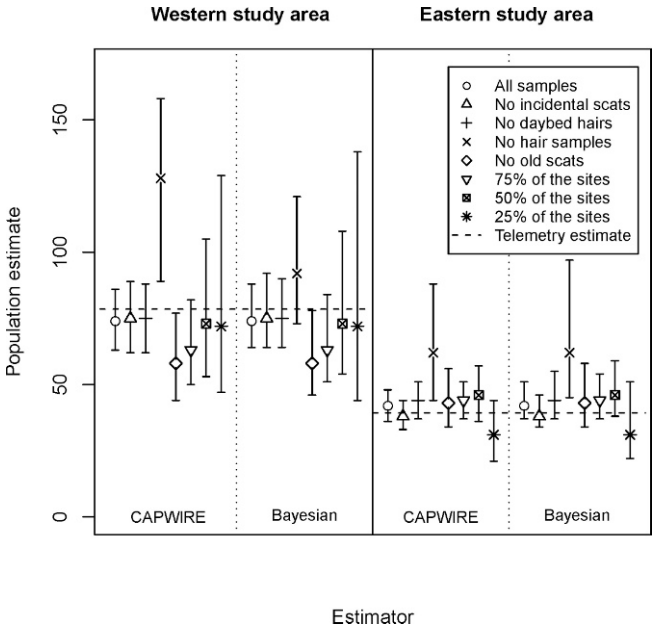

Figure 3. The effect of reduced sampling on the 2008 population estimates of gray wolves in 2 areas (western and eastern study areas) of central Idaho, USA. The CAPWIRE and Bayesian, single-session population estimates and the 95% confidence intervals and 95% credibility intervals are shown for each reduced-sampling scenario. The horizontal, dashed line depicts the 2008 telemetry estimate.

To further evaluate the sampling intensity needed for accurate population estimates, we found the average number of detections per individual to be an accurate predictor of performance of the population estimators. We saw reduced performance when average number of observations per individual was <1.7. In all cases when 95% confidence intervals or 95% CrIs did not overlap the telemetry estimate, the number of observations per individual was <1.7. When number of observations per individual dropped to <1.6, 95% confidence intervals or 95% CrIs were >2 times the average width of the 95% confidence intervals and 95% CrIs from trials where number of observations per individual was >1.7.

Cost of labor and supplies for genetic analysis was US$9,569 in 2007 (US$39/sample collected) and US$21,766 in 2008 (US$16/sample collected; Table 3). Cost per wolf detected was lower in 2007 (US$162/wolf) than in 2008 (US$222/wolf), but cost per genotype was much higher in 2007 (US$76/sample) than in 2008 (US$31/sample; Table 3). Of the subsampling scenarios, we saved 35% of total cost and per-wolf cost by removing all hair samples, and we saved 35–68% of total cost and 22–41% of per-wolf cost by reducing the number of sites visited. Cost per genotype was only reduced when we removed daybed hair samples or all hair samples (Table 3).

DISCUSSION

Developing accurate methods for surveying and monitoring carnivores is an important goal of wildlife management, and noninvasive sampling approaches are playing an increasingly important role (Long et al. 2008). The combination of traditional sign survey methods with DNA analysis of hair and feces provides new opportunities for wildlife biologists. We introduce a new, noninvasive sampling approach for

wolf population monitoring that combines resource-selection modeling, sign-survey approaches, and genetic analysis to detect wolves and estimate population sizes and densities. We demonstrated that our targeted sampling scheme was efficient and generated data that produced accurate population estimates. We detected 122 wolves with NGS, which was >4.5 times the number of radiocollared wolves in our study area. Additionally, we tracked relative wolf density among study areas using the minimum count of wolves. Finally, we illustrated how NGS could be used as a practical and cost-effective tool for monitoring and management of wolves because reduced effort during sampling would decrease cost with little effect on population estimates.

Survey and Detection

The strategy of visiting highly probable rendezvous sites was an efficient sampling method for our large study area where access was challenging. We were more likely to collect wolf samples from predicted rendezvous sites than from areas outside of predicted sites. For example, 48% of the incidental scat samples collected outside of predicted rendezvous sites was coyote scat. When evaluating scat samples collected at unoccupied, predicted rendezvous sites, only 25% were coyote scats, and <1% of scat samples collected from occupied rendezvous sites were coyote in origin. Thus, visiting predicted rendezvous sites increased our sampling efficiency because we were more likely to detect our target species. Targeted NGS at rendezvous sites can be continually improved as additional known rendezvous sites are applied to the predictive model.

Probability of capture was similar between years, highlighting the consistency of our method. Similar rates have been observed using fecal DNA analysis of collared coyotes (*Canis latrans*) in a 15-km^2 California study area ($\hat{P} = 0.67$; Kohn et al. 1999) and fecal DNA surveys of brown bears (*Ursus arctos*) across a 7,328-km^2 study area in Sweden ($\hat{P} = 0.49$–0.64; Solberg et al. 2006). The highest $\hat{P}$ reported for a fecal DNA study was 100% for collared coyotes in a 1,000-km^2 area of Alaska (Prugh et al. 2005). Although $\hat{P}$ was lower in our study than the $\hat{P}$ from the similar canid study, we conducted our study over a much larger area (11,335 km^2).

Minimum Count, Density, and Population Estimates

Analysis of scat samples that appeared old was the most important sampling strategy for increasing the minimum count of wolves. We believe that scats can become quickly desiccated in the dry, summer conditions of central Idaho and are often misidentified as old. Other studies have shown that dry climates are preferred for DNA preservation (Waits and Paetkau 2005, Murphy et al. 2007), and our high amplification success (89%, compared with 77% amplification success for all other scat samples) supports the hypothesis that these scats were relatively fresh and recently desiccated. Furthermore, collecting incidental scats was a low-cost addition to the 2008 sampling, with the potential to identify lone wolves.

Field-based sign-survey approaches have historically been an important monitoring method for wildlife (Heinemeyer et al. 2008). We putatively detected wolves with field sign survey of scat and hair at 20% of sites. However, DNA analysis revealed a lower percentage of sites occupied by wolves based on NGS alone. We did not accurately detect a trend in the proportion of sites putatively occupied by wolves in high-density and low-density areas until genetic analysis resolved the minimum number of wolves in each GMU. Therefore, genetic analysis was important for accurately assessing wolf density in our study areas, and our results suggest that researchers should be cautious about using sign survey without DNA analysis to track trends in wolf density. Furthermore, genetic markers can be used to track changes in the genetic health of the wolf population by monitoring population parameters like genetic diversity and effective population size (Schwartz et al. 2006, vonHoldt et al. 2008).

The accuracy of the population estimation using fecal DNA analysis of wolves has been questioned in the literature (Creel et al. 2003). We believe that our rigorous error-checking during genotyping was sufficient to assume that no errors remained in the data set, and this inference is corroborated by comparisons to field data that demonstrated genetic-based population estimates were not inflated. We designed our study to minimize closure violations by sampling for a short period (<1 month) after pups were born and when wolf packs were localized at rendezvous sites. Although it was not possible to accurately determine the age of scat samples, our data set was unlikely to be adversely affected by inclusion of old scat samples because significant DNA degradation has been observed in wolf scats after 3 days (Santini et al. 2007). Therefore, we did not expect old scats (>1 month) to have produced a finalized genotype.

Multisession population estimators are the most common approach used in wildlife mark–recapture (Otis et al. 1978), but single-session population estimators, developed for noninvasive genetic data, are increasing in use (Miller et al. 2005, Puechmaille and Petit 2006). Single-session population estimators performed well in our study, but required use of 2 data types (i.e., scat and hair) to increase recapture rates. To meet the independence assumptions of the single-session models, we did not allow a wolf to be recaptured using the same DNA source (scat or hair) within a rendezvous site. Thus, we only recorded recaptures when they occurred between rendezvous sites or between sample types (scat and hair) within a rendezvous site. When comparing single-session population estimators, the Bayesian method was the most accurate. However, the CAPWIRE approach provides the important option of modeling with unequal rates of capture probability (Miller et al. 2005). In addition, it is important to obtain an average of ≥ 1.7 detections per individual for accurate performance of all estimators.

Developing a Cost-Effective NGS Protocol

Collecting all scat samples, including incidentals and scats appearing old, as well as hair samples (excluding daybed hair) from all predicted rendezvous sites ensures the highest minimum count and the most precise population estimates.

Hairs collected from daybeds were not an important addition, and they would not need to be collected in the future. To obtain an accurate population estimate with a smaller budget, field crews could randomly select and visit half of the predicted sites and reduce overall field and laboratory costs substantially (40% savings in laboratory cost). Although hair samples were less likely to support detection of a consensus genotype and increased per-wolf and per-genotype costs, hair samples should continue to be collected because they add important recapture information for population estimates.

Laboratory costs per genotype were reduced by >50% from 2007 to 2008. Lower costs are possible when an efficient laboratory procedure is used. We removed poor-quality samples in the first step of analysis with a mitochondrial DNA species-identification test, which has been promoted as an important time and cost-saving step for NGS studies (Lucchini et al. 2002). In addition to time and cost savings, the species-identification test allowed for positive wolf identification for 19–51% of samples that did not produce a consensus genotype, providing valuable data for detection surveys and occupancy modeling.

The potential for hybridization between gray wolves and coyotes is an important consideration for implementation of our species-identification test. Earlier work has not detected any hybridization among gray wolves and coyotes in the Rocky Mountain region (Pilgrim et al. 1998). However, when hybridization was detected in the Great Lakes region, hybridization was hypothesized to occur only among coyote females and gray wolf males, which would lead to introgression of maternally inherited mitochondrial DNA from coyotes to gray wolves (Lehman et al. 1991). Therefore, the mitochondrial DNA species-identification test we used would categorize all hybrids as coyotes and remove these samples from the data set.

MANAGEMENT IMPLICATIONS

We demonstrated that NGS can be used to accurately detect trends in density and to estimate population size for recovering wolves in the NRM. Managers should consider adopting NGS methods for wolf population monitoring because only one sampling effort is necessary for accurate population estimates when scat and hair samples are collected simultaneously. Sampling must occur in the summer when packs are localized at rendezvous sites and scat size can be used to distinguish adults from pups. For long-term management, genetic monitoring could be used in open-population models to track survival and population trends over time. Additionally, estimating population genetic parameters across multiple generations will be useful for monitoring the health of the population (Schwartz et al. 2006). If sampling is thorough, a temporal heterogeneity model could be used with multiple data types, including a session of harvest, to produce accurate population estimates (Gervasi et al. 2008, Williams et al. 2009). Our results demonstrate the utility of NGS for population monitoring of wildlife that exist in low densities and in areas that are less accessible.

ACKNOWLEDGMENTS

We thank The Nez Perce Tribe, Idaho Department of Fish and Game, DeVlieg Small Project Grants, University of Idaho (UI) Student Grant Program, UI Environmental Science Program, The Mountaineers Foundation, Wolf Recovery Foundation, Defenders of Wildlife, Oregon Zoo Future for Wildlife Grants, and the Montana Integrative Learning Experience for Students Program at The University of Montana for funding and assistance. We also thank N. Balkenhol, J. Bohling, C. Goldberg, M. Meredith, S. Nadeau, and 2 anonymous reviewers for their assistance and helpful editorial comments, and M. Anderson, N. Carter, M. Conners, A. Fahnestock, B. Fannin, S. Howard, J. Joyce, R. Kalinowski, S. Longoria, T. Loya, D. Miles, B. Nelson, A. Roadman, L. Robinson, A. Sovie, and R. Wilbur for their dedication and perseverance in the field.

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Associate Editor: Gompper.