Developing and Evaluating Genetic Monitoring Tools for Sonoran Pronghorn (*Antilocapra americana sonoriensis*)

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AUTHORIZATION TO SUBMIT DISSERTATION

This dissertation of Susannah P. Woodruff, submitted for the degree of Doctorate of Philosophy with a Major in Natural Resources and titled "**Developing and Evaluating Genetic Monitoring Tools for Sonoran Pronghorn (*Antilocapra americana sonoriensis*)**," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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ABSTRACT

The Sonoran desert is home to the Sonoran pronghorn (*Antilocapra americana sonoriensis*), an endangered species with populations once numbering in the thousands and now reduced to fewer than 300. Monitoring of the population is limited to biennial counts which provide abundance estimates, but do not provide information on other demographic parameters. Pronghorn are sensitive to stress from physical capture making them good candidates for using noninvasive genetic methods. Noninvasive genetic sampling has commonly been utilized in carnivores, but is less developed in ungulates. We designed and implemented a method combining noninvasive genetic sampling and capture-recapture (NGS-CR) methods to monitor Sonoran pronghorn. One weakness of fecal DNA analysis methods is the difficulty of aging individuals with noninvasive genetic samples. We developed a model using several measures of pellet morphology to reliably classify pellets from fawn versus yearling and fawn versus adult using five-fold cross validation. We used our method of NGS-CR to estimate abundance and apparent annual survival and assessed the accuracy and precision of our estimates using capture-recapture simulations. While the inference of our estimates was limited to the population using watering holes (drinkers), our results indicate this methodology provided reasonable and precise abundance estimates though biased slightly low. Combining this method with radio-telemetry data would further improve the accuracy of the population estimate. As the population continues to expand, this method allows managers to monitor trends in abundance and survival as an indicator of the population's trajectory, as opposed to current aerial survey methods, which provide abundance estimates, but are costly and do not provide information on survival or other demographic parameters.

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DEDICATION

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CHAPTER 1 – RAPID SPECIES IDENTIFICATION OF SONORAN PRONGHORN (*ANTILOCAPRA AMERICANA SONORIENSIS*) FROM FECAL PELLET DNA

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Abstract

The Sonoran pronghorn (*Antilocapra americana sonoriensis*) is a subspecies of pronghorn found exclusively in the Sonoran Desert of Arizona and Mexico. Sonoran pronghorn persist at low densities and are geographically isolated from other pronghorn populations. Numbers have declined in recent decades, but the population has rebounded from a low of fewer than 50 animals in 2003 to an estimated 150 individuals in 2012; however, little is known about population demographics beyond abundance estimates. We developed a species identification test that uses mitochondrial DNA (mtDNA) species-specific primers to distinguish between sympatric Sonoran pronghorn and mule deer (*Odocoileus hemionus*) using DNA extracted from fecal pellets. We accurately identified each species in 100% of the reference samples. We also evaluate the rate of DNA degradation in pronghorn fecal samples ranging from 1 day to 124 days old and document that mtDNA species identification success rates were 100% through day 14. Success rates dropped to 95% by day 21, 45% on day 60 and 10% by day 124. This new test will be a valuable tool for documenting the presence of Sonoran pronghorn across their current range and can also be used for other pronghorn populations.

1.1 Introduction

In 1991 the Sonoran pronghorn population in Arizona was estimated at 250 animals; but by 2003, after several years of below-average rainfall, changing climatic conditions, and a

severe drought in 2002, the population estimate dropped to 21 animals (Bright and Hervert 2003). Following the 2002 drought, a captive breeding pen for Sonoran pronghorn was initiated on the Cabeza Prieta National Wildlife Refuge (CPNWR) to facilitate recovery efforts (Otte 2006). Natural surface water in the region is limited (USFWS 2010, Bagne and Finch 2012); therefore, old livestock water tanks and building catchments were improved and developed into artificial water sources (drinkers) for pronghorn and desert bighorn sheep (*Ovis canadensis nelsoni*). Today, approximately 159 individuals exist in the U.S. population, and 2 separate populations in Sonora, Mexico contain approximately 220 total individuals.

Sympatric ungulate species include mule deer (*Odocoileus hemionus*) and desert bighorn sheep; however, pronghorn and bighorn sheep rarely overlap because of differences in habitat selection preferences (Krausman et al. 1989, Hervert et al. 2005, Wallace and Marsh 2005). Similarities between pronghorn and mule deer pellets make it impossible to distinguish by visual inspection (Johnson and MacCracken 1978). In this paper, we report the design of a mitochondrial (mtDNA) species identification test to distinguish between Sonoran pronghorn and mule deer. Additionally we performed sex identification analysis on samples to test for potential differences in polymerase chain reaction (PCR) success by sex. We applied this method to fecal pellets collected in the captive pen and in the wild at supplemental feed and water sites maintained by USFWS and Arizona Game and Fish Department. Additionally, we evaluate the impact of time (1–124 days), age class (fawn vs. adult), and sex on PCR success rates for DNA extracted from fecal pellets.

1.2 Materials and Methods

The study area included the captive Sonoran pronghorn pen on CPNWR and 2 nearby drinkers. The region was characterized by wide alluvial valleys divided by fault-block

mountains. Elevation varied from 610 m to 1,219 m, but pronghorn were typically found below 900 m. Scrub vegetation communities throughout pronghorn range varied with the topography, elevation, proximity to ephemeral washes, and frequency and amount of precipitation. Vegetation was characterized by Arizona Upland and Lower Colorado River Valley subdivisions (Brown 1982) and consisted primarily of open hardwood–mixed cactus woodlands or mesquite shrublands with sparse vegetation cover (Shreve and Wiggins 1964).

This area was one of the hottest and driest regions of North America, with average summer high temperatures >38° C (INRMP 2003). From June to October, temperatures on CPNWR could be >32° C for >100 consecutive days (USFWS 2002). April to June was the dry season—most precipitation fell in winter and late summer with monsoon rains. Rainfall varied dramatically and declined from east to west; average annual precipitation was approximately 20 cm on the eastern edge and 7.5 cm annually on the western edge of the refuge (USFWS 2002). Climate shifts in the past 25 years have led to warmer, drier conditions (Weiss and Overpeck 2005, Kimball et al. 2010) and winter rains, which once started in October, often now arrive in December (Kimball et al. 2010).

To design and optimize the test, we collected reference samples consisting of 20 tissue samples from hunter-killed mule deer at a meat processing facility in Yuma, Arizona, and 12 pronghorn blood samples, which were obtained during Arizona Game and Fish Department capture operations. All samples were collected in accordance with University of Idaho Institutional Animal Care and Use permit 2013-79. In July 2012, we collected 20 pronghorn fecal pellet samples (10 presumed [by size] adult, 10 presumed fawn) within the CPNWR captive pen; 9 additional fecal pellet samples (all presumed adult) were collected from drinkers in the CPNWR (all ≤24 hr old). There are currently no published studies

documenting size differences in fecal pellets of fawn and adult pronghorn, but these differences have been documented for other ungulates (Ezcurra and Gallina 1981, Bubenik 1982, MacCracken and Van Ballenberge 1987, Sanchez-Rojas et al. 2004).

To evaluate the broader applicability of the test to other subspecies, we also analyzed 10 pronghorn (*A. americana*) tissue samples collected from 0.5-cm ear-tissue punches on the National Bison Range, Montana, USA (Dunn et al. 2010). To evaluate possible cross-amplification with other species in the study area, we tested the primer set on DNA of 5 bighorn sheep samples (blood) from Idaho, USA, and 4 desert bighorn sheep (0.5-cm ear-tissue punches) from Arizona collected during capture operations. However, we note that bighorn sheep have not been documented on remote cameras at the pronghorn drinkers.

Using different latex gloves for each sample to avoid cross-contamination of DNA, we placed 3 pellets from each of the 20 fecal samples in a separate coin envelope, sealed, and labeled the envelope. All samples were stored in silica desiccant to reduce DNA degradation prior to analysis. The remaining samples from each pellet pile were transferred to a 'pallet pellet holder' (Figure 1) constructed to ensure samples were kept separate from each other and were not washed away by rain for the duration of the degradation study. We kept samples for the degradation study near the captive pen in ambient field conditions for 124 days from 4 July 2012 through 5 November 2012. We collected 3 pellets from each sample for DNA extraction at 3 days, 5 days, 7 days, 14 days, 21 days, 60 days, and 124 days to evaluate the rate of DNA degradation over time. Additionally, we tracked rainfall and temperature during the period using local weather stations (http://www.earthonly.com/ajo/weather/). Average high temperatures were approximately 39° C from day 1 to 60 and 33° C from days 60 to 124. Rainfall from day 1 to 124 totaled 16.4 cm with 1.9 cm falling between day 14 and day 21

(Table 1).

We extracted DNA from the fecal samples using the QIAamp DNA Stool Mini Kit (Qiagen, Inc., Valencia, CA) in a laboratory dedicated to low-quantity DNA samples. All 3 pellets from each sample (within a time point) were extracted and considered a single sample. Prior research on pygmy rabbit (*Brachylagus idahoensis*) pellets indicated that extraction of 4 pellets/sample was needed to ensure sufficient collection of DNA (Adams et al. 2011). We used 3 pellets/sample because of the larger size of pronghorn pellets compared with pygmy rabbit pellets. The tissue and blood samples were extracted using a DNeasy Blood and Tissue Kit (Qiagen, Inc.) with an overnight Proteinase K digestion in a laboratory dedicated to high-quantity DNA samples. A negative control was included in each DNA extraction to monitor for contamination.

Cytochrome-b (cyt-b) coding regions are commonly used targets for species identification tests (Parson et al. 2000, Bradley and Baker 2003, Branicki et al. 2003), and we designed our test to use a species-specific primer approach (Dubey et al. 2009, Meganathan et al. 2010, Stewart et al. 2010, Adams et al. 2011, Gunderina 2012). We developed a 3-primer mtDNA cyt-b primer set (ProngID F2, SOPH R2, MuleD R2; Table 2) to distinguish Sonoran pronghorn DNA from mule deer DNA (*Odocoileus hemionus, O. h. hemionus, O. h. californicus*, *O. h. eremicus*) using representative cyt-b sequences obtained from GenBank (accession nos.: GU175434, FJ188817, FJ188847, FJ188823, FJ188857, FJ188725, FJ188745, FJ188836, FJ188824, FJ188858, FJ188820, FJ188882, FJ188775, FJ188749, FJ188881, FJ188795, FJ188773, FJ188784, FJ188793, FJ188794, FJ188782, FJ188800, FJ188785, FJ188786, FJ188796). Using these sequences, we designed a forward primer common to both species and species-specific reverse primers. The reverse primers create

products of a specific base-pair (bp) length for each species (Sonoran pronghorn: 129 bp; mule deer: 176 bp). The 7-μL PCR reaction contained 0.6 μM ProngID F2, 0.3 μM SOPH R2, 0.3 μM MuleD R2, 1× Qiagen Master Mix, 0.5× Qiagen Q solution, and 1 μL DNA extract. The PCR profile includes an initial denaturation step at 95° C for 10 minutes followed by a touchdown of 15 cycles at 94° C for 30 seconds, 63° C for 30 seconds (0.5° C decrease during each cycle), and 72° C for 60 seconds, followed by 35 cycles at 94° C for 30 seconds, 55° C for 30 seconds, and 72° C for 60 seconds before a cool-down at 4° C for 10 minutes. We analyzed PCR products using a QIAxcel automated capillary electrophoresis instrument and visualized using QIAxcel screen gel software (Qiagen, Inc.). This instrument can analyze 5-bp–5-kb (kilobase) DNA fragments and differentiate 3–5-bp differences in fragment size for fragments under 500 bp (Qiagen, Inc.). All samples with a visible PCR product showing the expected fragment length, with concentrations >0.1 ng/μL, were scored as successful amplifications. We re-analyzed samples that failed in the first PCR. Samples with 2 unsuccessful amplifications were considered failed samples.

We determined sex using KY1/KY2 primers (Brinkman and Hundertmark 2009) in a separate PCR reaction. We identified sex only for purposes of evaluating differences in success rates by sex, because we were not testing success rates of nuclear DNA (nDNA) in this manuscript. Analyses of nuclear DNA success rates will be presented in a different manuscript focused on identifying optimal sampling intervals for mark–recapture analyses (S.P. Woodruff unpublished data). Thus, we tested sex identification (ID) only on day-1 samples and success rate was 95% (19 out of 20). Polymerase chain reaction conditions were 0.05 μM KY1 and KY2 primers, 1× Qiagen Master Mix, 0.7× Qiagen Q-Solution, and 1 μL DNA in a 7-μL reaction. The PCR profile was an initial denaturation of 94° C for 15 minutes,

followed by a touchdown of 13 cycles with a 30-second denaturation at 94° C, 90-second annealing step at 65° C decreasing 0.4° C each cycle, and 60-second extension at 72° C. Then, 32 cycles of a 30-second denaturation at 94° C, a 90-second annealing step at 60° C, and a 60-second extension at 72° C. The cycle finished with a 30-minute final extension at 60° C. We included a negative control in all PCRs to test for contamination, and a positive pronghorn control was included in all PCRs to ensure the PCR was working properly.

Statistical Analyses

We examined the effects of sample age, age class (fawn or adult), and sex on mtDNA PCR success rate using a mixed-effects logistic regression model, implemented using SAS/STAT PROC GLIMMIX (SAS Institute Inc. 2011). We specified the fixed effects as sample age (transformed to the natural-log scale), age class, and sex. We specified a random effect for sample to account for sample-specific effects and statistical dependencies among pseudo-replicates within each sample. To improve estimates for sample-specific effects, we specified a single model for both mtDNA and nDNA PCR success because their success rates are likely statistically dependent. We included type of DNA (mtDNA vs. nDNA) and locus length for the nDNA markers as additional fixed effects to allow differences in success rates between the types of DNA and among the nDNA markers. Data from nDNA markers are included to develop an appropriate statistical model for the joint distribution of PCR success for both types of DNA to enhance the analyses of each type of DNA individually. A single model for both types of DNA is potentially more powerful than 2 separate models. However, because the focus here is on PCR success for mtDNA, these effects and the nDNA data will not be further discussed herein (nDNA results are reported in Woodruff et al., in prep). We obtained parameter estimates with maximum likelihood using adaptive Gauss–Hermite

quadrature to numerically approximate the intractable integral in the likelihood function. We computed tests and confidence intervals using Wald test statistics and standard errors.

1.3 Results

No DNA contamination was detected in any of the negative controls. The PCR-based mtDNA species ID test successfully distinguished pronghorn and mule deer DNA. All mule deer and pronghorn blood and tissue reference samples amplified with 100% success, and species was identified correctly in all samples. Known pronghorn fecal samples ($n = 20$) collected from inside the captive pen within 1 day of defecation also amplified species correctly 100% of the time, as did all 10 pronghorn samples from the National Bison Range in Montana. Nine fecal samples (≤24 hr old) of unknown species origin, which were collected in the wild at drinkers, amplified as pronghorn. Reference tissue samples from bighorn sheep were indistinguishable from mule deer.

For the DNA degradation experiment, PCR success rates were 100% through day 14 and dropped to 95% at day 21; however, they declined notably to 50% and 10% by day 60 and day 124, respectively. Overall, after two PCR attempts, 82% (131/160) of the samples amplified successfully (Table 3). Three out of 34 samples that were unsuccessful in the first PCR successfully amplified in a second PCR. Statistical analyses indicated a significant effect of exposure time on PCR success rates (Wald $\chi^2 = 178.14$, $P < 0.001$). A 95% confidence interval for the effect of age estimated that for each unit increase in the age (on the natural-log scale) of a typical sample (i.e., at the modal value of the random effect for sample), the odds of the probability of a successful PCR decreased between 93% and 97% (Figure 2). Neither sex (Wald $\chi^2 = 0.14$, $P = 0.710$) nor age class (Wald $\chi^2 = 2.77$, $P = 0.100$) had a statistically significant effect on success rates.

1.4 Discussion

Indirect methods, such as non-invasive genetic sampling, often provide a more efficient, less expensive way of sampling and monitoring wildlife (Waits and Paetkau 2005, Lukacs et al. 2009). MtDNA species ID tests have been successfully used in documentation of species presence (Riddle et al. 2003, Adams et al. 2011), distribution (McKelvey et al. 1999, Palomares et al. 2002), and range expansion (Valiere et al. 2003, Gajardo et al. 2004). They have also been used to identify species in predation studies (Onorato et al. 2006, Mumma et al. 2013), and diet analysis (Farrell et al. 2000, Symondson 2002, Deagle et al. 2005, King et al. 2008). Efficient and accurate methods documenting presence of a species can be critical in wildlife management, especially in rare or cryptic species (Foran et al. 1997), or in expanding populations.

The mtDNA species identification method described here is simple, technically straightforward, and relatively inexpensive (US$6/sample supply cost). Documenting the presence of Sonoran pronghorn using a species ID fecal DNA test has multiple benefits. First, pronghorn and mule deer pellets are not distinguishable in the field (Johnson and MacCracken 1978), and mule deer are sympatric with pronghorn across their range. Although bighorn sheep and pronghorn rarely, if ever, overlap spatially in our study area, the test provides the ability to correctly distinguish pronghorn pellets from bighorn pellets.

Currently, presence of pronghorn is confirmed through radio collared individuals and/or remote cameras; thus, detection is limited because only a small proportion (currently 12.5%) of the population is collared, and remote cameras are largely deployed only in areas of known or suspected pronghorn use, such as drinkers (Hervert et al. 1997). This non-invasive genetic sampling approach greatly expands the ability of managers to monitor for the presence

of this species and detect occupation of new areas. Our results demonstrate high success rates (≥95%) for fecal DNA species ID up to 21 days after deposition and 50% success rates after 3 months of exposure to summer field conditions.

Compared with other studies using fecal pellets to amplify mtDNA, our success rates are very high. In a summer DNA degradation study of pygmy rabbit pellets, DeMay et al. (2013) reported 94.4% success in 1-day-old samples dropping to 66.7% in 21-day-old samples and only 7.7% after 60 days for mtDNA. In the DeMay et al. (2013) study, pellets were exposed to a high temperature of 37.5° C and average high temperature was 30.7° C. In a winter study of eastern cottontail (*Sylvilagus floridanus*) and snowshoe hare (*Lepus americanus*), mtDNA amplification from fecal pellets was 60% after 42 days, but for New England cottontail (*S. transitionalis*) pellets, success rates were only 10% after day 7 (Kovach et al. 2003). In the New England cottontail study, temperatures were drastically cooler (−1° C to −20° C) and there was appreciable precipitation (rain and snow).

Factors such as age of the sample (i.e., no. of days post-deposition), diet, environmental conditions (ultraviolet radiation, rain), and storage and preservation method affect DNA amplification success (Murphy et al. 2003, 2007; Beja-Pereira et al. 2009; Adams et al. 2011; Panasci et al. 2011), and all must be taken into account when conducting research using fecal DNA (Farrell et al. 2000, Piggott 2004). Previous research has shown that cold and/or dry conditions (Farrell et al. 2000, Lucchini et al. 2002, Maudet et al. 2004, Brinkman et al. 2010, Adams et al. 2011) and the freshest samples (Farrell et al. 2000; Piggott 2004) produce the highest quality DNA, resulting in higher success rates.

Similar to both Murphy et al. (2007) and DeMay et al. (2013), length of time since deposition had the strongest effect on DNA degradation rate of pronghorn fecal pellets, with

older samples having lower amplification success. It should be noted that the effect of age represents an aggregate of sample age as well as the cumulative effects of temperature, rainfall, and other environmental effects that were common to all samples. This was a limitation of our study, and a follow-up study using a design that would follow samples under varying conditions would avoid confounding age with environmental factors. Our results were inconclusive as to whether rainfall affected success rates, but effects of wet conditions on PCR success rates shown in other studies (Farrell et al. 2000, Piggott 2004, Brinkman et al. 2010) highlight the importance of sampling during the dry season. Although we did not find a difference in DNA amplification success rates by sex, effects of individual sex on PCR success are rarely reported, and only one study has documented an impact of sex on PCR success (DeMay et al. 2013). Age class was not statistically significant in our analyses, but presumptive fawn samples had slightly higher success rates than presumptive adults. However, we speculate that we lacked the power to detect the effect of age class because of low sample sizes. We are unsure of the reason for greater success in fawns because we hypothesized that adults would have greater success because of greater surface area of the pellets. The trend shows the opposite potentially due to differences in diet or other unknown effects. No other studies have evaluated the effects of age class in fecal DNA PCR success rates.

Management Implications

MtDNA species identification from fecal pellets provides a rapid, relatively inexpensive method of documenting presence of pronghorn without the potential for false identification, as could be the case in visual identification of pellets or tracks. As a federally listed endangered species, accurate population estimates and distribution are paramount to successful recovery.

The main goal of developing these species-specific primers is to distinguish Sonoran pronghorn and mule deer DNA in fecal pellets or other non-invasively collected samples (i.e., hair or diet analysis of carnivore scat). Additionally, we demonstrate the applicability of this test to pronghorn in other regions. We are currently using this test in Arizona to separate pronghorn and mule deer pellet samples for ongoing research using non-invasive genetic sampling to evaluate the status of Sonoran pronghorn throughout their geographic range. Use of these methods could potentially increase knowledge of Sonoran pronghorn occupancy and spatial use across their range, and confirmation of pronghorn presence in previously unoccupied areas (such as east of Arizona State Highway 85) could further down-listing efforts (USFWS 2010).

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Table 1.1. Temperature, daily rainfall, and total rainfall from Day 1–124 during degradation study for Sonoran pronghorn fecal pellets collected in July 2012 in a captive pen, Cabeza Prieta National Wildlife Refuge, Ajo, Arizona, USA.

Date	Time point	Highest temp[a] (° C)	Avg. temp[b]	Cumulative rainfall (cm)
4 Jul	Day 1	35.1	29.6	0
6 Jul	Day 3	39.5	31.1	0
8 Jul	Day 5	42.7	33.5	0
10 Jul	Day 7	44.1	37.4	0
17 Jul	Day 14	41.7	31.0	1.5
24 Jul	Day 21	40.9	32.5	3.4
4 Sep	Day 60	45.11	32.4	9.6
12 Nov	Day 124	39.72	26.1	16.4

[a] Highest temp. since last time point

[b] Avg. temp. since last time point

Table 1.2. Species-specific primers designed to amplify pronghorn and mule deer for fecal pellets collected in July 2012 in a captive pen, Cabeza Prieta National Wildlife Refuge, Ajo, Arizona, USA. The forward primer is used for both species, while the reverse primers are species-specific.

Species	Primer name	Primer Sequence 5'-3'	Product Length (bp)
Both	ProngID F2	TTCCTCCACGAAACAGGATC	
A.a. sonoriensis	SOPH R2	GTATTATTAGGGCTAAGATTATT	126
O. hemionus	MuleD R2	GTATAATTGTCTGGGTCTCCG	179

Table 1.3. Number of successful and % of successful samples per time point from Day 1 to Day 124 for the first and second polymerase chain reaction (PCR) attempt during mtDNA degradation study for Sonoran pronghorn fecal pellets collected in July 2012 in a captive pen, Cabeza Prieta National Wildlife Refuge, Ajo, Arizona, USA. NA = not applicable.

	1st PCR			2nd PCR		
Time point	***N***	**No successful samples**	**% Successful samples**	***N***	**No. Successful samples**	**% Successful samples**
Day 1	20	20	100%	0	NA	NA
Day 3	20	20	100%	0	NA	NA
Day 5	20	20	100%	0	NA	NA
Day 7	20	20	100%	0	NA	NA
Day 14	20	18	90%	2	2	100%
Day 21	20	18	90%	2	1	50%
Day 60	20	8	40%	12	2	17%
Day 124	20	2	10%	18	0	0%

Figure 1.1. Pellet pallet constructed to ensure 20 pronghorn pellet samples were kept separate from each other and were not washed away by rain for the duration of study, 4 July 2012 through 5 November 2012 Ajo, AZ, USA.

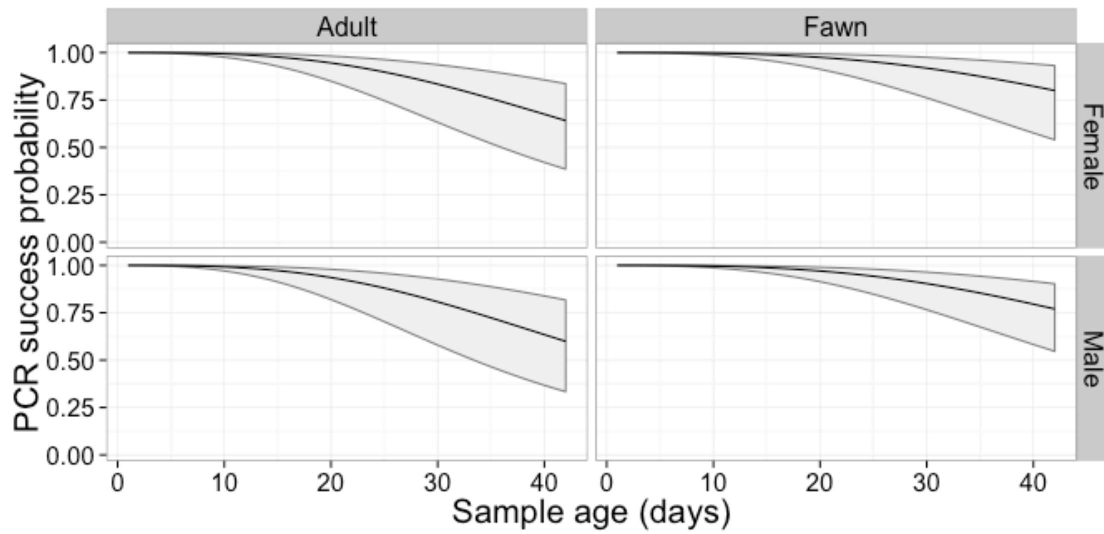

Figure 1.2. Estimated PCR success probability for a modal sample as a function of sample age, age class, and sex for Sonoran pronghorn fecal pellets collected in July 2012 in a captive pen, Cabeza Prieta National Wildlife Refuge, Ajo, Arizona, USA. The bands represent a 95% confidence interval for the predicted probability.

CHAPTER 2-EVALUATING THE INTERACTION OF FECAL PELLET DEPOSITION RATES AND DNA DEGRADATION RATES TO OPTIMIZE SAMPLING DESIGN FOR DNA-BASED MARK-RECAPTURE ANALYSIS OF SONORAN PRONGHORN

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Abstract

Knowledge of population demographics is important for species management but can be challenging in low-density, wide-ranging species. Population monitoring of the endangered Sonoran pronghorn (*Antilocapra americana sonoriensis*) is critical for assessing the success of recovery efforts, and noninvasive DNA sampling (NDS) could be more cost-effective and less intrusive than traditional methods. We evaluated faecal pellet deposition rates and faecal DNA degradation rates to maximize sampling efficiency for DNA-based mark-recapture analyses. Deposition data was collected at five watering holes using sampling intervals of one to seven days and averaged one pellet pile per pronghorn per day. To evaluate nuclear DNA (nDNA) degradation, 20 faecal samples were exposed to local environmental conditions and sampled at eight time points from one to 124 days. Average amplification success rates for six nDNA microsatellite loci were 81% for samples on day one, 63% by day seven, 2% by day 14, and 0% by day 60. We evaluated the efficiency of different sampling intervals (1-10 days) by estimating the number of successful samples, success rate of individual identification, and laboratory costs per successful sample. Cost per successful sample increased and success and efficiency declined as the sampling interval increased. Results indicate NDS of faecal pellets is a feasible method for individual identification, population estimation, and demographic

monitoring of Sonoran pronghorn. We recommend collecting samples less than seven days old and estimate that a sampling interval of four to seven days in summer conditions (i.e., extreme heat and exposure to UV light) will achieve desired sample sizes for mark-recapture analysis while also maximizing efficiency.

2.1 Introduction

Population estimation is important for management, especially with endangered species, but can be challenging in low density, wide-ranging species. Accurate estimates of population abundance rely on maximizing detectability and minimizing individual heterogeneity in detection probability, especially when employing noninvasive genetic sampling (NDS) methods (Borchers *et al.* 2002; Lukacs & Burnham 2005). To be successful, sampling must be conducted at a time and location that maximizes the probability of detection. NDS approaches can provide accurate population estimates, eliminate capture and post-capture related stress on animals, and improve cost efficiency over traditional methods (e.g., Marucco *et al*. 2009; Brøseth *et al*. 2010; De Barba *et al.* 2010; Stenglein *et al*. 2010; Stansbury *et al.* 2014). Faecal DNA approaches are well developed in monitoring carnivores (Prugh *et al.* 2005; Meijer *et al.* 2008; Marucco *et al.* 2009; Stenglein *et al*. 2010); however, these methods are less common in population estimation of ungulates (Harris *et al.* 2010; Brinkman *et al.* 2011; Poole *et al.* 2011; Ebert *et al.* 2012a, 2012b).

Faecal DNA analysis has become a viable method for estimating population size, sex ratio, parentage, relatedness, and genetic structure through individual identification of species that are rare and/or difficult to capture (Mills *et al.* 2000; Creel *et al.* 2003; Waits & Paetkau 2005; Schwartz *et al.* 2007; De Barba *et al.* 2010). Many studies have been successful in conducting individual identification of wildlife by amplifying nuclear DNA (nDNA) obtained

from epithelial cells shed in faeces (e.g., Marucco *et al.* 2009; De Barba *et al.* 2010; Stansbury *et al.* 2014; Wultsch *et al.* 2014). Environmental conditions (Lucchini *et al.* 2002; Piggott 2004; Murphy *et al.* 2007; Santini *et al.* 2007), age of the sample (Piggott 2004; Murphy *et al.* 2007; Santini *et al.* 2007; Beja-Pereira *et al.* 2009), diet (Murphy *et al.* 2003; Panasci *et al.* 2011), and storage and preservation method (Piggott & Taylor 2003; Panasci *et al.* 2011) can all contribute to the rate of DNA degradation. DNA degradation rates influence faecal DNA amplification success rates and microsatellite genotyping error rates (Broquet & Petit 2004; Pompanon *et* al. 2005), which together impact the success rates for individual identification. Genotyping errors can lead to inflated population estimates (Creel *et al.* 2003; Waits & Leberg 2000; Lukacs *et al.* 2009), and biased population estimates can have major effects on the management of rare or endangered species (Taberlet *et al.* 1999).

An important factor in faecal DNA sampling design is deposition rate, which can be affected by moisture content in forage, season, diet, sex, and age of target species (Smith 1964; Neff 1968; Irby 1981; Rogers 1987; Sawyer *et al.* 1990). Research on multiple species of wild ungulates indicates average daily deposition rates range from eight to 52 pellet piles per individual per day (Neff 1968; Rogers 1987; Sawyer *et al.* 1990; Miller & Drake 2004). Scat persistence, or removal rate, also affects the number of samples available. Removal rates are higher in wet than dry conditions due to increased microbial activity (Kie *et al.* 2003; McConkey 2005; van Vliet *et al.* 2008; Norris & Michalski 2010), and rain events that wash away samples (Harestad & Bunnell 1987) or increase degradation rates (Brinkman *et al*. 2010, 2011). Additionally, the number and density of individuals can influence removal rates with samples often being trampled or rendered unusable in areas of high animal activity or density, e.g., artificial feeders or drinkers (Harestad & Bunnell 1987).

DNA degradation rates determine how long after deposition an individual can be detected from a faecal sample with molecular methods (i.e., successful genotyping). Research indicates that cold, dry conditions (Farrell et al 2000; Lucchini *et al.* 2002; Adams *et al.* 2011) and collection of the most recently deposited samples (Farrell *et al.* 2000; Piggott 2004) maintain the highest DNA quality and quantity. As success rates differ between species, study area, and season, it is essential to implement pilot studies to determine DNA degradation rates, genotyping errors, and number of replicates needed for reliable genotypes prior to conducting research (Taberlet *et al.* 1999; Piggott 2004). Additionally, collecting fresh samples can be difficult in rare or wide-ranging species, and thus knowledge of the time post-deposition that DNA remains detectable is critical for appropriate study design.

We use the Sonoran pronghorn (*Antilocapra americana sonoriensis*) as a model system for optimizing sampling design for NDS mark-recapture methods for population monitoring by assessing deposition and DNA degradation rates for faecal pellets. The Sonoran pronghorn is found exclusively in the Sonoran Desert of Arizona and Mexico and was listed under the US Endangered Species Act of 1973 (USFWS 1998). In the mid 1800s, the U.S. population is believed to have been in the thousands (O'Gara & Yoakum 2004), but by 1991, Arizona's population was estimated at 250 animals. However, after several years of below-average rainfall, changing climatic conditions, and a severe drought, the 2003 U.S. population estimate was just 21 individuals (Bright & Hervert 2003). A captive breeding pen for Sonoran pronghorn was initiated on the Cabeza Prieta National Wildlife Refuge (CPNWR) to facilitate recovery efforts (Otte 2006). By 2012, the U.S. population had recovered to an estimated 159 individuals (95% CI: 112-432). The current range of Sonoran

pronghorn in the U.S. covers approximately 11,000 km^2 (USFWS 2010), and most of the U.S. population resides in southwest Arizona.

While there are no federal delisting criteria for Sonoran pronghorn, criteria for downlisting to threatened requires either 1) a stable population of approximately 300 adult Sonoran pronghorn in one U.S. population and a second separate U.S. population, or 2) population numbers that are determined to be adequate to maintain a long-term, viable population (USFWS 1998). Current surveys and monitoring of wild pronghorn consist of locating radio-collared individuals opportunistically from the ground, bi-monthly monitoring flights, and a biennial aerial population count (Hervert *et al*. 1997; USFWS 2010). While this provides population estimates, it is costly and does not provide information on survival, reproduction, or genetic diversity, which are attained with NDS methods.

Often the primary motive behind sampling design in wildlife management is minimizing cost, which does not necessarily correlate with ideal sampling for optimal NDS-CR design. Our methods aim to optimize both cost and recovery of target DNA from faecal samples. Our goal was to determine the optimal sampling design for monitoring Sonoran pronghorn using faecal DNA and mark-recapture techniques by estimating faecal pellet deposition rates and DNA degradation rates. During the hot, dry months (April-June), Sonoran pronghorn congregate at artificial drinkers (Hervert *et al*. 1997; USFWS 2010), presenting the opportunity for targeted sampling, which should reduce sampling effort and increase the number of samples collected. We anticipated collection of multiple pellet piles per individual per day at drinkers due to the length of time spent at the feed stations, and we expected rapid DNA degradation due to exposure to intense ultraviolet light and high temperatures. Therefore, we predicted a one to three week interval would be the optimal gap

between sampling sessions to obtain adequate samples sizes for mark-recapture analysis and maximize efficiency for both field and laboratory (Brinkman *et al.* 2010, DeMay *et al.* 2013).

2.2 Methods

Study Area

The study area includes the captive Sonoran pronghorn pen and two drinkers on the CPNWR, and three drinkers on the Barry M. Goldwater Range (BMGR). State and federal personnel provide supplemental feed for pronghorn at these sites weekly during the dry months (Figure 1). Feeding and watering begins in April or May and ends in October or November depending on the amount of rainfall (J. Atkinson, personal communication).

The study area has average high temperatures of over 38° C in summer and is one of the hottest and driest regions of North America (INRMP 2003). From June to October, temperatures on CPNWR can exceed 32° C for more than 100 consecutive days (USFWS 2002). The dry season extends from April to June as most precipitation falls in winter and late summer. Average annual precipitation on the CPNWR ranges from 20 cm in the east to 7.5 cm in the west (USFWS 2002).

Estimating Deposition Rates

In July 2012, we collected deposition data for pronghorn at two drinkers on CPNWR and three drinkers on BMGR. First, we cleared all faecal pellets at a radius of 25 – 40 m around drinkers and feeding areas. Circle diameters varied between sites as the layout of feed and water sites differed. Faecal pellet piles were counted at an interval of one to seven days. Three sites were sampled once on day 1 (n=1) and day 7 (n=2), while two sites were sampled multiple times on day 1, 2 and 6, and day 3 and 7, respectively (Table 1). Sampling location

and interval were limited by requirements to minimize pronghorn disturbance and travel to drinkers with agency personnel. We counted all discrete pellet piles in the cleared circle.

To estimate the expected number of pellet piles available for sampling each day, we averaged number of pellet piles per pronghorn per day across sites (Table 1). The number of pronghorn using each drinker is estimated by agency personnel from remote camera data and is based on the maximum number seen on camera at one time.

DNA Degradation

To ensure collection of samples less than 24 hours old, the area around feed stations in the captive pen on CPNWR was cleared of pellets on 3 July 2012. On 4 July 2012, we returned and collected 20 pellet piles from ten presumed adult (five males, five females) and 10 presumed fawn (five males, four females, one unknown sex). To minimize the potential for wasted effort in the laboratory, we excluded piles that appeared to be from >1 individual based on pellet shape, color, and size, as these were likely mixed samples. Fawn and adult samples were classified based on visual inspection of size and morphology (thus, “presumed”). While there are currently no published studies documenting size differences in faecal pellets of fawn and adult pronghorn, differences have been documented for other ungulates (Ezcurra & Gallina 1981, Bubenik 1982, MacCracken & Van Ballenberge 1987, Sanchez-Rojas *et al.* 2004). Pellet piles were exposed to local environmental conditions near the captive pen for 124 days from 4 July 2012 through 5 November 2012. Three pellets were collected from each sample at days 1, 3, 5, 7, 14, 21, 60, and 124, placed in paper coin envelopes, and stored at room temperature in a plastic Ziploc bag with one cup of silica desiccant (Fisher catalogue no. S161-212) to reduce DNA degradation prior to analysis. We tracked rainfall and temperature during the sampling period using local weather stations

(http://www.earthonly.com/ajo/weather/) (Table S2.1). Average high temperatures were ~39 ˚C from day 1 to 60 and 33 ˚C from days 60 to 124, and total rainfall from day 1 to 124 was 16.4 cm (Table S2.1).

DNA Extraction and PCR Amplification

DNA was extracted using three pellets from each sample and samples were extracted in a random order across time points to minimize bias introduced during sample processing. Preserved samples were extracted seven to 25 days post-collection to minimize DNA degradation during storage. Previous studies of DNA preservation on silica for ungulate faecal samples showed higher nDNA concentration, fewer genotyping errors, and greater success achieving a consensus genotype compared to other storage methods (Soto-Calderon *et al*. 2009), and no consistent support for an effect of storage time on DNA amplification success on faecal pellets (Soto-Calderon *et al*. 2009, DeMay *et al*. 2013). All extractions were performed using a Qiagen QIAamp DNA stool mini kit (Qiagen Inc., Valencia, CA.) in a laboratory dedicated to processing low quantity DNA samples. Extraction protocols followed those described in Adams *et al.* (2011) with the modification of adding a larger volume (3200 μl) of ASL buffer in step one to ensure pellets were completely immersed in buffer. Each set of extractions contained a negative control to monitor for contamination.

Six nDNA microsatellite loci previously used for pronghorn ranging in size from 113-270 base pairs (bp) were amplified in a single multiplex PCR reaction (Table S2.2). Sex identification was determined by a separate PCR using KY1/KY2 primers (Brinkman & Hundertmark 2009) as described in Woodruff *et al.* (2014). A negative and positive control was included in each PCR. PCR products were analyzed using an ABI 3130xl capillary machine (Applied Biosystems, Foster City, CA) and visualized in Genemapper 3.7 (Applied

Biosystems, Foster City, CA). All samples that failed to amplify in the first PCR were re-amplified (Murphy *et al.* 2007). We performed a minimum of two replicates per sample from each time point, and consensus genotypes were determined using replicates from multiple time points. A PCR replicate for a locus was deemed successful if DNA amplified in the expected size range with an intensity of ≥100 fluorescent units, even if it did not match the consensus genotype. PCR success rates were estimated as number of successful PCRs across all loci per time point divided by total number of PCRs conducted across all loci. In an earlier study, we assessed mitochondrial DNA (mtDNA) amplification success rates for the same samples by amplifying a 129 bp mtDNA locus Woodruff *et al.* in press).

Genotyping Error Rates

Two replicates were performed per time point; however, three to eight repetitions were performed per sample to obtain a consensus genotype for each of the six microsatellite loci using replicates from multiple time points. Genotyping errors were calculated following Broquet & Petit (2004). We classified errors as false alleles (FA) when alleles not found in the consensus genotype were present (Taberlet *et al.* 1999; Broquet *et al.* 2007) and as allelic dropout (ADO) if, in a heterozygous individual, there was failure to amplify one of two alleles (Taberlet *et al.* 1996).

Statistical Analysis

PCR success was modeled using a mixed-effects binary logistic regression model. Fixed effects were specified for sample age in days transformed to $\log_2$ (days), age (adult or fawn), sex, locus length (represented by the midpoint in base pair size range for all observed alleles), and marker type (mtDNA or nDNA). PCR success rates for mtDNA and nDNA were analyzed simultaneously to improve statistical precision of estimating sample-specific random

effects; however, only nDNA are reported here since mtDNA results are reported in Woodruff *et al.* (in press). To account for multiple observations per sample (i.e., markers within samples) a random effect was specified for sample. Maximum likelihood was used to obtain point estimates for model parameters using adaptive Gauss-Hermite quadrature to approximate the integral in the log-likelihood function. Reported tests and confidence intervals were also based on standard asymptotic results for the sampling distributions of the maximum likelihood estimators. SAS/STAT PROC GLIMMIX (SAS Institute Inc. 2011) was used for all calculations.

Evaluating Sampling Interval Efficiency for Mark-Recapture Analyses

A successful sample (successful individual ID genotyping) was defined as a sample that amplified at a minimum of four loci. Probability of identity siblings (P_{ID} sibs) (Waits et al. 2001) was conducted using GenAlEx 6.5 software (Peakall and Smouse 2012) and was 0.023 for these six loci. We acknowledge that 4-6 loci will not be sufficient for distinguishing some first-degree relatives; however, when applying this method in the field, we require consensus genotypes at seven of 10 loci (70%) for individual ID. Here we use the four out of six threshold to indicate the proportion of loci that need to amplify to obtain an individual ID.

To evaluate optimal sampling intervals for mark-recapture analysis, we assumed 15 pronghorn per site (range: 6-25 per site), an estimated deposition rate of one pellet pile per pronghorn per day, and an average sample removal rate of 10% per day. We acknowledge that removal rates vary by drinker; however, we used this average value of removal rates at East Release (Table 1) for modeling purposes. Thus, the number of samples available for collection is a product of the number of pronghorn using the site and the number of days in the interval, minus 10% per day. For example, with 15 pronghorn and a sampling interval of

four days, 15 samples are 1 day old, 14 are 2 days old, 13 are 3 days old, 12 are 4 days old, and so on. We then used our model-based predicted PCR success rates to estimate the number and percent of samples that could be successfully genotyped at each sampling interval from 1 to 10 days. At every sampling interval, each samples was assigned a specific predicted PCR success dependent on sample age. For simplicity, and because there were negligible differences between different sex and age classes, only adult females PCR success rates are shown in the figure.

Additionally, we measured efficiency in terms of cost per successful sample, assuming a cost of $60 per collected sample to cover laboratory supplies and labor. Cost per successful sample was calculated as total cost of analyzing all collected samples divided by number of predicted successful samples. We did not estimate field costs because future sampling will be conducted as part of the regular duties of field personnel when they visit drinkers to provide feed. To estimate an optimal sampling interval for our study system, we set the following criteria: 1) Total number of collected samples for each sampling event should be three times the number of pronghorn estimated to use the site (i.e., ≥45 samples if there are 15 pronghorn present), and 2) individual ID success rates (successful genotyping) should be >80%. The target number of samples was chosen to ensure sufficient recaptures and maximize performance of mark-recapture estimators (Solberg *et al*. 2006); individual ID success rate of 80% was chosen to maximize efficiency and minimize cost per sample.

2.3 Results

Deposition

The average number of faecal pellet piles deposited per day ranged from four to 43, and deposition rates varied depending on temperature and rainfall, number of days between

observations, and number of pronghorn estimated to use the site (Table 1). The average number of pellet piles per pronghorn per day decreased as number of days between observations increased, and the number of pellet piles counted at drinkers decreased after major rain events (Table 1). The average number of pellet piles per pronghorn per day was 1.11 (range: 0.77-1.72), excluding sites with major rain events (Table 1).

Degradation and PCR Success

PCR success rates for nDNA microsatellites were 81% and 80% at days 1 and 3, respectively, and dropped to 69%, 63%, 2%, and 0.01%, at days 5, 7, 14, and 21, respectively (Table 2). None of the microsatellite loci amplified at the 60 or 124 day time points (STable S2.3). Based on the mixed-effects logistic regression model for PCR success, we found a significant effect for age of sample ($Z = -13.35$, $p < 0.0001$), with the odds (odds = probability/(1-probability)) of PCR success decreasing by approximately 88% every time sample age is doubled. Longer loci had significantly lower PCR success probabilities ($Z = -2.34$, $p = 0.0193$), with the odds of success decreasing by a factor of approximately 0.5% per unit (base pair) increase in length. No statistically significant differences were detected in PCR success probabilities between samples from animals of different sex or age; however, the trend in estimates suggests a higher success rate for samples from fawns and females (Table 2 and Figure 2). DNA contamination was not detected in any negative controls.

Genotyping Error Rates

Average ADO rates were high (21%) at day 1, and per locus ADO rates ranged from 4% to 33%. Average rate of ADO increased to 23% at day 3, 53% by day 7, 88% by day 14, and 100% by day 21. Average FA rates were lower at 1% (locus range: 0-5%) in samples less

than 1 day old, 4% (locus range: 0-9%) for day 7, and 100% by day 14 (Tables 3 and S2.4). We did not detect a significant impact of locus length on genotyping error rates.

Sampling Interval Efficiency for Mark-Recapture

For a simulated population of 15 pronghorn, the estimated number of successful samples was 15 on day 1 increasing to 62 successful samples by day 10 (Figure 3). Predicted individual ID success was 100% through day 3, dropping to 94% by day 5, and 75% by day 10 (Figure 3). Target sample size (≥3x number of individuals using the drinker) was reached on day 4, and a sampling interval of ≤7 days assured the proportion of successfully genotyped samples was >80%. Cost per successful sample increased from $60 per successful sample on day 1 (100% efficiency) to $61 per sample by day 4 (98% efficiency) to >$71/sample by day 7 (84% efficiency) (Table 4).

2.4 Discussion

Appropriate sampling methods are of primary importance, and knowledge of the species' biology and behavior helps dictate sampling design (Lukacs & Burnham 2005; Puechmaille & Petit 2007). NDS approaches typically use standardized transect sampling (Kohn *et al.* 1999; Prugh *et al*. 2005; Kendall *et al.* 2009; DeBarba *et al.* 2010) or targeted sampling methods (Prigioni *et al*. 2006; Puechmaille & Petit 2007; Rudnick *et al*. 2008; Robinson *et al.* 2009; Stenglein *et al*. 2010). Abundance estimates using standardized transect sampling methods are the most commonly used approach and perform well in randomly distributed populations (Buckland *et al.* 2001; Williams *et al.* 2002); however, less focus has been placed on targeted sampling designs. Pronghorn provide a good model system for evaluating a targeted sampling design because they congregate at drinkers during drought periods. In this study, our goal was to develop an efficient temporal sampling design for

mark-recapture that accounted for sample deposition rates and DNA degradation rates while also minimizing disturbance to pronghorn. Much research has been done on faecal DNA degradation rates; however, this is one of the first studies (but see Lonsinger *et al*. 2014) to consider the combination of faecal deposition, DNA degradation rates, and cost in mark-recapture sampling design, and we expect this approach to be valuable for optimizing mark-recapture sampling design in other systems.

Factors Influencing Deposition at Drinkers

Estimates of faecal deposition rates are a function of the number of deposition events that occur in the focal area and the sample removal rate. Our study evaluated deposition at drinkers and feeding sites, which are likely to have high and concentrated levels of deposition (Irby 1981). Factors that likely influenced removal in our study system were insect activity, rain, mixing of pellet piles, and stomping of pellet piles by pronghorn or humans. Estimates of deposition rates varied depending on the number of days between observations, maximum number of animals observed on camera at each site, removal rate, and local weather conditions.

Our data indicated an average deposition rate of one pellet pile per pronghorn per day and a removal rate of approximately 10% per day. Average number of pellet piles per pronghorn per day decreased as number of days between observations increased, presumably due to increased removal rates. Deposition data from two sites (Point of Pintas and Devil Hills) showed considerably fewer pellet piles than expected (Table 1), during a time when significant rain (29.46 mm) fell at these sites. At Point of Pintas, with an interval of seven days and seven pronghorn present, we expected 44 pellet piles and counted 31, and at Devil Hills, with an interval of seven days and 11 pronghorn using the site, we expected 69 pellet

piles and counted only 30. We postulate this decrease in pellet piles could be attributed to removal by rain (washing the pellets away) and less time spent at the drinkers as a result of increased availability of native forage and water due to rain. Additionally, deposition rates at Charlie Bell were lower than expected. Remote camera data indicates high levels of human activity at drinkers decreases pronghorn activity in the area (USFWS 2010) and could result in lower deposition at the drinker. Charlie Bell experiences high levels of human activity compared to other drinkers.

Factors Influencing DNA Degradation

Similar to other studies, we found the age of pellets dramatically affected PCR success rates (Piggott 2004; Murphy *et al.* 2007; Brinkman *et al*. 2010; DeMay *et al.* 2013) likely due to rapid DNA degradation caused by exposure to intense ultraviolet light and high temperatures. As expected, nDNA success rates were lower than mtDNA success rates reported previously for the same samples which were 100% up to day 14, dropping to 95% by day 21, 45% on day 60, and 10% by day 124 (Woodruff *et al.* 2014). There are few studies of nDNA degradation rates for pellet-type faecal samples with comparable environmental conditions; however, our success rates are slightly lower at day 1 than other pellet studies with similar amplicon sizes (Maudet *et al*. 2004; DeMay *et al*. 2013) and lower than one pellet study with longer amplicon lengths (Brinkman *et al.* 2010). From day 5 to 7, our success rates were higher (64% vs. 43%) than those of pygmy rabbit (*Brachylagus idahoensis*) pellets under similar dry, hot conditions, yet our 21 day old samples failed nearly completely, while pygmy rabbit samples maintained 29% success (DeMay *et al*. 2013). Studies in southeast Alaska of Sitka black-tailed deer (*Odocoileus hemionus sitkensis*) pellets protected from rainfall had >90% PCR success rates for 1 to 28 days, while those exposed to

rain showed a significant decrease over time to approximately 70% by day 28 (Brinkman *et al.* 2010). The higher PCR success rate for the deer suggests that other factors, such as diet, environment, and/or lab protocols, have greater influence on amplification success than locus length.

Our samples showed the greatest decline from day 7 to 14 with PCR success falling to 2% by day 14 and indicating analyses using nDNA, such as individual identification and parentage, should be conducted only on samples ≤7 days old. The first rain of the sampling period fell during the 7 to 14 day interval (total 1.5 cm) potentially indicating an effect of precipitation on degradation rates. However, in this study, the effect of age represents a combination of sample age, as well as the collective effects of rainfall, temperature, and other environmental effects that were common to all samples. We acknowledge this was a limitation of our study and additional research using a design that evaluates samples exposed to varying environmental conditions over time would avoid confounding age with environmental factors. While our results were inconclusive as to whether rainfall impacted success rates, impacts of wet conditions on PCR success rates shown in other studies (Farrell *et al*. 2000; Piggott 2004; Murphy *et al*. 2007; Brinkman *et al*. 2010, 2011) highlights the importance of sampling during the dry season. Similar to other studies (Broquet *et al*. 2007; DeMay *et al*. 2013) longer locus length significantly decreased PCR success rates.

While age class was not statistically significant in our analyses, we found slightly higher success rates in fawn and adult female samples than adult male samples. However, due to our low sample sizes, we speculate that we lacked the power to detect the effect of age class. We had hypothesized that adults would have greater success because of greater surface area of the pellets. The trend of higher success in females and fawns is potentially due to

differences in diet, hormones, and metabolic rates. No other studies have evaluated the effects of age class in faecal DNA PCR success rates, and this may be a useful direction for future analyses.

Time since deposition also affected genotyping error rates, and there was an increase in average ADO and FA rates over time. ADO rates were high compared to other studies (Brinkman *et al.* 2010; DeMay *et al.* 2013). While average ADO rates changed only moderately from day 1 to day 3, some per locus error rates changed considerably, with ADO rates of one locus (ADCYC) increasing from 4% to 50%.

Maximizing Sampling Design for Mark-Recapture Analyses

Our primary goal was to determine an optimal sampling interval between mark-recapture sessions. Specifically, we wanted a long enough interval to ensure samples could be collected at a rate of three times the number of individuals estimated to use the site and short enough to ensure successful genotyping of individuals of at least 80%, while balancing pronghorn disturbance and financial cost. During the hot dry months when pronghorn are stressed due to heat and lack of forage and free-standing water, minimizing pronghorn disturbance is a critical concern. To minimize pronghorn disturbance and collect more samples in each sampling session a longer time interval between sessions is desirable. However, with longer sampling intervals, number of samples removed increases, and PCR success rates decrease, thus increasing cost. For our study system, we estimate we can obtain the desired number of samples with a sampling interval of ≥4 days and maintain individual identification success rates above 80% from days 1 to 7 (Figure 3).

Another metric of efficiency is cost per successful sample. Cost per successful sample using predicted number of successful samples (Table 4) ranged from \$60 (1 day interval) to

$79.49 (10 day interval), an increase of 32%. Our data indicates the optimal sampling interval would be 4 to 7 days to achieve the target sample size and individual ID success rates while minimizing cost per successful sample and disturbance to pronghorn.

Implications for Monitoring

The small population size and large range of Sonoran pronghorn present major challenges for faecal DNA sampling. Our proposed method of sampling at drinkers should allow collection of a large number of samples from most of the individuals visiting the drinkers and provide a more accurate population estimate than is feasible with methods such as remote cameras. One potential weakness of our targeted sampling approach is that some individuals may not use drinkers, and AZGFD personnel posit this is around 30% of the total population (J. Hervert, personal communication).

Our research was conducted in summer, which is likely the most ideal season for estimating abundance for Sonoran pronghorn using NDS methods. During cooler, wetter times of year pronghorn are spread out over a large spatial area (11,000 km^2), and thus, sampling should be completed prior to the onset of monsoon season in July. Our results indicate rapid nDNA degradation occurs in the intense ultraviolet light and high temperatures experienced in summer conditions, thus, collection of fresh samples will maximize success. However, mtDNA species identification could be for useful for presence/absence information and occupancy analyses, and mtDNA success rates remain over 40% for up to three months (Woodruff *et al.* 2014).

We initially predicted an intermediate interval of approximately 1 to 3 weeks would be the optimal gap between sampling sessions to maximize efficiency in the field and laboratory. Based on individual ID success, a sampling interval of 1 to 7 days would be sufficient to

optimize amplification success rates; however, an interval of 1 to 3 days would likely give too small a sample size, and local managers attempt to limit disturbance of pronghorn at drinkers to once per week. Sampling every 4 to 5 days is the ideal balance between efficiency of cost, DNA degradation, and deposition. However, in order to minimize disturbance, we propose using a 7 day sampling interval and synchronizing weekly agency personnel visits for stocking feed and water with faecal DNA sample collection. We expect that sampling at drinkers during the driest times of year, which coincides with supplemental feeding and increased drinker use, will present the best opportunity for optimum sampling efficiency.

Conclusions

While no sampling design will fit every study, our results highlight the need to consider multiple factors in sampling design including faecal deposition rates, removal rates, DNA degradation rates, species' biology and behavior, and the possible disturbance caused by sampling. As noted by other authors (Taberlet *et al*. 1999; Piggott 2004), conducting a pilot study prior to initiation of sampling is critical to informing researchers of site- and species-specific differences and will facilitate optimal sample collection approaches and maximize sample collection and DNA amplification success rates. While faecal DNA mark-recapture methods have rarely been used for population estimation of ungulates, we believe that with effective sampling design and implementation, it is a promising approach. Based on faecal degradation rates, an NDS study of this species, or others that are uncommon and broadly distributed, might not be feasible. Therefore, the opportunity to collect fresh faecal samples where the target species is congregated (naturally or due to human intervention) allows an NDS study to be conducted. These methods could be applicable to other ungulate species and study systems where animals congregate at centralized locations, such as in African savanna

ecosystems, where, for example, zebra (*Equus quagga*), sable antelope (*Hippotragus niger*), waterbuck (*Kobus ellipsiprymnus),* and other species gather at natural and artificial watering sites in the dry season (Cain *et al*. 2012) presenting an opportunity for targeted sampling of a typically broadly distributed species.

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Table 2.1. Deposition rates for Sonoran Pronghorn faecal pellets collected in Cabeza Prieta National Wildlife Refuge and Barry M. Goldwater Range, Arizona, USA during July 2012.

Drinker	Sampling Interval (days)	# Pellet Piles	Average Pellet Piles/Day	Est. # pronghorn present	Average # piles/pronghorn/ day
East Release	1	9	9	7	1.29
Charlie Bell	1	7	7	9	0.78
East Release	2	19	10	8	1.19
TACNA 1B	3	129	43	25	1.72
East Release	6	37	6	8	0.77
TACNA 1B	7	135	19	25	0.77
Point of Pintas	7	31	4	7	0.63*
Devil Hills	7	30	4	11	0.39*

*outliers are due to rain events

Table 2.2. A comparison of male/female and adult/fawn PCR success rate (%) for nDNA microsatellite amplification in Sonoran Pronghorn faecal pellets of varying ages (1-21 days exposure).

Day	All Samples PCR success (N=180)	Male* PCR success (N=42)	Female* PCR success (N=60)	Fawn PCR success (N=54)	Adult PCR success (N= 68)
1	81	98	95	98	96
3	80	90	92	100	79
5	69	67	76	77	65
7	63	62	69	73	57
14	2	8	2	8	3.7
21	0.01	8	2	8	3.7

*includes both adults and fawns

Table 2.3. Observed rates of allelic dropout (ADO) and false alleles (FA) in nDNA extracted from Sonoran pronghorn pellets 1–21 days post deposition.

	Sample Age (days)					
	1	**3**	**5**	**7**	**14**	**21**
ADO (%)	21	23	35	53	88	100
FA (%)	1	3	13	4	33	100

* PCR success was zero for samples aged 60 and 124 days so error rates were not calculated.

Table 2.4. Cost per successful sample for sampling intervals of 1–10 days estimated assuming 15 pronghorn using a drinker, a deposition rate of 1 sample/pronghorn/day, and a removal rate of 10% /day.

Day	# Samples*	% Successful samples	# Successful samples*	Cost ($) per successful sample	% Increase in cost
1	15	100	15	60.00	0
2	29	100	29	60.01	0
3	41	100	41	60.19	1.0
4	51	98	50	61.18	3.1
5	60	94	57	63.65	8.7
6	68	89	65	67.43	16.3
7	74	84	68	71.64	23.5
8	78	80	71	75.38	29.9
9	81	77	73	79.09	35.1
10	83	75	62	79.49	37.0

*Rounded values

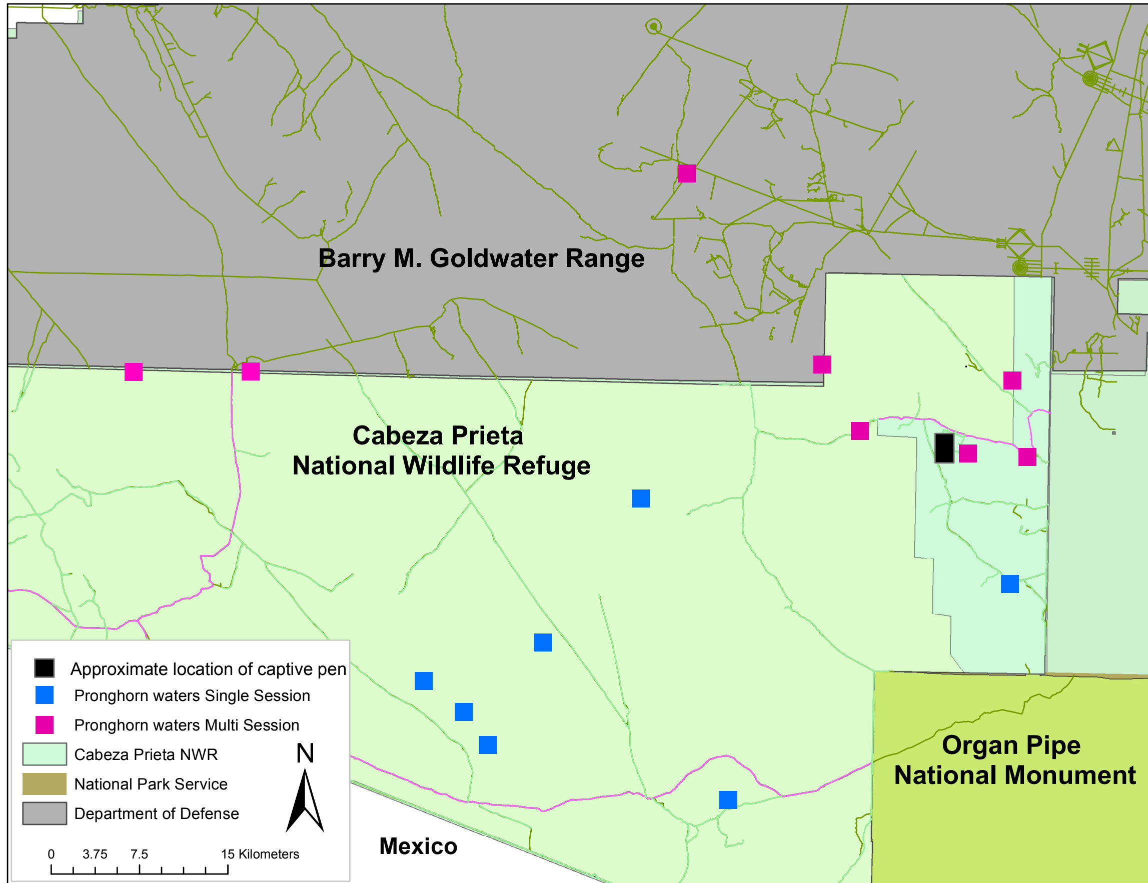

Figure 2.1. Map of the study area showing Cabeza Prieta National Wildlife Refuge and Barry M. Goldwater Range, Arizona, USA. Sampling locations are indicated by pink and blue boxes.

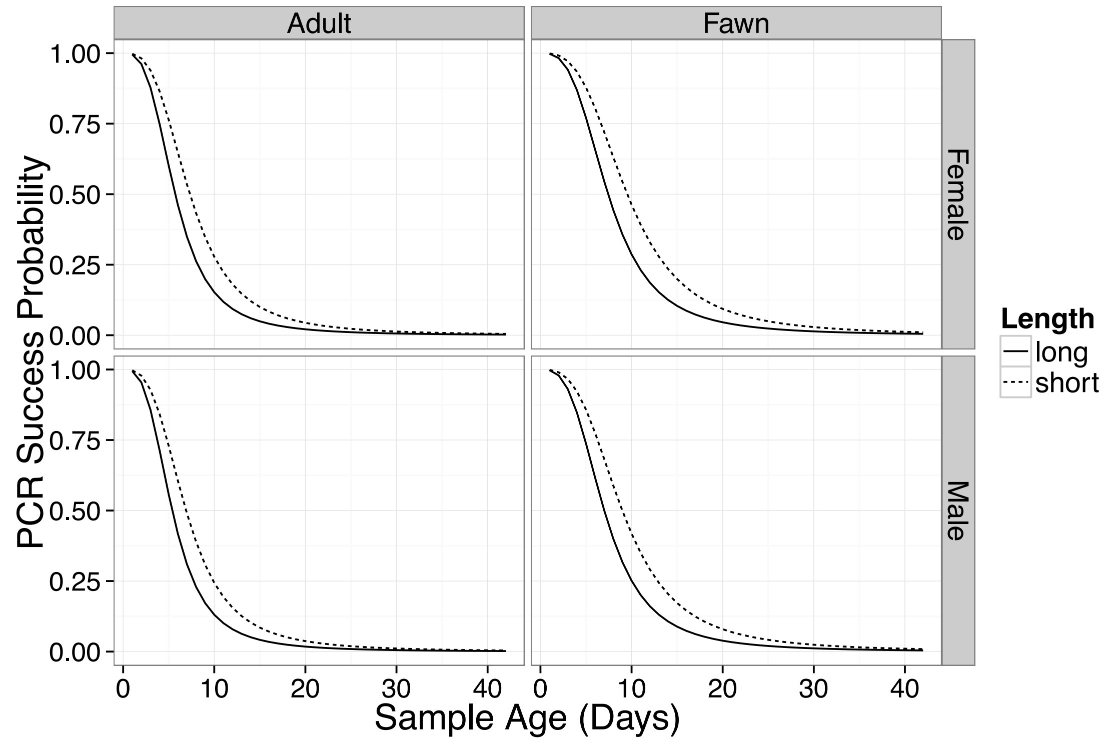

Figure 2.2. Model-based median predicted PCR success probability of Sonoran pronghorn faecal pellets by sample age, individual pronghorn age and sex, and locus length conditional on the random effect for age at its mean value. For simplicity only curves for the shortest (PRM6506) and longest (ADCYC) loci lengths are shown.

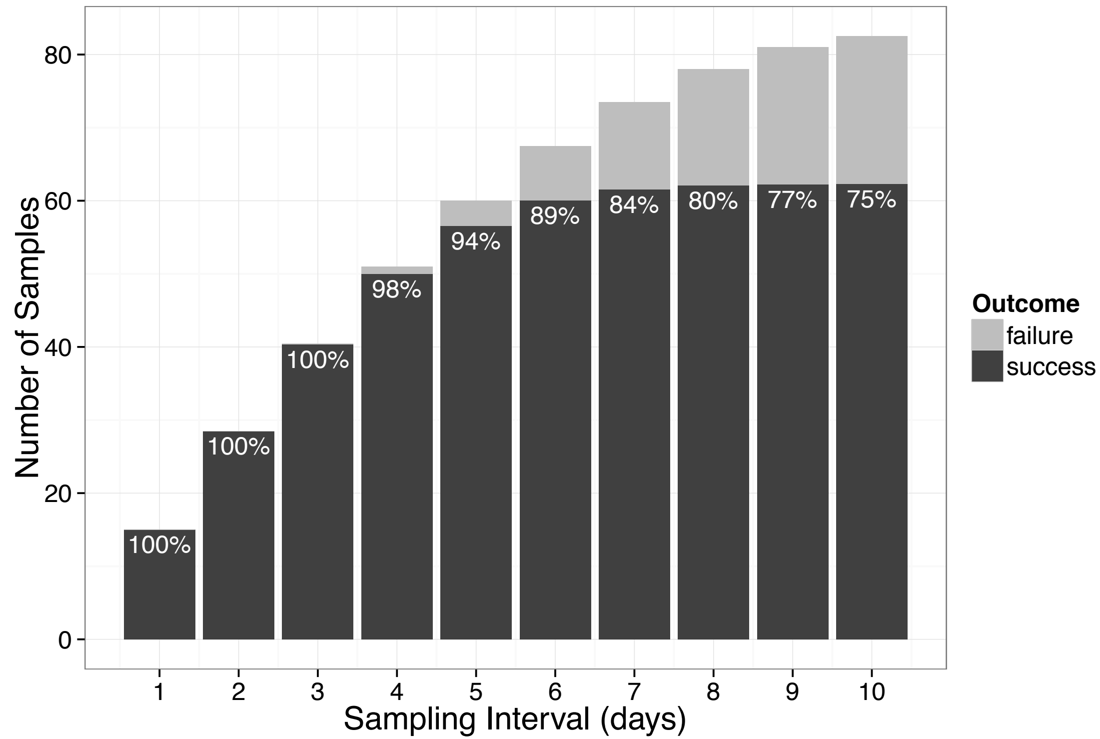

Figure 2.3. Expected number of failed and successful Sonoran pronghorn faecal pellets samples by sampling interval (days). The percentages are the relative efficiency (i.e., percent of expected successful samples).

2.6 Supplemental data

Table S2.1. Temperature and total rainfall from Day 1 to Day 124 during a degradation study for Sonoran pronghorn faecal pellets collected July to November 2012 in a captive pen, Cabeza Prieta National Wildlife Refuge, Ajo, Arizona, USA.

Date	Time point	Highest Temp* (˚C)	Avg Temp**	Cumulative Rainfall (cm)
4 July	Day 1	35.1	29.6	0
6 July	Day 3	39.5	31.1	0
8 July	Day 5	42.7	33.5	0
10 July	Day 7	44.1	37.4	0
17 July	Day 14	41.7	31.0	1.5
24 July	Day 21	40.9	32.5	3.4
4 Sept.	Day 60	45.11	32.4	9.6
12 Nov.	Day 124	39.72	26.1	16.4

*Highest temperature since last time point

**Avg. temperature since last time point

PCR Conditions and Primers

Six nuclear DNA microsatellite loci ranging in size from 113–270 base pairs (bp) were amplified in a single multiplex reaction (Supplemental Table S2) (Lou 1998; Carling *et al.* 2003; Stephen *et al.* 2005). The 7 µl PCR reaction contained 1x Qiagen Master Mix, 0.5x Qiagen Q-Solution, 0.22µM ADCYC, 0.04µM PRM6506, 0.025 µM Aam1, 0.025 µM Aam2, 0.05 µM Aam4, 0.05 µM Aam6, and 1.5 µl DNA extract. The PCR profile includes an initial denaturation of 95°C for 15 minutes, followed by a touchdown of 13 cycles with a 30 second denaturation at 94°C, 90 second annealing step at 61°C decreasing 0.3°C each cycle, and a 60 second extension at 72°C, followed by forty-two cycles of a 30 second denaturation at 94°C, a 90 second annealing step at 57°C, and a 60 second extension at 72°C. The cycle finished with a 30 minute final extension at 60°C.

Table S2.2. Primer set and allele size ranges for microsatellite loci

Locus	Primer set (5'-3')	Size range (bp)	Source
ADCYC	AAAGTGACACAACAGCTTCTCCAG AACGAGTGTCCTAGTTTGGCTGT	258-282	Lou 1998
PRM6506	TAGCAACTTGAGCATGGCAC GAAGCTTCAGCCTAGCCAGT	114-140	Stephen *et al.* 2005
Aam1	CCCGCGCCACAGCCAGTTCTT GGGCCAGGGGAGGGACTCTCAA	218-222	Carling *et al.* 2003
Aam2	CCTGCCCCCTTGCATGATTAT TAGCCACCTAACTCCCCTTCCATTG	141-159	Carling *et al.* 2003
Aam4	GAGCCTGGCAGGTTACAGTCTA TCCCCTCCCAATAAAAAAAGAT	224-262	Carling *et al.* 2003
Aam6	AGGCATTTAGTTATT TGTGTC CCCGCATCTCCTACAG	124-130	Carling *et al.* 2003

Table S2.3. Per locus PCR success rates by locus length in nDNA extracted from Sonoran pronghorn pellets ranging in age from 1 to 21 days post deposition. PCR success was zero for samples aged 60 and 124 days so error rates were not calculated.

Day	1	3	5	7	14	21
Locus (bp)						
ADCYC (240-280bp)	77%	86.4%	47.8%	52.2%	0%	0.03%
PRM6506 (104-150)	69.7%	75.0%	71.4%	72.7%	3.0%	0.03%
Aam1 (200-240)	87.1%	75.0%	77.3%	65.2%	0%	0%
Aam2 (120-175)	93.8%	87.5%	86.4%	63.6	0%	0%
Aam4 (210-280)	77.4%	75.0%	63.4%	52.2%	6.1%	0%
Aam6 (110-150)	84.4%	79.2%	66.7%	72.7%	3.0%	0%

Table S2.4. Per locus rates of allelic dropout (ADO) and false alleles (FA) in nDNA extracted from Sonoran pronghorn pellets ranging in age from 1 to 21 days post deposition. PCR success was zero for samples aged 60 and 124 days so error rates were not calculated.

Day	1		3		5		7		14		21	
Locus	**ADO**	**FA**	**ADO**	**FA**	**ADO**	**FA**	**ADO**	**FA**	**ADO**	**FA**	**ADO**	**FA**
ADCYC	0.04	0	0.5	0	0.75	0.2	0.43	0.09	NA	NA	1	1
PRM6506	0.14	0	0.42	0.59	0.5	0.06	0.42	0	1	0	1	0
Aam1	0.28	0.08	0.5	0	0.5	0	0.54	0.07	NA	NA	NA	NA
Aam2	0.32	0.03	0.23	0	0.36	0	0.33	0.07	1	0	1	1
Aam4	0.29	0.05	0.13	0	0.38	0.25	0.29	0	0.5	1	NA	NA
Aam6	0.33	0	0.33	0.05	0.63	0	0.73	0	1	NA	NA	NA

NA indicates failure of all samples.

CHAPTER 3-EXAMINING THE USE OF FAECAL PELLET MORPHOMETRY TO DIFFERENTIATE AGE CLASSES IN SONORAN PRONGHORN

In Review: *Wildlife Biology* Authors – Susannah P. Woodruff, Timothy R. Johnson, and Lisette P. Waits

Abstract

Managers require knowledge of population demographics, yet for low-density, wide-ranging species procuring demographic information is challenging. While accurate abundance estimates can be costly and difficult to obtain, recruitment and survival trends can be used as an alternative indicator of a population's trajectory. Physical capture has been the traditional practice for obtaining demographic parameters, yet capture-related stress can lead to reduced levels of fitness, impaired locomotion, or even mortality for some species. Use of noninvasive sampling methods provides an alternative to physical capture, but these methods often lack the ability to age the individual. Population monitoring of endangered Sonoran pronghorn (*Antilocapra americana sonoriensis*) is critical for assessing the success of recovery efforts, and monitoring survival and recruitment by age class would provide information on the trajectory of population growth. We measured Sonoran pronghorn faecal pellets collected post-fawning and matched to known age animals using fecal DNA genotyping to determine the feasibility of distinguishing age class by pellet dimensions. Based on cross-validation with logistic regression predictive models, we estimated a 98% probability of correct classification of fawn versus yearling and fawn versus adult using pellet width as a single explanatory variable. We could not, however, distinguish between yearling and adult. We additionally evaluated our ability to classify age class of faecal pellets by visual assessment only, and this approach was unreliable. Thus, we recommend measuring pellets for more accurate age

classification. This measurement method is simple, inexpensive, and shows potential for use in wild populations of pronghorn to discriminate fawns from other age classes, and when combined with individual identification using faecal DNA, could provide better knowledge of recruitment and age-specific survival.

3.1 Introduction

A comprehensive understanding of population demographic metrics, such as abundance, survival, and recruitment, is important for species management, yet these parameter estimates are often expensive and difficult to obtain. Alternatively, managers can monitor trends in recruitment and survival as an indicator of a population's trajectory, which, in some cases, may be more easily attained, particularly for low-density, wide-ranging species (Peek 2003, DeCesare et al. 2012). For some species, recruitment can be difficult to document as juveniles quickly become the same size as adults, and visual assessment of age is often incorrect (Smith 1988, Garel et al. 2006). Survival among age classes varies broadly, and thus knowledge of age structure is essential to accurately assess population demographics. It is widely accepted that for ungulates, adults generally have higher survival rates and elasticity than juveniles, and adult females typically have higher survival rates than adult males (Gaillard et al. 1998, 2000). However, due to high variability, juvenile survival typically has a greater impact on population dynamics (Gaillard et al. 1998, 2000, Raithel et al. 2007, Harris et al. 2008). Thus for endangered populations, estimates of juvenile survival are often a strong indicator of population health and viability and a valuable metric for managers.

Assigning age to an individual to track it throughout its lifetime usually involves capture and handling which can be expensive, dangerous, and potentially injurious or lethal to the animal (Murray and Fuller 2000, Arnemo et al. 2006, Solberg et al. 2006). Consequently,

the use of noninvasively obtained DNA samples (e.g., feces, hair, saliva) as a tool for measuring population parameters has become common in mammal populations, and methods such as faecal DNA microsatellite analysis have proven to be a useful tool for estimating abundance, survival, sex ratio, relatedness, and genetic structure in species that are uncommon and/or difficult to capture (Waits and Paetkau 2005, Schwartz et al. 2007, Beja-Pereira et al. 2009, De Barba et al. 2010). One weakness of this method, however, is the difficulty to age individuals with noninvasive genetic samples, yet understanding the age structure of a population is central to understanding age-specific survival and recruitment.

Morphological measurements of feces (i.e., pellets, bolus) have been used to determine age class in a variety of ungulate species (Table 1). Successful age classification in these studies varied from 75–100% depending on age class, statistical method used, and study species. The ability to distinguish age classes greatly improves the applicability of noninvasive genetic sampling and thus would be especially useful in a monitoring program designed to measure key demographic parameters such as population size, survival rates, and recruitment. Here, we examine the use of faecal pellet dimensions of Sonoran pronghorn (*Antilocapra americana sonoriensis*) to distinguish between fawns, yearlings, and adults using known-age individuals to validate our models.

Sonoran pronghorn exist exclusively in the Sonoran Desert and are federally listed as endangered under the Endangered Species Act (USFWS 1998) and as "most endangered" under CITES Appendix 1 (Hoffman et al. 2008). While believed to number in the thousands in the 1800s (O'Gara and Yoakum 2004), the population declined from 250 animals in 1991 to an estimated 21 individuals in 2002 in the United States (US) range (USFWS 2015) purportedly due to drought, habitat loss, and fragmentation due to fencing and human activity

along the US–Mexico border (USFWS 1998, O'Gara and Yoakum 2004, Wilson et al. 2010). Subsequently, a 1.6 km^2 captive breeding pen was established on the Cabeza Prieta National Wildlife Refuge (CPNWR) to facilitate recovery efforts, and captive individuals are released annually into the wild (Otte 2006). In summer 2013, there were approximately 100 captive individuals in the pen. Population estimates for the wild population are derived from a biennial aerial count, and the population size in 2014 was estimated at 202 individuals (95% CI: 171–334; USFWS 2015). The aerial survey does not provide recruitment estimates as not all individuals observed during the survey are classified to sex or age class due to the potential for disturbance, and survival probability is not estimated (J. J. Hervert, Arizona Game and Fish Department, personal communication).

Pronghorn are sensitive to stress and mortality from capture, and myopathy is not an uncommon consequence during capture and handling (Chalmers and Barrett 1977, Bright and Hervert 2005, Yoakum et al. 2013); thus, noninvasive sampling methods are a promising alternative. Our specific objectives were to determine if pellet size and shape could be used to distinguish age class and to test our ability to determine age class by visual assessment of pellet size and morphology in the field. We predicted an increase in morphometric measurements with age class (i.e., adult pellets larger than yearling and fawn and yearling pellets larger than fawn). We also predicted we would be able to assign coarse age class (i.e., fawn = ≤1 year or non-fawn = ≥1 year) in the field by visual classification.

3.2 Methods

Study Area

In the summers of 2012–2014, we collected samples from individuals in the captive pen and 12 developed watering holes (hereafter drinkers), and nine sites not associated with

drinkers on Organ Pipe National Monument (ORPI), CPNWR, and the adjacent Barry M. Goldwater Range (BMGR) in southwest Arizona, USA. The 1.6 km^2 captive pen is split into a north and south pen with a total population of ~100 free ranging individuals (USFWS 2015). Arizona Game and Fish Department (AZGFD) and US Fish and Wildlife Service (USFWS) personnel monitor captive pronghorn daily and provide alfalfa at feed stations when needed. Fawning starts in the pen in mid-February and most fawns are born in March and April (Wilson et al. 2008). Wild pronghorn are fed alfalfa weekly during the dry months at six of the 12 drinkers outside the pen. Feeding and watering typically begin in April or May and continue through October or November depending on annual rainfall amounts (J. Atkinson, U.S. Fish and Wildlife Service, personal communication). In the wild, fawns are typically born between February and June with one birth reported as early as January in 2013 (Bright and Hervert 2005, USFWS 2015).

Sample Collection

Annual capture operations are conducted in the captive pen by AZGFD and USFWS, during which individuals are captured, radio-collared, and a blood sample is collected. Fawns in the pen are tracked from birth when possible and captured fawns are ear-tagged, and radio-collared if re-captured in subsequent captures. Young of the year are easily identified and classified as fawn (0–11 months), and individuals captured as fawns in the previous year are known yearlings (12–23 months). However, not all fawns are caught during capture operations and consequently, an individual may not be handled until it is >1 year old and is potentially misclassified as to actual age. Thus, we classified all non-fawns as adult. Some captured (captive) individuals are subsequently released into the wild, at a ratio of approximately two males to one female (UWFWS 2015). To obtain DNA, 58 blood samples

(hereafter reference samples) were collected from captured individuals in December 2012 and 2013 when feasible (i.e., if health and safety of the animal was not at risk due to stress) (USFWS 2015). These samples provided a genotype of an individual of known age for later matching to genotypes obtained from faecal pellets collected in the pen.

In May 2012 in the captive pen, we collected five faecal pellets (Morden et al. 2011) less than 24 hours old from each of 185 faecal pellet piles in three pellet size groups defined visually as small, medium, and large (size of pellet, not size of pile). While we recognize our size classification is subjective, we wanted to ensure collection of all age and sex classes, and this size classification was used only to structure collection and was not part of the analyses. To ensure collection of samples less than 24 hours old, we cleared pellets from the area around feed stations in the captive pen on the day prior to collection. We excluded piles that appeared to be from more than one individual based on pellet shape, color, and size to minimize the potential for wasted effort in the laboratory, as these are likely mixed samples. We placed pellets in paper coin envelopes and stored them at room temperature in a plastic Ziploc bag with ~ 250 ml of silica desiccant (Fisher catalogue no. S161-212) to minimize DNA degradation prior to analysis. To determine the age of the individual from which the faecal sample was collected, we matched 7–16 locus microsatellite genotypes (described below) of faecal samples to the reference samples.

Due to high rates of capture myopathy, only limited capture and radio-collaring of wild Sonoran pronghorn occurs, and the majority of marked animals in the wild are captive released individuals (USFWS 2015). To test our ability to assign age class, fawn or non-fawn (≥1 year old), in the wild population, we measured samples collected from wild pronghorn in May and June of both 2013 and 2014 as part of a larger mark-recapture study (Woodruff et al.

In Review). Samples were initially field-classified as fawn or non-fawn based on visual assessment of size and morphology. We had two sources of known age wild individuals: 1) genotypes of known age individuals released from the captive pen obtained from reference samples, which consisted of two known adults captive released in 2012 and sampled in the wild in 2013, and 2) 15 individuals recaptured in 2014 from faecal pellet genetic analysis in 2013. All samples were collected in accordance with methods approved by the University of Idaho Institutional Animal Care and Use Committee (permit no. 2013-79).

DNA Extraction and Genotyping

DNA extraction from some (n = 10) blood samples was conducted using a Qiagen DNeasy blood and tissue kit. For the others (n = 48), DNA was extracted by overnight lysis with ProteinaseK (10mg/mL) at 55 °C, followed by a modified protocol based upon the standard phenol/chloroform extraction and isopropanol/sodium acetate precipitation (Sambrook et al. 1989). We used Phase Lock gel tubes (5-Prime) to aid in the separation between organic and aqueous phases and resuspended the DNA in Low TE (10mMTris-pH 8.0, .01mM EDTA). Faecal pellet DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Inc.) following methods described in Adams et al. (2011) and Woodruff et al. (2015). For individual ID, ten nuclear DNA (nDNA) microsatellite loci ranging in size from 90–278 base pairs and one sex ID locus were amplified in a single multiplex reaction (Lou 1998, Carling et al. 2003, Munguia-Vega et al. 2013). The 7 µl PCR reaction contained 1x Qiagen Master Mix, 0.5x Qiagen Q-Solution, 1.71µM Anam97, 0.04µM Anam50, 0.07 µM Anam82, 0.01 µM Anam79, 0.86 µM Aam13, 0.43 µM Aam11, 0.14 µM ADCYC, 0.26 µM Aam10, 0.04 µM Aam1, 0.04 µM Aam2, 0.29 µM KY (sex ID), and 1.5 µl DNA extract. The PCR profile included an initial denaturation of 95 °C for 15 minutes, followed by a

touchdown of 20 cycles with a 30 second denaturation at 94 °C, 90 second annealing step at 63 °C decreasing 0.5 °C each cycle, and a 60 second extension at 72 °C, followed by 34 cycles of a 30 second denaturation at 94 °C a 90 second annealing step at 53 °C, and a 60 second extension at 72 °C. The cycle finished with a 30 minute final extension at 60 °C.

We initially screened all samples with two PCR replicates to assess sample quality, and samples failing to amplify at ≥5 loci were dropped from additional genotyping to remove low quality, error-prone samples from the dataset. To obtain a consensus genotype, three to eight PCR replicates were performed per sample. Consensus genotypes were based on multiple runs of a sample as follows: 1) For homozygotes, the allele was present at least three times, and 2) for heterozygotes, we had to see each allele at least two times. We repeated this testing and evaluating process until we obtained a consensus genotype at a minimum of seven loci to meet the matching criteria of ≤0.01 Probability of Identity Siblings (P(ID)sibs) (Waits et al. 2001). Consensus genotypes were determined in Microsoft Access (Skrbinsek 2010), and matching and P(ID)sibs analysis was conducted in GenAlEx 6.5 (Peakall and Smouse 2006). Within the captive pen, faecal pellet genotypes were first matched to other faecal pellet genotypes. Then, unique genotypes were matched to genotypes of reference (blood) samples for individuals of known age. In the wild population, faecal pellet genotypes were matched to other faecal pellet genotypes, and individuals redetected in year two were then known to be ≥1 year old (non-fawn).

Measurements

Using digital calipers (Control Company, Friendswood, TX), we measured (mm) maximum length (L), maximum width (W), and calculated length-to-width ratio (L/W), and approximate volume (V: $4/3\pi\,(L/2)(W/2)^2$; volume of an ellipsoid), where W is used for

width and diameter, of each of the five pellets per sample from the captive pen. We used the mean measurements of the five pellets to represent the sample. From wild individuals, we attempted to measure five pellets from at least one pellet group from each individual (confirmed by DNA analysis). However, we did not always have enough pellets post-extraction for measuring, and thus not all individuals had a measured sample. Samples with fewer than two pellets were discarded and we excluded broken, split, or partial pellets (Zahratka and Buskirk 2007). To evaluate the effect of time and desiccation on the measurements, we re-measured 16 samples after seven days of exposure to local field conditions and reran the calculations.

Statistical Analysis

We conducted all statistical analyses using R statistical programming language (R version 3.1.2). We used t-tests with an alpha of 0.05 to examine differences in pellet measurements between captive and wild individuals and between measured and re-measured samples. We used logistic regression and evaluated the predictive accuracy of models using each measurement (e.g., length, volume) as a single predictor variable, as well as all models using combinations of the predictor variables (e.g., length and volume) to distinguish between age classes. Predictive accuracy was estimated using randomized five-fold cross-validation with 100 replications and models were also ranked using Akaike's Information Criterion corrected for small sample size (AIC_c). To avoid numerical errors due to complete or quasi-complete separation, the model parameters were estimated using bias-reduced maximum likelihood (Firth 1993, Kosmidis and Firth 2009). Probability of correct classification was compared to a null model (i.e., no explanatory variables) where the predictive accuracy

depends only on the base rate of classes in the population (e.g., if 60% of the population are adults, then prediction accuracy would be 60% if every sample were called adult).

For age class, cross validation analyses indicated that it was not possible to reliably distinguish between yearlings and adults based on pellet morphology significantly better than using the base rate (probability = 0.69–0.72; null model probability = 0.67), so our age class analysis focused on separate analyses distinguishing fawn from yearling and fawn from adult. We used only known age captive individuals and known age captive released wild individuals for age class analysis. Finally, we combined yearlings and adults into a single age class (non-fawn) and tested the accuracy of our visual field-classification of wild samples by age class, i.e., fawn or non-fawn. We used a fitted model from known age individuals (captive fawns and captive and wild non-fawns) to predict age class of wild samples. We used models with single explanatory variables of width and volume because these were two of the highest AIC_c ranked models for predicting age class (see Results). In cases where the model-predicted age class differed in multiple samples from the same individual (i.e., one predicted adult, one predicted fawn), we ran an additional model (length + length-width ratio).

3.3 Results

We collected 185 pellet groups from 58 small, 69 medium, and 58 large faecal pellet piles from the captive pen (Figure S3.1). From these, we confirmed consensus genotypes for 176 which represented 67 individuals or approximately 85% of the pronghorn in the pen in 2012 (USFWS 2015). During physical capture, we obtained 58 reference samples over two years from known age individuals. We genotyped and matched 33 of the reference samples (four adults, 17 yearlings, 12 fawns) to 87 faecal samples (Table 2). The remaining 98 pellet piles were not matched to known age individuals and were not included in our analyses. All

samples collected in the captive pen were measured, and the number of samples per individual averaged 2.8 (range 1–8). We did not find significant differences between measurements of fresh pellets and pellets re-measured after seven days ($P > 0.05$).

Pellet Size and Age Class

For both captive and wild, length, width, and volume of yearling and adult (or non-fawn) pellets were greater than fawns, but length-width ratio was larger for fawns, indicating fawn pellets were rounder than either yearling or adult pellets (Figure 1 and Table 3a). Probability of correct age class classification for captive fawn versus yearling using a single variable was 0.98 for width only and was the highest AIC_c ranked model (Table 4). Other single variable models and combinations of ≥2 explanatory variables performed similarly with predictive probability ranging from 0.91 to 0.98 and delta AIC_cs from 1.66 to 7.16. Length as a single explanatory variable was the lowest ranked model with probability of correct classification of 0.77 and delta AIC_c value of 7.16. The null model (i.e., the model with no explanatory variables) had probability of correct classification of 0.32 (i.e., if we guess fawn, we will be right 32% of the time because 32% of our samples were fawn).

The highest AIC_c ranked model for captive fawn versus adult again included width only with probability of correct age class classification of 0.98. The volume only model and combinations of ≥2 explanatory variables performed similarly with predictive probability ranging from 0.93 to 0.97 and delta AIC_cs from 1.46 to 8.41. Length and length-width ratio as single explanatory variables were the lowest ranked models with probability of correct classification of 0.85 and 0.77 and delta AIC_c values of 17.77 and 26.65, respectively. The null model (i.e., the model with no explanatory variables) had probability of correct classification of 0.50 (i.e., 50% of samples were adult and 50% were fawn).

Captive versus Wild Measurements

Mean length, width, and volume of wild non-fawns (≥1 year as evidenced by recapture) were smaller than those of captive non-fawns (Figure 2). Mean length-width ratio, however, was consistent for non-fawn captive (1.59) and wild (1.68) individuals. Length, width and volume were significantly different between captive and wild non-fawns (Table 3b). For fawns, only width was significantly different between captive and wild individuals.

Prediction of Age Class from Wild Samples

In 2014, we recaptured 15 individuals (n = 39 samples), thus known to be non-fawn (≥1 year old). All samples from recaptured individuals were correctly model predicted as non-fawn in either the width only or volume only model (Table 5).

Ten unique individuals (a total of 16 samples) had conflicting model predicted age class (i.e., one model predicted non-fawn, one predicted fawn). For eight of these individuals, we measured multiple samples, and we took the majority model-predicted classification (i.e., four model-predicted as non-fawn, two model-predicted as fawn, we called it non-fawn). The two other individuals were single captures (i.e., we had only one sample) for which we ran an additional fitted model (length + length-width ratio) and used the age class that was predicted twice (i.e., volume and width models both predicted fawn, and length + length-width ratio predicted adult, we called the individual fawn).

Correct visual field-classification by age class varied considerably between years. In 2013, we measured 128 samples from 76% (n = 72 individuals) of the total wild individuals we captured (i.e., detected their genotype) (Table 2). Ninety-six percent of visual field-based classifications matched the model predictions. In 2014, we measured 130 samples from 64

individuals, and 65% of samples were correctly field-classified by visual observation (i.e., matched the model prediction).

In 2013, only one sample that was visually field-classified as non-fawn (n = 119) was model predicted as fawn, whereas three samples visually field-classified as fawn were subsequently model predicted as non-fawn (Table 5). Similarly, in 2014, 98% (n = 62) of samples visually field-classified as non-fawn were model predicted as non-fawn, but only 33% (n = 22) of samples field-classified as fawn were model predicted fawn. Nearly all samples (46 of 49 in the years combined) incorrectly visually field-classified as fawn were male.

3.4 Discussion

Understanding age-specific survival rates is critical to management and identifying long-term trends in population growth, particularly in endangered species, yet documenting age-specific survival relies on knowing age structure. We successfully demonstrated the ability to distinguish between fawns and yearlings or adults using morphometric pellet measurements of Sonoran pronghorn. While we did not have enough predictive power to separate yearlings and adults, an understanding of recruitment rates and fawn survival is important in determining the trajectory of the population. This method provides a reliable way to document fawns, and the use of faecal DNA for individual identification further strengthens the method's utility as individuals can be tracked over multiple years of sampling.

Pellet Size and Age Class

Defining pellets of captive pronghorn by size (small, medium, large) was a mechanism to facilitate collection of all age and sex classes and was not used in the analysis, but our results indicate this was an appropriate collection method for obtaining a range of age classes

for our analyses. As expected, pellet size was larger for yearlings and adults than for fawns. The probability of correctly assigning age class models was similar for fawns and yearlings and fawns and adults and performed notably better than the null models (null probability 0.32 and 0.50, respectively). Contrary to other studies (Delibes-Mattos et al. 2009, Morden et al. 2011), our cross-validation analysis showed that single measure models assigned age class with roughly the same accuracy as models with multiple variables. Our small sample size (n = 4) for adults is perhaps the reason we could not distinguish between adults and yearlings. Collecting and measuring faecal pellets from known adults would potentially allow for this distinction.

Captive versus Wild

Similar to other research, our results suggest that the most accurate predictive models are built from samples collected from the target population (MacCracken and Van Ballenberghe 1987, Chapman 2004, Morden et al. 2011). Differences in diet (e.g., seasonal variation, captive vs. wild) can affect size of faecal output (Campos-Arceiz et al. 2008, Morden et al. 2011) and defecation rate (Rogers 1987, Mayle et al. 1996, Chapman 2004, Ferretti et al. 2014) with captive individuals showing reduced defecation rates (Irby 1981, Asa et al. 1985, Rogers 1987, Kitchen and Martin 1996, Chapman 2004). Brashares and Arcese (1999a, b) suggest dominant male oribi (*Ourebia ourebi*) restrict defecation volume and occurrence in order to increase the frequency of territory marking events. More defecation events would presumably translate into less faecal matter in each defecation event. Consequently, there could be differences in defecation rate, and potentially pellet size, between captive and wild individuals, as well as fed and unfed individuals in the wild.

However, we believe that our approach to develop predictive models using captive individuals was justified and effective for multiple reasons. First, in our study system, any faecal DNA sampling will be conducted at watering holes, nearly 50% of which also provide supplemental feed. Second, we were able to test our methods on 15 known age wild animals and 100% were correct in model predictions. Third, our results illustrate significant differences in pellet size between fawn and non-fawn in both the captive and wild populations, with wild fawn having the smallest measurements and thus having a lower chance of being misclassified as non-fawn compared to captive animals. Fourth, length-width ratios between captive and wild animals were not significantly different and indicated that pellet size changed consistently across measurements from captive to wild.

Accuracy of Visual-based Field Classification

Our results suggest that age classification based on visual assessment is imprecise and likely influenced by individual differences in observers. In 2013, we had two people collecting samples who were trained simultaneously and extensively. In 2014, four new personnel collected samples and training was limited. Based on model predictions, visual-based field classification was more accurate in 2013 (96% correct) compared to 2014 (65% correct). Samples were more often field-classified as fawn and model predicted as, or known to be, non-fawn (n = 19) than vice versa (n = 2). While we captured twice as many males as females (Woodruff et al. In Review), 94% (46 of 49) of samples incorrectly visually field-classified as fawn were male suggesting adult males have smaller faecal pellets and are more difficult to classify than adult females. In 2014, two redetected individuals made up 24% (n = 11) of the samples incorrectly field-classified as fawn. One of these individuals was field-classified and model predicted as fawn in 2013 and would putatively be a yearling in 2014

perhaps providing explanation for the misclassification. The other individual was captured at a site with supplemental feed and field-classified and model predicted as non-fawn in 2013. However, in 2014 this individual was recaptured at a site with no supplemental feed and was field-classified as fawn in all samples, but model predicted as adult in 64% (n = 7) of samples. While based on only a single individual, this strengthens the idea that fed individuals produce larger pellets. Thus, we recommend measuring pellets when trying to determine age class to increase accuracy. We also note that developing a model based on measurements from wild pronghorn would strengthen predictive power. However, we posit that when obtaining samples from wild individuals is not feasible, samples from captive animals provide a reasonable surrogate.

Management Implications

In species that are highly sensitive to capture and disturbance, like Sonoran pronghorn, noninvasive genetic sampling methods are an appealing alternative approach for obtaining critical demographic data. Managers of Sonoran pronghorn do not have current sex and age-based survival estimates because these data cannot be obtained with the current aerial survey approach used for population estimation (USFWS 2015). Because we measured samples collected in May and June and relatively soon after fawning, we do not know at what age fawn samples are no longer distinguishable from adults. However, faecal DNA sampling for population monitoring is likely feasible only during these months when pronghorn are congregating at watering holes due to the hot, dry conditions (Woodruff et al. In Review). Accuracy of age classification could potentially be improved by developing separate models for sites with and without supplemental feed, as our limited data (not shown) indicates pellets from individuals at sites with supplemental feed are larger. We also note that while seven

days of weathering did not alter the pellet measurements, results could differ if pellets are older than seven days. Additional pilot studies are needed to evaluate this. However, other research shows older Sonoran pronghorn pellets have lower DNA amplification success rates, and if the objective is to use these measurement methods in conjunction with individual identification from faecal DNA, pellets older than seven days will be of little use (Woodruff et al. 2015).

While we could not determine sex using morphological methods (data not shown), sex is easily ascertained using genetic methods, and our method shows strong potential for use in wild populations of pronghorn to distinguish fawns from yearlings or adults. The ability to distinguish age structure using faecal pellet measurements greatly improves the applicability of faecal pellet collection and has significant implications for management of the Sonoran pronghorn population. We recommend analyzing pellet using both single width and volume variable models and running the combined model (length + length-width ratio) if the two single variable models disagree. A long-term monitoring scheme combining pellet measurements with individual identification using DNA would allow the identification of fawns that would remain known age individuals for the remainder of the monitoring years. This would provide a powerful tool for documenting the population's age and sex structure, recruitment, and age-specific survival rates.

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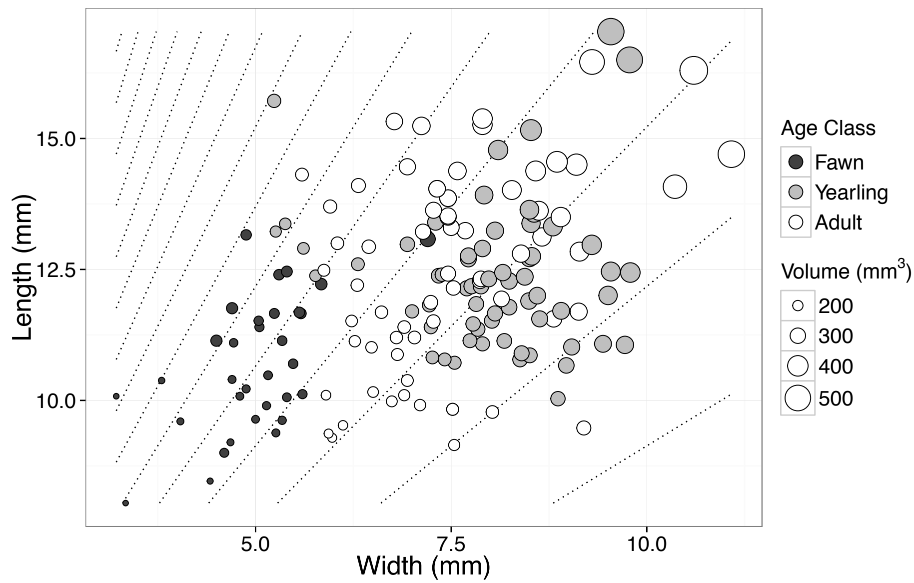

Figure 3.1. Scatterplot of faecal pellet measurements of captive and wild Sonoran pronghorn collected May and June 2013 on the Cabeza Prieta National Wildlife Refuge, Arizona, USA. Captive individuals are known age and wild are as predicted by models. The dotted lines represent contours of constant length-width ratio, with points in the upper left representing pellets that are relatively long and narrow, while lower right points represent pellets that are relatively short and wide.

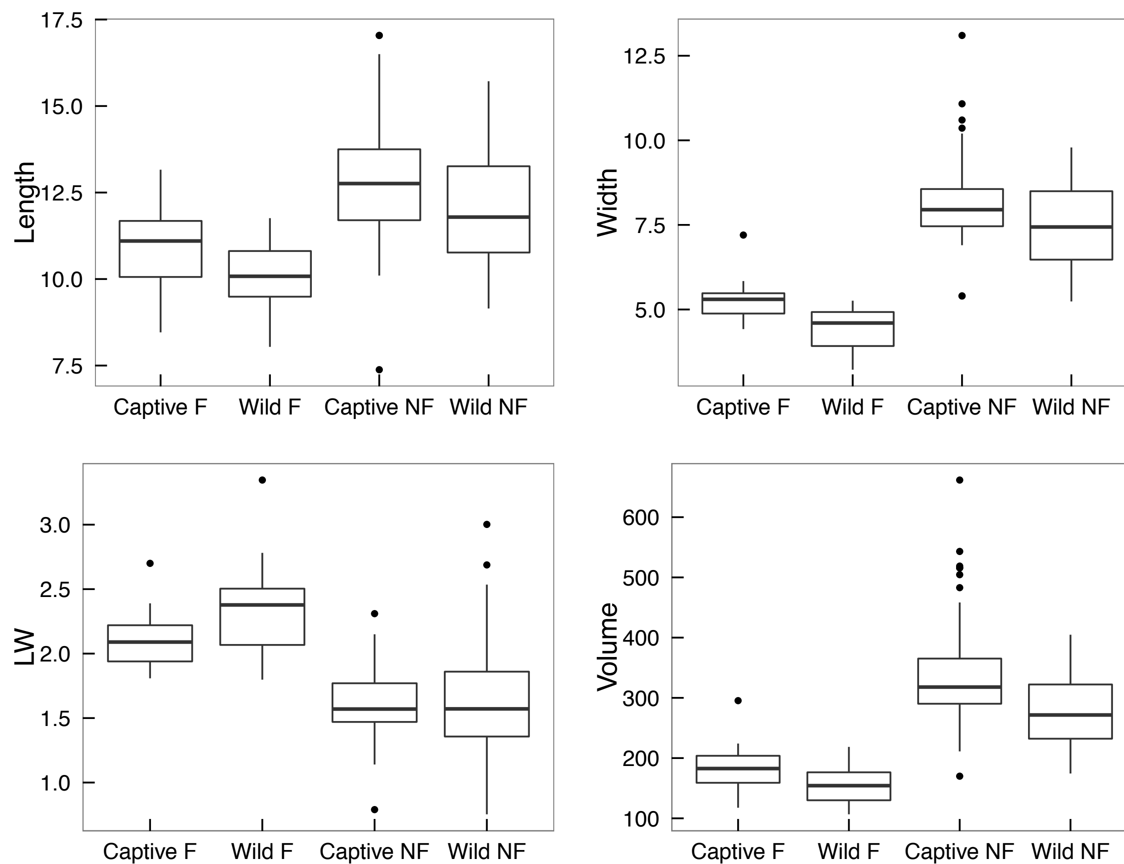

Figure 3.2. Box plots showing mean measurements of Sonoran pronghorn faecal pellets collected in May and June 2012–2014 in Arizona, USA, from captive and wild fawn (F) and non-fawn (NF) for length, width, length-width ratio, and volume. The box signifies the upper and lower quartiles and the median is represented by the thick black line. Black dots represent outliers

Table 3.1. Summary of ungulate studies using fecal pellet measurements to distinguish between age classes.

Species	Study	Age Classes Discriminated
White-tailed deer (*Odocoileus virgianianus*)	Ezcurra and Gallina 1981	Yearling, adult
Elk (*Cervus elaphus*)	Bubenik 1982, Alvarez 1994	Juvenile, adult
Moose (*Alces alces*)	MacCracken and Van Ballenberge 1987	Yearling, adult
Manipur brow-antlered deer (*Cervus eldi eldi*)	Khan and Goyal 1993	5 age classes
Fallow deer (*Dama dama*)	Alvarez 1994	Calf, juvenile, adult
Sumatran elephant (*Elephas maximus sumatranus*)	Reilly 2002, Tyson et al. 2002	Juvenile, sub-adult, adult
Mule deer (*Odocoileus hemionus*)	Sanchez-Rojas et al. 2004	Yearling, adult
Greater bilby (*Macrotis lagotis*)	Southgate 2005	Immature, mature
Boreal caribou (*Rangifer tarandus caribou*)	Ball 2010	Calf, yearling, adult
Svalbard reindeer (Rangifer tarandus platyrhynchus)	Morden et al. 2011	Calf yearling, adult

Table 3.2. Total number of samples measured in both the captive and wild Sonoran pronghorn populations in summer 2013 and 2014, Arizona, USA. Captive samples are known age class from individual ID genotype matching. Wild individuals classified as adult are non-fawn (i.e., yearlings and adults) and were field (visual) classified.

	Captive		Wild	
Age class	Male	Female	Male	Female
Fawn	14	7	67	16
Yearling	40	5	---	---
Adult	12	9	113	62

Table 3.3a. Descriptive statistics and associated standard errors (SE) of faecal pellets from captive and wild Sonoran pronghorn collected May and June 2012– 2014, Arizona, USA. Wild non-fawn individuals include only recaptured individuals, and wild fawns were determined using model predictions. b) P-values from t-tests comparing differences in faecal pellet measurements between captive and wild Sonoran pronghorn.

a)

Age class (sample size)	Length (SE)	Width (SE)	LW[a] ratio (SE)	Volume[b] (SE)
Captive adult (21)	14.00 (0.32)	8.30 (0.28)	1.71 (0.05)	518.87 (44.98)
Captive yearling (45)	12.48 (0.24)	8.21 (0.14)	1.55 (0.03)	454.05 (26.67)
Wild non-fawn (64)	11.98 (1.63)	7.36 (1.22)	1.68 (0.43)	346.70 (189.56)
Captive fawn (21)	10.97 (0.22)	4.98 (0.13)	2.19 (0.05)	161.87 (8.35)
Wild fawn (11)	10.44 (0.18)	4.78 (0.16)	2.26 (0.07)	163.60 (6.50)

b)

Captive/Wild non-fawn	0.03*	0.005*	0.18	1.19e-07*
Captive/Wild fawn[c]	0.10	0.02*	0.09	0.99

[a] Length-width ratio

[b] Volume= V: $4/3\pi (L/2)(W/2)^2$; volume of an ellipsoid

Table 3.4. Results of the logistic regression models evaluating the influence of pellet measurements on the ability to distinguish a) captive Sonoran pronghorn fawn vs. yearling and b) fawn vs. adult. Only models with delta $AIC_c \leq 2$ are shown. Pellets were collected in the captive pen on the Cabeza Prieta National Wildlife Refuge, Arizona, USA in May and June 2013 and 2014. We included single dimension measurements as well as all combinations of measurements.

a)

	β (SE)					
Intercept	Length[a]	Width[b]	LW Ratio[c]	Volume[d]	CVP[e]	AIC_c[f]
20.48 (6.05)	--	-3.12 (0.87)	--	--	0.98	12.07
-13.59 (10.82)	-0.99 (0.52)	--	13.96 (4.83)	--	0.97	13.73
12.63 (6.34)	0.93 (0.86)	-3.60 (1.21)	--	--	0.97	13.75
12.59 (11.20)	--	--	-9.28 (5.50)	-0.02 (0.01)	0.97	13.78
0.71 (15.81)	--	-1.88 (0.96)	6.36 (6.00)	--	0.97	13.78
32.50 (10.55)	--	-6.34 (2.13)	--	0.32 (0.01)	0.98	13.94

b)

Intercept	Length[a]	Width[b]	LW Ratio[c]	Volume[d]	CVP[e]	AIC_c[f]
19.18 (6.10)	--	-2.91 (0.90)	--	--	0.98	11.93
7.45 (2.26)	--	--	--	-0.03 (0.01)	0.95	13.39

[a] Length of pellet measured from tip to tip

[b] Pellet width at widest part of pellet

[c] Length-width ratio

[d] Volume = V: $4/3\pi\ (L/2)(W/2)^2$; volume of an ellipsoid

[e] Cross-validation probability is the probability of the model correctly assigning age class to a sample

[f] Akaike's Information Criterion corrected for small sample size

Table 3.5. Comparison of visual field classification and measurement prediction for pellet samples collected for wild pronghorn in 2013 and 2014, Arizona, USA.

		Measurement prediction	
Field Classification	# Measured	# Non-fawn (%)	# Fawn (%)
Adult	122	119 (98)	3 (2)
Fawn	64	46 (72)	18 (28)

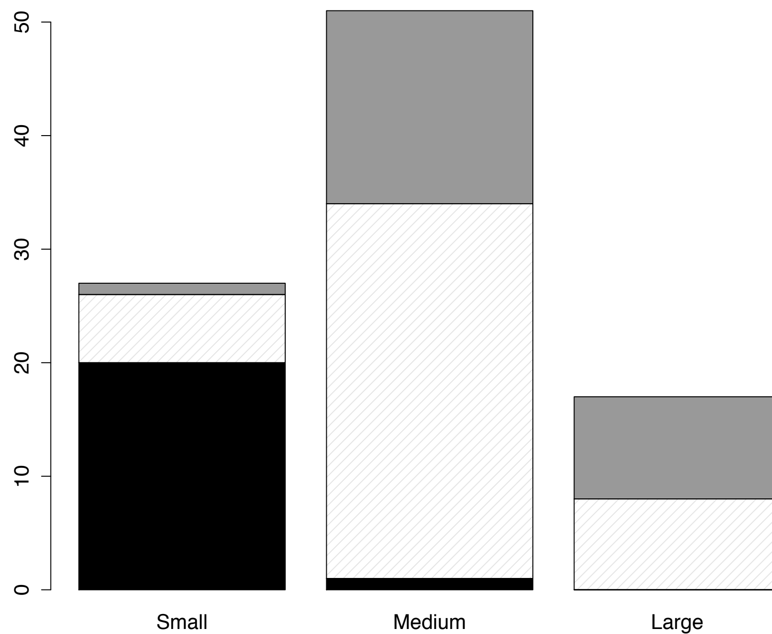

Figure S3.1. Breakdown of pellets by size. Fawn is black, yearlings are light gray, and adults are dark gray, unclassified are hashed. Small: 74% fawn (n = 20), 22% yearling (n = 6) and 4% adult (n = 1); medium: 2% fawn (n = 1), 65% yearling (n = 33), 33% adult (n = 17); large: 47% yearling (n = 8), 53% adult (n = 9).

CHAPTER 4-ESTIMATING ENDANGERED SONORAN PRONGHORN (*ANTILOCAPRA AMERICANA SONORIENSIS*) ABUNDANCE AND SURVIVAL USING FECAL DNA AND CAPTURE-RECAPTURE METHODS

In Revision: *Conservation Biology* Authors – Susannah P. Woodruff, Paul M. Lukacs, David A. Christianson, and Lisette P. Waits

Abstract

Population abundance estimates are important for management, but can be challenging in low density, wide-ranging, and endangered species, such as Sonoran pronghorn (*Antilocapra americana sonoriensis*). The Sonoran pronghorn population has been increasing; however, population estimates are currently derived from a biennial aerial count which does not provide survival or recruitment estimates. We identified individuals using noninvasively collected fecal DNA and used robust design capture-recapture sampling to estimate survival and abundance for Sonoran pronghorn in the United States between 2013 and 2014. Separate population estimates were generated for developed water holes (drinkers) and drinker and non-drinker locations in 2014. The population using drinkers remained stable at 116 individuals (95% CI: 102–131) and 121 individuals (95% CI: 112–132) in 2013 and 2014. The population estimate for all locations was 144 individuals (95% CI: 132–157). Adults had higher annual survival probabilities (0.83, 95% CI: 0.69-0.92) than fawns (0.41, 95% CI: 0.21-0.65). This new method of using targeted sampling and non-invasive genetic sampling capture-recapture with Sonoran pronghorn fecal DNA proved successful. Our results provided the first survival estimates for this population in over 2 decades as well as precise estimates of the population using the drinkers. This method also presents opportunities for targeted sampling in other systems for broadly distributed species, such as in African savanna

ecosystems, where many species congregate at watering sites.

4.1 Introduction

Reliable population estimates are central to wildlife management and are a key component in determining conservation status and recovery success (IUCN 2012). However, obtaining reliable population estimates can be challenging, especially in low-density, wide-ranging species. Traditional methods of obtaining population estimates can require physical capture which can be expensive and dangerous. Also, capture-related stress can lead to reduced levels of fitness, compromised immune system, impaired locomotion, or even mortality (see Murray & Fuller 2000; Arnemo et al. 2006; Solberg et al. 2006). Consequently, there has been increasing use of noninvasive sampling methods, such as track surveys, remote cameras, and collection of DNA samples to monitor wildlife populations (Silviera et al. 2003; Waits & Paetkau 2005).

Fecal DNA microsatellite analysis has proven to be a useful tool for estimating population size, sex ratio, parentage, relatedness, and genetic structure (Waits & Paetkau 2005; Schwartz et al. 2007). Combining noninvasive genetic sampling and capture-recapture methods (NGS-CR) to estimate demographic parameters is well developed in monitoring carnivores (Prugh et al. 2005; Stenglein et al. 2010; Stansbury et al. 2014) and has been expanding in ungulates (Brinkman et al. 2011; Poole et al. 2011; Ebert et al. 2012a, b; Goode et al. 2014). These approaches eliminate capture and capture-related stress on animals, provide accurate population estimates, and can improve cost efficiency over traditional methods (e.g., DeBarba et al. 2010; Stenglein et al. 2010; Stansbury et al. 2014).

One endangered ungulate species for which noninvasive monitoring methods could be useful is the Sonoran pronghorn (*Antilocapra americana sonoriensis*). The Sonoran

pronghorn is found exclusively in the Sonoran Desert of Arizona, United States (US) and Mexico, and was listed as endangered under the US Endangered Species Act of 1973 (USFWS 1998). By 2002, with changing climatic conditions, severe drought, and threats from human activities the US population had fallen to an estimated 21 individuals (Bright & Hervert 2003). To facilitate recovery efforts, a captive breeding pen was established on the Cabeza Prieta National Wildlife Refuge (CPNWR) and captive individuals are released annually into the wild. Federal downlisting criteria requires either 1) a stable population of approximately 300 adult Sonoran pronghorn in one US population and a second separate U.S. population, or 2) a population size that is determined to be adequate to maintain a long-term, viable population (USFWS 1998). At present, abundance estimates are based on biennial aerial counts using sightability models to account for imperfect detection (USFWS 2010). The US population estimate was 159 (95% CI: 112–432) and 202 individuals (95% CI: 171–334) in December 2012 and 2014, respectively (J. Hervert personal communication). While the current approach provides population estimates and trends, high costs prohibit annual population estimates, and the method does not provide information on survival, reproduction, or genetic diversity.

Pronghorn are sensitive to stress, and mortality from capture myopathy is not uncommon during capture and handling (Chalmers & Barrett 1977; Bright & Hervert 2005), thus, they are good candidates for NGS-CR monitoring. Our overall goal was to evaluate the feasibility of using a NGS-CR approach to monitor pronghorn annual survival and population size. In 2013 and 2014, we designed and implemented a targeted sampling strategy for collection of fecal samples (Woodruff et al. 2015) focusing sample collection during the hot, dry months (April–June) when Sonoran pronghorn congregate at artificial water sources or

'drinkers' (USFWS 2010). Our first objective was to estimate annual survival probabilities for different sex and age classes. We expected to find higher annual survival probability for adults than fawns. Our second objective was to generate a population estimate and associated confidence interval for comparison with the current estimate for this population. Observation of radio-collared pronghorn using drinkers from 2006–2013 suggested only 70% of the population would be sampled at drinkers (J. Hervert, personal communication). Thus if our noninvasive genetic sampling methods were effective, we expected to estimate populations approximately 30% lower than aerial counts (see Methods).

4.2 Methods

Study Area

The study area encompasses *c* 7000 km^2 on the CPNWR, Barry M. Goldwater Range (BMGR) and Organ Pipe National Monument (ORPI) (Fig. 1). The region is characterized by wide alluvial valleys divided by fault-block mountains. Elevation varies from 610 m to 1219 m, but pronghorn are typically found below 900 m. Vegetation consists primarily of mesquite shrublands with sparse vegetation cover or open hardwood–mixed cactus woodlands (Shreve & Wiggins 1964). The average annual precipitation ranges from 7.5 cm in the west to 20 cm in the east (USFWS 2002a). The dry season extends from April to June and temperatures in the study area average over 38 °C in summer (INRMP 2003).

Natural surface water in the region is limited (USFWS 2010). Quitoboquito Spring, located on ORPI, is the only naturally occurring year-round water source in current Sonoran pronghorn range (USFWS 2011). Motion-sensing cameras documented no pronghorn use of this spring during this study (D.C., unpublished data) likely due to close proximity to a busy highway and high human use. Numerous artificial water sources, or 'drinkers', provide

supplemental water for Sonoran pronghorn. Drinkers are either gravity fed or filled manually by state and federal personnel. From April through July, supplemental alfalfa hay is provided weekly for pronghorn at 6 drinkers.

Sample Collection

Sonoran pronghorn are distributed over approximately 11 000 km^2 (USFWS 2010), but low population density makes detection on the periphery of their range difficult. To increase efficiency, we implemented a targeted sampling approach (Puechmaille & Petit 2007; Rudnick et al. 2008; Stenglein et al. 2010). Radio telemetry and aerial surveys have shown that Sonoran pronghorn use areas close to natural and developed water sources more often than random locations (deVos & Miller 2005; Morgart et al. 2005) making targeted sampling at drinkers a practical method for sampling a majority of the population. In May and June 2013 and 2014, we attempted to collect fecal samples 3 times (6 total) at an interval of 7 days at all developed drinkers likely to be used by pronghorn (17) (Table S1 in Supporting Information) to maximize the chance of obtaining usable DNA (Woodruff et al. 2014; Woodruff et al. 2015). However, actual sampling locations, frequencies, and intervals were limited by logistic constraints on agency access, minimizing pronghorn disturbance, terrain, and level of pronghorn use. Because of these limitations, we classified each sampling site into 1 of 2 session types: single-session and multi-session. Based on relocations of >100 radio-collared pronghorn from 2006–2013, approximately 30% of the population never visited a drinker during summer (J. Hervert, personal communication) but the true level of drinker visitation is unknown. Therefore in 2014, we targeted groups with radio-collared individuals located away from (>1 km) drinkers and opportunistically collected samples from 9 locations after observing defecation events (Fig. 1). Based on 2013 sampling results and weather

conditions (i.e., wetter conditions and better forage), we started 2014 sampling 12 days later than in 2013.

Samples were collected from the area within 50 m of drinkers (Woodruff et al. 2015), and we excluded piles that appeared to be from >1 individual based on pellet shape, color, and size, as these were likely mixed samples. At multi-session sites the collection area was divided into 4 quadrants (Q1-Q4) for later subsampling (Appendix S1). For each sampling event, we attempted to collect samples at a rate of 3 times the number of pronghorn counted at the drinker just prior to sampling (based on direct observation and motion-sensing cameras). We chose this target number of samples to ensure sufficient recaptures/redetections and maximize performance of capture-recapture estimators (Solberg *et al.* 2006). At single-session drinkers, we sampled from all acceptable pellet piles. At least 6 pellets were collected from each sample, placed in paper coin envelopes, and stored at room temperature in a plastic Ziploc bag with (~250 ml) of silica desiccant (Fisher catalogue no. S161-212) to reduce DNA degradation. All remaining pellet groups were crushed or scattered to avoid resampling when searching the same area during a later sampling period, and thus, we assumed samples collected in the next occasion were deposited during the interim period.

We classified samples by freshness (F1: Freshest; visibly wet on outside of pellet; F2: Less fresh; wet/moist on inside of pellet only, crushes easily; F3: Oldest; no moisture, crumbles when crushed) for later subsampling. We used chi-square analysis (R version 3.1.2, www.r-project.org) to assess individual ID success by freshness and session (1–3).

Genetic Analysis

We analyzed all samples from single-session sites and approximately 2 times the number of individuals estimated to be using the drinker from multi-session sites starting with

the freshest samples. The subsampling regime, DNA extraction method, PCR and genotyping methods, and probability of identity calculations are described in Appendix S1. Genotyping errors were calculated following Broquet & Petit (2004). As genotyping errors can lead to inflated population estimates (Waits & Leberg 2000; Lukacs et al. 2009), we reanalyzed samples mismatching at 1 or 2 loci following methods similar to Kendall *et al.* (2009). Samples with continuing ambiguity were amplified in a second multiplex with an additional 6 loci to refute or confirm a match (Appendix S1).

Population Abundance and Survival Estimation

We field classified samples based on visual inspection of size and morphology as adult (≥1 year old) or fawn (<1 year old). For 76% of individuals we cross-checked our age assignment against a fitted cross-validation model developed from multiple pellet measurements (length, width, length/width ratio, and volume) of known age individuals (S.P.W, unpublished data).

Each successfully amplified sample was considered a detection. We generated an encounter history for each individual indicating detected (1) or not (0) in each sampling session. We counted only a single detection per individual per sampling session. We collapsed sessions 3 and 4 into a single session since only 3 sites were sampled 4 times.

Adult

We applied Huggins' robust design in the development of 4 biologically appropriate models of survival and abundance (Tables 3, S5) which allowed varying survival and detection probabilities with time and variation in capture and recapture probability, (Pollock 1982; Pollock et al. 1990; Huggins 1989; Kendall et al. 1997) in Program MARK (White & Burnham 1999). We used Huggins' models because our models included an individual

covariate, and we believe we sampled a substantial proportion of the population. The population was assumed to be demographically and geographically closed within the annual sampling occasions during which we estimated capture/detection (p) and recapture/redetection (c) probabilities, and abundance (N). Across years, gains (birth and immigration) and losses (death and emigration) in the population are expected and we estimated annual apparent survival (φ). For survival, we modeled equal survival probability for male and female adults as well as varying survival probabilities by sex. Additionally, we modeled the individual covariate fed/not fed on survival probability because supplemental feed is not provided at all sites.

Data from single and multi-session sites were combined in the same model. While redetection of individuals from single-session drinkers was possible at multi-session drinkers and vice versa, we never detected the same individual at both single and multi-session drinkers (i.e., no single-session individuals were redetected within the same year). We hypothesized that drinker visitation could differ by sex and estimated detection and redetection probabilities and abundance in groups (males and females at both single-session and multi-session sites) resulting in 4 estimates. To evaluate differences between sessions in a single year, we modeled time varying detection and redetection probabilities. To estimate detection probability at single session sites, we used the model-estimated mean detection probability from multi-session sites by sex (i.e., mean of males' detection probability at multi-session sites equals detection probability for males at single-session sites). Mean detection probability by sex was estimated across all of the data using the means coding in the design matrix. Before fitting models, parameter estimates for single-session site redetection probabilities were fixed at zero and emigration parameters (δ) were also fixed to zero due to

lack of precision (Lukacs *et al.* 2009). Additionally, because we expanded our sampling extent in 2014, we performed analyses on drinker locations only (i.e., no individuals from non-drinker sites were included) from 2013 and 2014, as well as on all locations.

Fawn

We used separate closed capture models in each year to estimate fawn detection and redetection probabilities and abundance in 2013 and 2014, again, modeling drinker only and all locations separately. We used a full likelihood robust design model to estimate annual apparent survival for the 2013 fawn cohort. We included models with and without time varying detection and redetection probabilities. We estimated fawn survival over the interval from approximately 3 months to 15 months of age, largely outside the highly vulnerable neonatal period (i.e., < 1 month of age). Survival in neonates is highly stochastic, and analysis of survival in older fawns that have survived the neonatal period may be more sensitive to current environmental conditions or management practices and provide a more meaningful indicator of current population growth.

All adult and fawn models assumed equal detection and redetection probability (i.e., no behavioral effect), as it is unlikely that the initial detection would affect subsequent detections. We did not detect fawns at single-session sites in 2013; however, in 2014 we did and used the model-estimated mean fawn detection probability at multi-session sites for single-session sites. We used Akaike's Information Criteria (AIC_c) corrected for small sample size to evaluate relative support for each model. We model averaged parameter estimates and standard errors over all models (Burnham & Anderson 2002). We summed population estimates for each group (6 groups: single and multi-session for each of adult male, adult

female, and fawn) and calculated standard errors for abundance using the Delta method (Seber 1982).

4.3 Results

Sample Collection and Genetic Analysis

From 13 drinkers, we collected 730 samples in 2013 and 980 samples in 2014 and extracted 634 and 692 samples in 2013 and 2014. We also collected and extracted 79 non-drinker samples in 2014 (Table S2). Four single-session drinkers had no indication of recent pronghorn use.

In 2013 and 2014, 75% (n = 474) and 72% (n = 555) of pronghorn samples achieved a consensus genotype for individual ID. Samples classified as freshest (F1) had the highest individual ID success rates. Individual ID success rates increased in later sessions in 2013, but were generally consistent across sessions in 2014 (Appendix S1, Tables 1, S3, S4).

Allelic dropout rates were higher than false alleles and overall genotyping error rates were slightly higher in 2014 (Table 1). Our final dataset contained no individuals mismatched at only 1 locus and 1 pair of samples that mismatched at 2 out of 10 loci. We amplified these samples at an additional 6 loci resulting in 4 mismatches at the 16 loci, and they were determined to be 2 individuals.

Population Abundance and Survival Estimation

We had 474 detections of 91 individuals (50 adult males, 24 adult females, 17 fawns) in 2013 and 555 detections of 127 individuals (69 adult males, 38 adult females, 20 fawns) in 2014. Twenty-one of the individuals (8 adult males, 10 adult females, 3 fawns) in 2014 were detected at 9 non-drinker locations, 4 of which were also detected at drinker locations (Table 2). Sixty-three individuals detected in 2013 were also detected in 2014. The number of

detections per individual (i.e., samples) in a year ranged from 1 to 32, and 33.7% and 26.0% were single detections in 2013 and 2014 (Fig. 2). At drinkers we detected 2.5–3 times more adult male than female samples, and the average number of detections per individual was 6.1 and 3.6 for adult males and females.

Adult detection probability and survival

For drinker only and all locations, the top model was the same and included equal survival (φ) by sex and equal detection (*p*) and redetection (*c*) probabilities both varying by time and group (Tables 3, S5). Males had higher detection probabilities (range: 0.64–0.76) than females (range: 0.36–0.61) in all occasions across years (Fig. 3). In 2013, detection probabilities were highest in the last (third) session increasing from 0.66 in the first session to 0.76 in the third session for males and from 0.37 to 0.49 during the same time for females. On the contrary, detection probabilities were highest for both males and females in the first session in 2014 and remained relatively constant over time. Annual apparent adult survival probability was 0.83 (95% CI: 0.69–0.92).

Fawn detection probability and survival

Across years, and for drinker only and all locations, the top model for abundance estimation included equal detection and redetection probabilities with time variation (Tables 3, S5). Consistent with adults, detection probabilities were highest in the third session in year 1 and highest in the first session in year 2 (Fig. 3). The best AIC_c ranked model for fawn survival had constant and equal detection and redetection probabilities (Tables 3, S5). The second ranked model differed only in that detection and redetection probabilities were time varying. As expected, fawn survival probability was lower than adult survival at 0.41 (95% CI: 0.21–0.65).

Population estimation

At drinker locations, the models produced (summed) population estimates of 116 individuals (95% CI: 101–132) in 2013 and 121 individuals (95% CI: 112–132) in 2014. For all locations (2014 only), the population estimate was 144 individuals (95% CI: 132–157). Population estimates indicated a bias towards males with 1.4–1.6 times more adult males than adult females. When comparing annual population estimates from drinker locations, the results suggest little to no change in population size. As expected, the population estimate at the expanded geographic location was larger as more individuals were sampled.

4.4 Discussion

This new method of using NGS-CR with Sonoran pronghorn fecal DNA proved successful and provided the first survival estimates for this population as well as precise estimates of the population using the drinkers. Targeted sampling enabled us to collect many pellet groups and high detection probabilities resulted in high precision (Burnham *et al.* 1987). Capture-recapture analysis assumes that all individuals in the population have equal probability of capture, (i.e., no individual heterogeneity; Link 2004; Lukacs & Burnham 2005), yet individual heterogeneity is inherent in capture probability through differences in sex and age class, dominance, or individual location in relation to the “trap” (Ebert et al. 2010). We recognize that while our estimates were precise, they are likely biased low as they represent only the portion of the population visiting the drinkers (i.e., individuals closer to the “trap”).

Genetic Analysis

As predicted, the samples classified as freshest had the highest individual ID success rates. We predicted slightly lower success rates in the first session as we did not clear the sites

of fecal pellets prior to our first sampling session, and thus some pellets may have been more than 1 week old. In 2013 but not 2014, success rates increased in the later sessions. We don't believe this affects the population closure assumption as our pilot studies indicated low nDNA PCR success rates (2–28%) by day 14 and 0% by day 60 (Woodruff et al. 2015; S.P.W. unpublished data).

While we had moderate to high genotyping errors (average ADO = 12.1%, average FA = 3.4), we feel confident this did not result in misidentification because after conducting 3–8 replicates per sample and correcting for errors, all samples had ≤3 mismatches. In 2014, individual ID success rates were lower and genotyping error rates were higher, but we could not identify any field protocols or environmental conditions that changed. One possible explanation is a change in extraction chemistry from 2013 to 2014. All samples were extracted using the Qiagen QIAamp DNA stool mini kit (Qiagen Inc., Valencia, CA.), but Qiagen changed the composition of the kits in 2014 from an InhibitEX™ tablet to a liquid InhibitEX™ buffer.

In both years, samples from 2 sites (Devil Hills and Point of Pintas) made up ~50% of the failed samples and 47% and 28% of the samples with genotyping errors in 2013 and 2014. These sites are located on the typically hotter western side of the BMGR. Site-wide, maximum daily temperatures ranged from 33.6–44.4 °C and daily maximum temperatures on the BMGR-West during sampling averaged 2.5 °C higher than locations to the east. Increased UV radiation and high temperatures are known to elevate the rate of DNA degradation (Nsubuga *et al.* 2004; Piggott 2004; Murphy *et al.* 2007; DeMay *et al.* 2013), and this may explain the lower success rates at these western sites. Decreasing the length of time between sampling occasions would allow for collection of more fresh pellets and reduce pellet

exposure to environmental conditions (Woodruff et al. 2015) but would increase effort and pronghorn disturbance.

Population Abundance and Survival Estimation

The relatively large spatial range and low-density of Sonoran pronghorn limited us to a targeted sampling design conducted during the hot, dry months to maximize detection probability. Year-to-date rainfall when we began sampling was higher in 2013 (3.4 cm) compared to 2014 (1.1 cm). Weather and range conditions likely play a significant role in detection probabilities as drinker visitation declines in relatively cooler, wetter conditions when there is adequate natural forage. To account for the increasing probability of detection and the identification of more individuals in later sessions in 2013 (51 and 77 individuals in the first and third sessions, respectively), we began sampling later in the season in 2014 (6 June 2014 compared to 25 May 2013). In contrast, detection probabilities were highest in the first session in 2014, and we identified 83 and 76 individuals in the first and third sessions, respectively. We suspect when we initiated sampling in 2013, the full impacts of the summer drought had not been realized and drinker use was not yet at the maximum. Our inconsistent detection probabilities across sessions also provide explanation as to why the best models all included time variation.

Detection probabilities were substantially higher in our study (0.36–0.76) compared to other ungulate studies (0.01–0.38; Ebert *et al.* 2009, 2012a, b; Poole *et al.* 2011; Goode *et al.* 2014), and were noticeably higher (1.45 times) in males compared to females. Contrary to similar ungulate studies (Ebert *et al.* 2009, 2012a, b; Poole *et al.* 2011), we detected twice as many adult males as adult females at drinkers with male:female ratios of 2.1:1. We detected approximately the same numbers of males and females at non-drinker locations and ratios

were 1.8:1 in 2014 when including non-drinker locations. The higher number of males compared to females (ratio: 1.4:1–1.6:1) was unexpected. Prior to the 2003 population crash, females constituted approximately 60% of the population (Wright & deVos 1986; Hughes & Smith 1990), and in 2014 telemetry flight data indicated male:female ratios were approximately 0.66:1 (J. Hervert, personal communication).

Due to our targeted sampling design, any inference from our estimates applies largely to the individuals using the drinkers. Twice as many males as females have been released from the captive pen potentially leading to a male bias using the drinkers, as released animals, conditioned to being provided supplemental feed and water, may use the feed stations more readily (USFWS 2015). Additionally, home range size and movement rates are likely to differ between sexes (Ockenfels et al. 1994; Clemente et al. 1995) which could affect the use, and representation, of sexes at drinkers. These results suggest that our sampling method was better at sampling the male fraction of the population. In the future, any extrapolation to the entire population, especially pertaining to male:female ratios, would need to include a sex ratio correction factor.

Males also may be defecating more frequently than females. Male duikers (*Sylvicapra grimmia*) have been shown to defecate more frequently for marking territory (Lunt et al. 2007; Lunt & Mhlanga 2011), and Brashares and Arcese (1999a, b) suggest dominant male oribi (*Ourebia ourebi*) restrict defecation occurrence in order to increase territory-marking frequency. However, Irby (1981) determined no significant differences in defecation rates between sexes of American pronghorn (*Antilocapra americana*). Further work is needed to determine whether defecation rates vary between sexes around drinkers and what effect this may have on population estimates.

Detection probability for fawns was generally lower than adults, and fawns had 4 of the 6 lowest detection probabilities. Nonetheless, the highest detection probability (0.79) was for fawns in the first session of 2014, which also had the highest detection probability for adult females (0.57). Detection probability of fawns is correlated with that of adult females as young are likely traveling with their mother. Typically, NGS-CR studies delineate sex with no analysis of detection probability or abundance by age, unless additional data is collected from cameras or physical capture. Our study benefited from the ability to determine an individual's age from fecal pellet measurements (S.P.W., unpublished data) allowing for finer scale delineation of demographic parameters.

In our study, age class best explained survival probability with fawns having much lower survival probabilities than adults, as predicted. Ungulate survival is generally lowest in the first year (Zager & White 2003; DelGiudice et al. 2006), and survival can be less than 10% for neonates ≤4 months of age (Franzmann et al. 1980; Ballard et al. 1991; Zager & White 2003). We had low survival probability (0.41) for fawns, although 2013 was considered a good year with the highest recruitment documented since the population crash (J. Hervert, personal communication). While our survival estimates represent only a single year of annual survival probabilities, they provide a baseline for continuing monitoring of survival trends. They are also the first survival estimates since the 1980s when mean adult survival was estimated at 0.93 during a period of above-average rainfall (deVos & Miller 2005). A 1996 population viability analysis (PVA) indicated probability of persistence for this population was strongly affected by adult survival rates but suggested fawn survival rates were perhaps even more significant (Hosack et al. 2002), while a more recent PVA indicated precipitation was the most important factor in population persistence (J. Horne unpublished

data). Fawn survival is innately tied to precipitation (Bright & Hervert 2005), and total rainfall in 2013 (~25 cm) was the highest in the past decade averaging 19 cm (range: 6–25 cm) annually (http:// http://www.earthonly.com/ajo/weather/).

Given our high detection probabilities, our survival estimate is likely unbiased, yet the precision (CI: 0.69–0.92) is low due to our relatively small sample size. Adult survival probabilities in this population did not differ by sex. While exceptions do exist (Van Vuren & Bray 1986; Fancy et al. 1994; Toïgo et al. 1997), adult survival probability of males is generally reported to be lower than females for ungulates (Clutton-Brock et al. 1982; Gaillard et al. 1993; Owen-Smith 1993; Loison et al. 1999), particularly in polygynous species due to competition for breeding opportunities (see Clutton-Brock & Isvaran 2007). However, Loisin et al. (1999) suggest when food resources are abundant, there may not be sex differences in survival. While food resources in this population are not “abundant”, the provision of supplemental food and water during the pre-rutting season when resources are the most limited may mitigate any differences in adult survival probabilities.

Population estimates from drinkers were 60% (121, 95% CI: 112–132) of the December 2014 biennial aerial count (202, 95% CI: 171–334), which was conducted over 11 days when individuals were spread out across their range. However, our upper CI is 77% of the lower CI from the aerial count. Our minimum count was 110 individuals at drinkers compared to 168 individuals from the aerial survey. The disagreement in population estimates likely arises from a lower detection probability in the aerial survey. The trade-off in the wider geographic sampling of the aerial survey is lower precision in both deteciton probability and population estimation. These results indicate that a substantial portion of the population is sampled by targeted sampling at drinkers and could be useful for determining population

trends. A captive breeding program continues to release collared animals into the population annually, and measuring the ratio of collared animals at drinker and non-drinker locations could be used to adjust estimates of population trend determined by NGS-CR.

Management Implications

Sonoran pronghorn in the US are geographically and genetically distinct from other pronghorn in North America. The long-term persistence of the population continues to be tenuous (USFWS 2010; J. Horne, unpublished data). While NGS-CR methods are less common for population estimation of ungulates, our results indicate this methodology is a viable monitoring method for Sonoran pronghorn. Genotyping for individual identification provides minimum counts and the ability to incorporate capture-recapture methods for precise demographic estimates. As the population continues to expand, this method allows managers to monitor trends in abundance and survival as an indicator of the population’s trajectory, as opposed to aerial survey methods, which provide abundance estimates, but are costly and do not provide information on survival or other demographic parameters. Combining NGS-CR data with information garnered from radio-collared individuals, such as mortality and movement data, would greatly improve the understanding of the population’s viability. This method also presents opportunities for targeted sampling in other systems for broadly distributed species, such as in African savanna ecosystems, where zebra (*Equus quagga*), sable antelope (*Hippotragus niger*), waterbuck (*Kobus ellipsiprymnus),* and other species congregate at watering sites in the dry season (Cain et al. 2012).

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Table 4.1. Sonoran pronghorn fecal pellet samples collected and analyzed in 2013 and 2014 and genotyping error rates are provided per year and by freshness category.

Year	Freshness	# Collected	# Analyzed	Success[a] (%)	ADO[b] (%)	FA[c] (%)
2013	Total	730	634	75	9.5	2.2
	F1[d]	57	57	96	10.4	2.1
	F2	350	332	82	9.3	2.2
	F3	323	245	68	9.5	2.3
2014[e]	Total	1059	771	72	15.7	4.6
	F1	62	57	86	10.8	3.4
	F2	346	356	77	15.9	4.2
	F3	651	358	69	16.4	5.3

[a]Individual ID success rates

[b]ADO = allelic dropout

[c]FA = false allele

[d]Freshness category: F1 = most fresh, F2 = moderately fresh, F3 = least fresh.

[e]Includes samples from drinker and non-drinker locations

Table 4.2. Number of detections and number of unique individual Sonoran pronghorn identified in 2013 and 2014. Pronghorn sampled at nondrinker locations in 2014 shown in parentheses.

Year					
2013			Adult Male	Adult Female	Fawn
	#Detections	474			
	# Individuals	91	50	24	17
2014	#Detections	555			
	# Individuals	127	69 (8)	38 (1)	20 (3)

Table 4.3. Results of robust design models (Huggins' p and c) and closed capture models (full likelihood p and c, fawns 2013 and 2014 cohorts) estimating apparent survival (φ), detection (*p*), and redetection (*c*) probabilities, and abundance (N) of Sonoran pronghorn adults and fawns (φ is 2013 cohort only) from drinker locations. Fed is an individual covariate indicating whether the individual was detected at a site with supplemental feed or a site with no feed. The group variable consists of four groups representing single- and multi session adult males and adult females.

	Model	AIC_c[a]	ΔAIC_c	AIC_c weight	K[b]
Adult (N and φ)	φ, p (time + group)[c] = c (time + group)	2047.89	0	0.54	9
	φ (fed), p (time + group) = c (time + group)	2049.85	1.96	0.20	10
	φ (sex), p (time + group) = c (time + group)	2049.98	2.09	0.19	10
	φ (sex + fed), p (time + group) = c (time + group)	2051.96	4.07	0.07	11
Fawn (N)[d]	p (time) = c (time)	2.77	0	0.65	4
	p = c	3.99	1.21	0.35	2
Fawn (φ)[e]	φ, p = c	36.47	0	0.58	4
	φ, p (time) = c (time)	37.13	0.66	0.42	8

[a]AIC, Akaike Information Criteria

[b]K = number of parameters

[c]Time represents sessions within a year.

[d]2014 only shown. Model ranking and AIC_c was similar in 2013

[e]Drinker only as non-drinker locations were not sampled in 2014

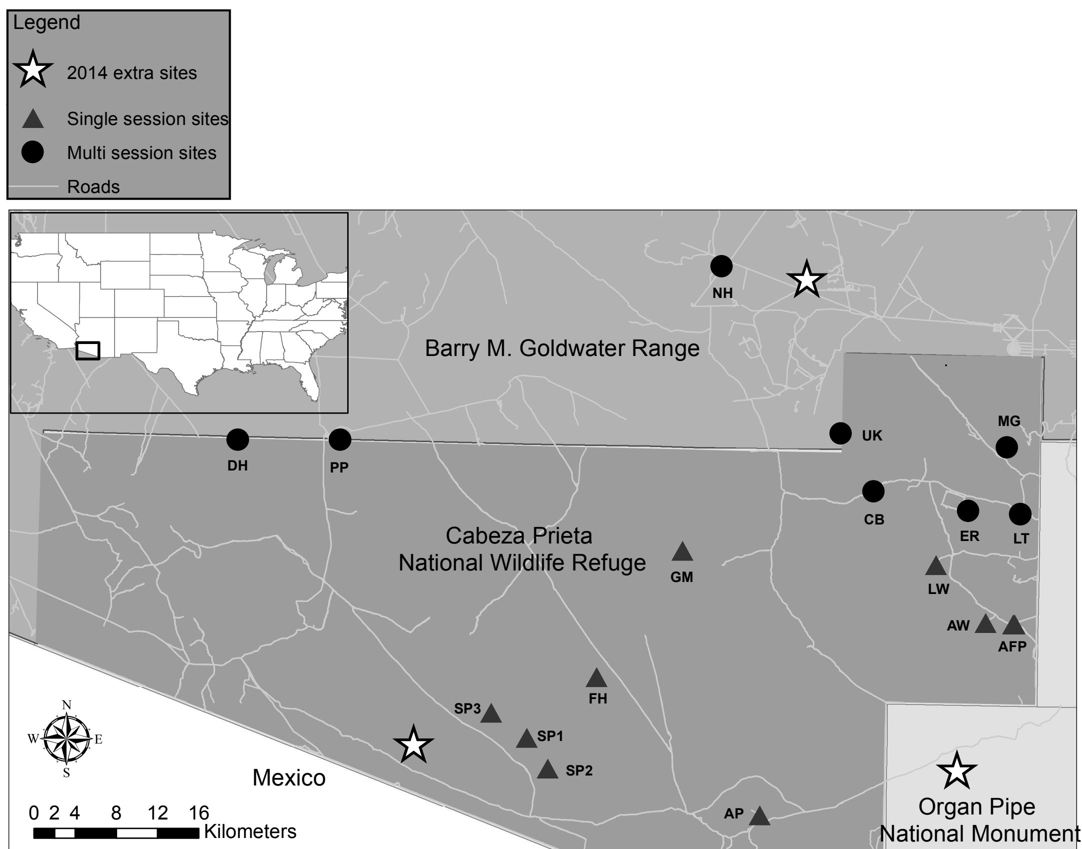

Figure 4.1. Map depicting Sonoran pronghorn fecal sampling locations (drinkers) in 2013 and 2014 on Barry M. Goldwater Range (BMGR–dark gray) and Cabeza Prieta National Wildlife Refuge (CPNWR–light gray), southern Arizona, USA. Circles represent drinkers sampled in multiple sessions, and triangles are drinkers which were sampled only a single time each year. Stars are non-drinker locations which were sampled only in 2014. The star on CPNWR was sampled a single time and the stars on BMGR and Organ Pipe National Monument represent approximate locations of multiple sampling occasions. Lines are roads. See Supporting Information for location names and abbreviations.

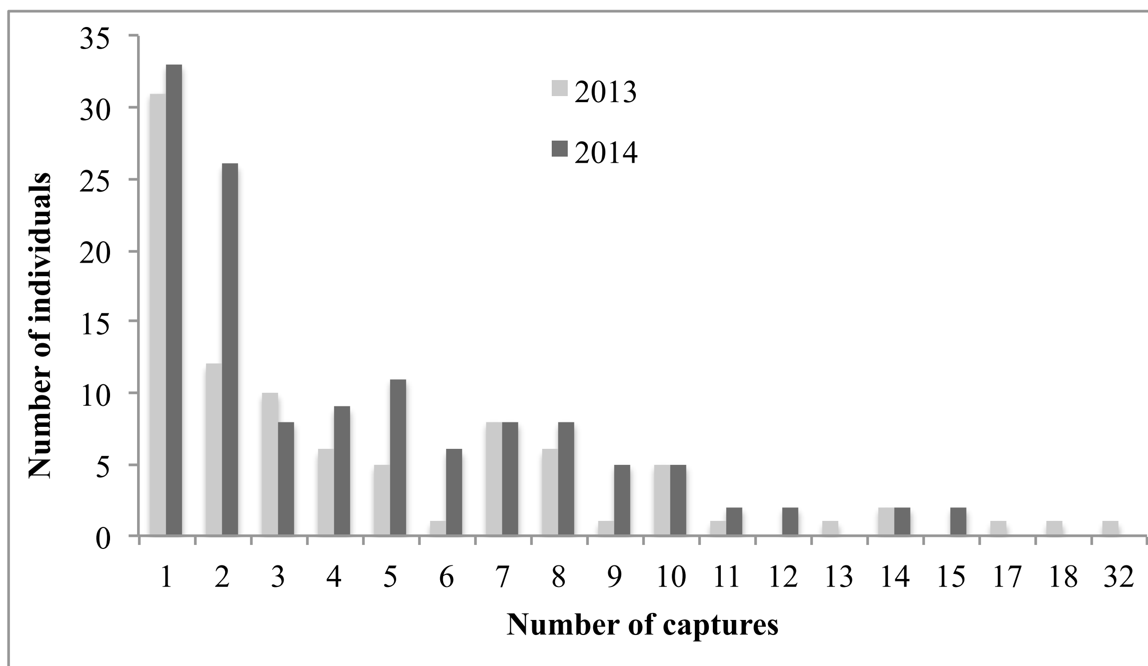

Figure 4.2. Capture distributions from noninvasive sampling data for Sonoran pronghorn in 2013 (light gray) and 2014 (dark gray). Note: many individuals were detected ≥1 time per session.

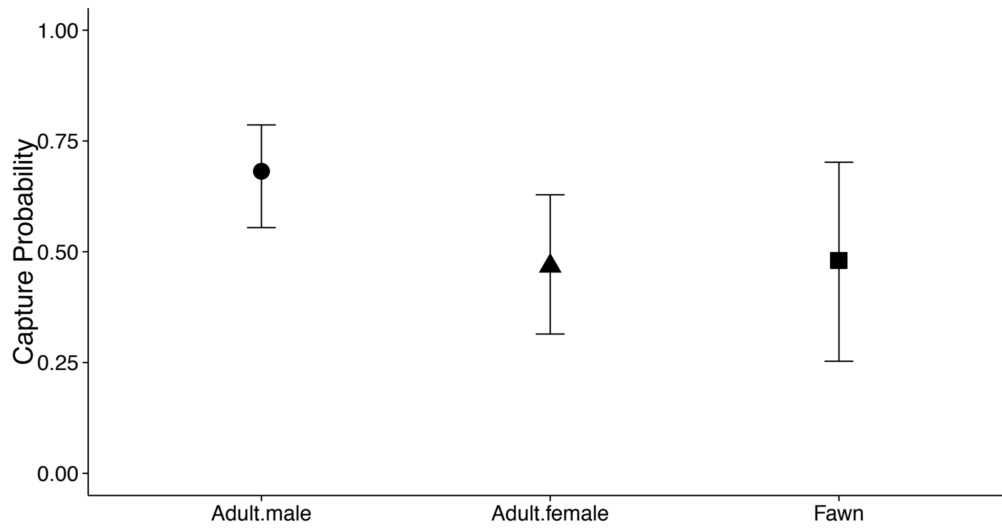

Figure 4.3. Average detection probability and 95% confidence intervals for adult males, adult females, and fawns from noninvasive sampling data for Sonoran pronghorn in 2013 and 2014.

4.6 Supporting Information

Genetic Laboratory Analysis

Subsampling method

We initially analyzed a subsample of approximately two times the number of individuals estimated to be using the drinker from multi-session sites and all samples from single-session sites. We first analyzed the freshest samples (F1s) (Lucchini *et al.* 2002) and then F2s. If we had too many F2s, we subsampled equally across quadrants choosing every other numbered sample. F3 samples were used only if necessary to reach target sample size and were subsampled across quadrants in the same manner. In 2013, we analyzed an additional 140 samples after determining that our minimum counts were less than 2 times the number of pronghorn observed on cameras at some drinkers.

Samples classified as freshest (F1) had the highest individual ID success of 96% and 86% and success dropped in less fresh samples to 82% and 77% for F2 and 68% and 69% for F3 at multi-session sites in 2013 and 2014, respectively (Tables 1, S3, S4). In 2013, individual ID success rates increased in later sessions (80%, 92%, 91%, 96% for sessions 1, 2, 3, and 4, respectively), but were generally consistent across sessions in 2014 (77%, 71%, and 74% for sessions 1, 2, and 3, respectively). Success rates were significantly different between sessions 1 and all other sessions in 2013, but there were no significant differences between sessions 2, 3, and 4 when compared to each other. In 2014, there was no difference in individual ID success rates between sessions (Table S4).

DNA extractions and analysis

DNA extraction followed methods described in Adams *et al.* (2011) and Woodruff, Johnson &Waits (2015) using the Qiagen QIAamp DNA stool mini kit (Qiagen

Inc., Valencia, CA.). For individual ID, ten nuclear DNA (nDNA) microsatellite loci previously used for pronghorn ranging in size from 90-278 base pairs (bp) (Lou 1998; Carling *et al.* 2003; Munguia-Vega *et al.* 2013) and one sex ID primer (Brinkman and Hundertmark 2009) were amplified in a single multiplex reaction. We chose these loci after generating microsatellite data from the population and evaluating observed and theoretical probability of identity statistics for unrelated individuals and siblings (see details below; Waits *et al*. 2001). The 7 μl PCR reaction contained 1x Qiagen Master Mix, 0.5x Qiagen Q-Solution, 1.71μM Anam97, 0.04μM Anam50, 0.07 μM Anam82, 0.01 μM Anam79, 0.86 μM Aam13, 0.43 μM Aam11, 0.14 μM ADCYC, 0.26 μM Aam10, 0.04 μM Aam1, 0.04 μM Aam2, 0.29 μM KY (sex ID), and 1.0 μl DNA extract. The PCR profile included an initial denaturation of 95 °C for 15 minutes, followed by a touchdown of 20 cycles with a 30 second denaturation at 94 °C, 90 second annealing step at 63 °C decreasing 0.5 °C each cycle, and a 60 second extension at 72 °C, followed by 34 cycles of a 30 second denaturation at 94 °C, a 90 second annealing step at 53 °C, and a 60 second extension at 72 °C. The cycle finished with a 30 minute final extension at 60 °C.

In 2014, Aam10 and Aam13 had significant spectral overlap when visualized in Genemapper making them difficult or impossible to call, and Anam79 had high allelic dropout of the longer alleles (209-215 bp) in the full multiplex, thus we ran them in a separate 3-primer multiplex. The 7 μl PCR reaction contained 1x Qiagen Master Mix, 0.5x Qiagen Q-Solution, 0.05 μM A106, 0.20 μM A12, and 0.01 μM Anam79, and 1.0 μl DNA extract and followed the same PCR profile as described above.

Samples with continued ambiguity post-matching were run in a second multiplex including six nDNA microsatellite loci previously used for pronghorn ranging in size from

113-304 base pairs (bp) (Carling *et al.* 2003; Munguia-Vega *et al.* 2013). The 7 μl PCR reaction contained 1x Qiagen Master Mix, 0.5x Qiagen Q-Solution, 0.01 μM Anam80, 0.11 μM Aam4, 0.03 μM Aam6, 0.07 μM Aam12, 0.09 μM Aam14, 0.21 μM Aam15, and 1.0 μl DNA extract. The PCR profile included an initial denaturation of 95° C for 15 minutes, followed by a touchdown of 15 cycles with a 30 second denaturation at 94 °C, 90 second annealing step at 63 °C decreasing 0.4 °C each cycle, and a 60 second extension at 72 °C, followed by 30 cycles of a 30 second denaturation at 94 °C, a 90 second annealing step at 57 °C, and a 60 second extension at 72 °C. The cycle finished with a 30 minute final extension at 60 °C.

A negative control and positive control were included in each PCR. PCR products were analyzed using an ABI 3130xl capillary machine (Applied Biosystems, Foster City, CA) and visualized in Genemapper 3.7 (Applied Biosystems, Foster City, CA). All samples that failed to amplify at ≥50% of loci were dropped from further analysis (Murphy *et al.* 2007).

All samples were screened initially with two replications to assess sample quality, and we dropped all samples failing to amplify at ≥50% loci (n = 5) from additional genotyping to remove low quality and error prone samples from the dataset. We determined consensus genotypes across three to eight repetitions of a sample at a minimum of seven loci. To confirm homozygotes, we required amplification of the allele at least three times, and for heterozygotes, each allele had to amplify at least two times. Consensus genotypes were determined in Microsoft Access (Skrbinsek 2010), and matching was conducted using the software GenAlEx 6.5 (Peakall & Smouse 2006).

The Probability of Identity (P_{ID}) is the probability that identical multilocus genotypes will be obtained for different individuals and is used to quantify the power of the loci in distinguishing individuals (Taberlet & Luikart 1999; Waits *et al.* 2001). However, to minimize the chance of underestimating the number of individuals in the population, we used the more conservative Probability of Identity Siblings (P_{ID} (sibs)) metric, defined as the probability that two full siblings drawn at random from a population share the same genotypes (Waits *et al.* 2001) and selected loci with a cumulative P_{ID} (sibs) ≤ 0.01. The observed P_{ID} (sibs) using all 10 loci was 3.7e-04, 6.2e-03 for the seven least powerful loci, and 9.8e-03 using the five most powerful loci (GenAlEx version 6.5, Peakall & Smouse 2006). Thus, be conservative, we required consensus genotypes at seven loci.

The average number of alleles per locus was 5.8 (range = 4–9; SD = 1.75) and mean expected heterozygosity of the 10 loci was 0.65 (range = 0.60–0.78; SD = 0.10).

Pronghorn are the primary ungulate species present at most drinkers. However, mule deer (*Odocoileus* hemionus) can visit the sites and similarities between pronghorn and deer pellets make it impossible to distinguish by visual inspection (Johnson & MacCracken 1978). Mule deer samples do not amplify at our selected microsatellite loci, so we performed mitochondrial DNA species ID on all failed samples (Woodruff *et al.* 2014) to discriminate between pronghorn and deer samples and calculate individual ID success rates for pronghorn samples only. Nine and 29 samples were mule deer in 2013 and 2014, respectively.

Table S4.1. Sampling design and dates for Sonoran pronghorn faecal pellet collection in 2013 and 2014

	2013			2014		
Location	Sess. 1	Sess. 2	Sess. 3 (4[a])	Sess. 4	Sess. 5	Sess. 6
East Release (ER), Morgart (MG)	May 25	May 31	June 5 (11)	June 9	June 16	June 23
Charlie Bell (CB)	May 31	June 6	June 11 (18)	June 6	June 13	June 20
Point of Pintas (PP), Devil Hills (DH)	June 3	June 10	June 17	June 3	June 12	June 18
Uken (UK), New Halliwill (NH)	May 31[b]	June 8	June 15[b]	June 15	June 22	June 28
Little Tule (LT)	May 31	June 6		June 7	June 20	
Adobe Well (AW), Adobe Forage Plot (AFP), Lower Well (LW)	May 30			June 14		
Granite Mountain (GM)	June 2			June 19		
Sierra Pintas (SP) 1, 2, 3	June 5			June 10		
Fawn Hills (FH)	June 11			June 24		
Antelope Parabolic (AP)	June 11			June 20		
3 Jack (3J)				June 8	June 15	June 28

[a]Day of Session 4 sampling which was combined with session 3 for capture-recapture input

[b]UK only. NH sampled only once in 2013 due to access limitations on Barry M. Goldwater Range

Table S4.2. Age group, freshness classification, and number of Sonoran pronghorn faecal samples collected at single and multi-session drinkers in 2013 and 2014 and non-drinker locations in 2014.

	Multi-session sites (Single-session sites)				
	Adults	Fawns	F1	F2	F3
2013	635 (35)	58 (2)	55 (2)	343 (7)	295 (28)
2014 (drinker)	787 (36)	211 (0)	54 (4)	335 (9)	609 (23)
2014 (non drinker)	24	1	4	2	19

F1 = freshest; F2 = less fresh; F3 = oldest

Table S4.3. Breakdown of faecal DNA individual ID success by year, single or multi-session, and freshness and sampling session. F1, F2, and F3 are freshness categories with F1 being the freshest and sessions are labeled S1-S4.

	Percent Success (n = # of samples)			
	Multi		Single	
	2013 (n)	2014	2013	2014
F1	96 (55)	86 (43)	100 (2)	100(9)
F2	82 (258)	77 (337)	50 (5)	69 (16)
F3	68 (158)	69 (275)	73 (15)	68 (59)
S1	80 (114)	77 (212)	61 (17)	75 (77)
S2	92 (124)	71 (124)	---	---
S3	91 (126)	74 (148)	---	---
S4	96 (45)	NA	---	---

Table S4.4. Individual ID success chi-square and p-values for pellet freshness and sampling session for Sonoran pronghorn faecal pellets. Asterisks represent a statistically significant result at p-value of 0.05. There was not a session 4 in 2014 which is represented by NA.

	2013		2014	
	χ^2	p	χ^2	p
F1 to F2	6.26	2.21e-06*	1.86	0.17
F2 to F2	12.67	3.30e-04*	4.99	0.03*
F1 to F3	16.54	4.76e-05*	5.75	0.02*
ALL	15.61	0.001*	2.26	0.32
S1 to S2	7.34	0.007*	1.88	.02*
S1 to S3	6.66	0.01*	0.58	0.45
S1 to S4	5.53	0.02*	NA	NA
S2 to S3	0.00	1.00	.023	0.63
S2 to S4		0.50[a]	NA	NA
S3 to S4		0.57[a]	NA	NA

[a]Odds ratio from Fisher's Exact Test

Table S4.5. Results of robust design models (Huggins' p and c) and closed capture models (full likelihood p and c, fawns 2013 and 2014 cohorts) estimating apparent survival (φ), detection (*p*), and redetection (*c*) probabilities, and abundance (N) of Sonoran pronghorn adults and fawns (φ is 2013 cohort only) from all locations. Fed is an individual covariate indicating whether the individual was detected at a site with supplemental feed or a site with no feed.

	Model	AIC_c[a]	ΔAIC_c	AIC_c weight	K[b]
Adult (N and φ)	φ, p (time + group)[c] = c (time + group)	6333.61	0	0.65	9
	φ (fed), p (time + group) = c (time + group)	6334.99	1.37	0.25	10
	φ (sex), p (time + group) = c (time + group)	6335.65	2.03	0.18	10
	φ (sex + fed), p (time + group) = c (time + group)	6337.03	3.42	0.09	11
Fawn (N)[d]	p (time) = c (time)	-8.22	0	0.98	4
	p = c	-0.58	7.64	.0.02	2
Fawn (φ)[e]	φ, p = c	36.47	0	0.58	4
	φ, p (time) = c (time)	37.13	0.66	0.42	8

[a]AIC, Akaike Information Criteria
[b]K = number of parameters
[c]Time represents sessions within a year.
[d]2014 only shown. Model ranking and AIC_c was similar in 2013
[e]Drinker only as non-drinker locations were not sampled in 2014

CHAPTER 5-A COMPARISON OF NONINVASIVE CAPTURE-RECAPTURE METHODS FOR ABUNDANCE ESTIMATION OF SONORAN PRONGHORN

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Abstract

Effective demographic monitoring is a central objective in endangered species management, yet low-density and sensitivity to disturbance can make sampling difficult. For some species, like Sonoran pronghorn (*Antilocapra americana sonoriensis*), noninvasive genetic sampling is a viable alternative to physical capture. Our first objective was determining the optimal sampling design for monitoring Sonoran pronghorn using noninvasive genetic sampling combined with capture-recapture. Second, we applied subsampling methods and the single-session *capwire* estimator to data previously analyzed in closed capture models. Last, we performed a cost-comparison between these and current aerial survey monitoring methods. We simulated encounter histories for true abundance of 100–300 individuals, a sample size of 0.33–3.33 samples/individual/session, in 1–3 sampling sessions. We explored trade-offs between sample size, number of sessions, and multi-session (CMR) versus single-session (*capwire*) closed capture-recapture abundance estimators, and the need for an accurate and precise estimate. In simulations, abundance was biased positively in *capwire* and negatively CMR. Bias increased with fewer samples/individual/session. Our empirical data had increased precision with more sessions. Our simulation results indicate our empirical estimates are reliable. We recommend collecting 1.5–2 samples/individual/session in ≥2 sessions and the use of a multi-session model, such as CMR. Cost per individual monitored in 2014 was ~$184 USD for NGS-CR methods and $599 USD for aerial

sightability methods. However, our results indicate that at the current estimated abundance (~200), the same level of precision (aerial CV ~ 21%) can be obtained using NGS-CR methods for ~$5800, or an annual cost savings of over $4000.

5.1 Introduction

Population monitoring of threatened or endangered species is necessary to verify recovery status and monitoring allows managers to document population growth and detect population declines. Capture-recapture modeling is one of the most commonly used approaches for estimating abundance and typically involves capturing individuals, marking them with a unique ID, and releasing them back into the population (Otis et al., 1978; Pollock et al., 1990; Williams et al., 2002; Amstrup et al., 2005). Individuals that are recaptured in a later session are identified from their unique ID, and using the recapture data, abundance and other demographic parameters can be estimated. Traditional methods of physical capture inherently involve risk and the potential for injury or mortality for both the animals and the researchers. Noninvasive genetic sampling combined with capture-recapture methods (NGS-CR) eliminate much of that risk, provide reliable population estimates, and can improve cost efficiency over traditional methods (e.g., Solberg et al., 2006; DeBarba et al., 2010*b*; Stansbury et al., 2014). Even with noninvasive approaches, obtaining adequate sample sizes and sufficient recapture rates can be difficult or costly with species that occur in low numbers or in low densities.

Effective sampling design for NGS-CR is crucial for accurate estimates. Abundance estimates generated from small sample sizes are often biased and are subject to low precision (Robson and Regier, 1964), and thus conducting sampling at a time and location that maximizes the probability of capture (or detection in NGS studies which do not physically

capture individuals) improves the chance of success. Accuracy and precision can be affected by multiple factors including number of sampling sessions and sample size, failure of poor quality samples, and analysis of a reduced number of samples (subsampling) (Burnham et al., 1987; Lukacs and Burnham, 2005; Settlage et al., 2008; Laufenberg et al., 2013). By evaluating potential biases related to specific capture-recapture methods and implementing a case-specific study design, researchers can attain the desired level of accuracy and precision.

In this study, we use capture-recapture analysis and simulation modeling to evaluate the sampling design of noninvasive genetic sampling methods for monitoring Sonoran pronghorn (*Antilocapra americana sonoriensis*) abundance on the Cabeza Prieta National Wildlife Refuge (CPNWR) and adjoining Barry M. Goldwater Range (BMGR). Endemic to the Sonoran Desert of Arizona, United States (US) and Mexico, Sonoran pronghorn are federally listed as “endangered” under the Endangered Species Act (USFWS, 2015) and the Mexican population is classified as “most endangered” under CITES Appendix 1 (Hoffman et al., 2008). Abundance is currently estimated from biennial aerial counts corrected with sightability models (Bright et al., 1999; USFWS, 2015). The US population has increased from an estimated 21 (95% CI: 18–33) individuals in 2002 to 202 (95% CI: 171–334) in 2014 (USFWS, 2015).

The overall goal of this study was to determine via simulation the optimal sample size (consensus genotypes obtained from fecal samples) required to yield precise abundance estimates and to evaluate the reliability of single and multi-session abundance estimators. Using data simulated with varying sampling intensity, we evaluated the differences in abundance estimates and associated precision using closed capture (CMR) models implemented in Program MARK (White and Burnham, 1999) and single-session capture-

recapture models in program *capwire* (Miller et al., 2005). Additionally, we applied subsampling techniques to a fecal genotyping data set collected from the Sonoran pronghorn population in Arizona, USA and estimated abundance using both capture-recapture estimators. Last, we performed a cost comparison between NGS-CR methods compared to traditional population estimation methods currently used for this species (i.e., aerial survey with sightability correction).

5.2 Methods

This study included simulated data as well as a previously analyzed data set from a closed population fecal DNA study of Sonoran pronghorn conducted on the Cabeza Prieta National Wildlife Refuge (CPNWR) and Barry M. Goldwater Range (BMGR), in southwestern Arizona, USA. We provide relevant details of the previously collected data, but see Woodruff et al. (In review) for a complete description of the study. Sampling design followed the assumptions of a closed population, and within each of two years, 2013 and 2014, we collected pronghorn fecal samples at 11 drinkers (developed water sites) during 1–3 sampling sessions per drinker.

Simulations

Using R (R Core Team 2015), we created simulations of closed populations emulating a noninvasive genetic sampling framework to estimate the optimal number of consensus genotypes needed for precise abundance estimates (CV≤10–20%; Pollock, 1990). Because we assumed all samples achieved a consensus genotype in simulations, number of samples collected refers to number of successful genotypes obtained (hereafter, samples). Approximately 75% of fecal samples achieved consensus genotypes and thus, actual number of samples collected would be approximately 33% higher. We used true population sizes of

100, 150, 200, 250, and 300 with a male:female ratio of 0.66:1 which reflects actual sex ratios in our study area (J. Hervert, Personal Communication). Previous research indicated deposition rates averaged one pellet pile per pronghorn per day in the sampling area (Woodruff et al., 2015); however, results of our empirical data indicated 2.5–3 times more male than female samples at the drinkers (Woodruff et al., In review) suggesting males have higher deposition rates than females. Thus, for 1–3 sessions we simulated mean deposition over a 7-day period with males having twice the mean deposition rate of females. We simulated sampling every seven days as, per current agency procedures, each drinker is visited approximately every seven days for restocking of supplemental feed. Per session, we randomly selected 50–600 samples with replacement, allowing individuals to be sampled more than once in a sampling session (i.e., 0–x times, where x is the total number of depositions by an individual; Table S5.1). Individuals with higher deposition rates were set to have a higher probability of being sampled. Our design reflected a consensus genotype rate of 0.33 to 3.33 times (per session) the number of individuals in the true population (i.e., 50–500 samples per session for 150 individuals). Not all ratios (e.g., 0.33 or 3.33 times) or number of sampling sessions were employed for every sampling design (Table S5.1).

Abundance Estimators

In multi-session models, populations are sampled during multiple sessions at distinct time points or within discrete sampling sessions (Chao, 2001). Although there may be multiple captures of the same individual within a sampling session, only a single capture per individual per sampling session is counted. In contrast, *capwire* uses the total number of captures for each individual and allows capture of an individual multiple times within sessions (Miller et al., 2005). Based on our previous analysis (Woodruff et al., In Review), we used a

closed capture (hereafter, CMR) design where capture probability (*p*) varied by group (sex) in MARK using the package RMark (Laake, 2013) in R (R Core Team, 2015) to build the multi-session model. The CMR model was a model with full likelihood parameterization with estimable parameters for capture and recapture probability as well as abundance. We implemented single-session sampling using the package *capwire* in R (Miller et al., 2005; Pennell et al., 2013). A likelihood-ratio test in *capwire* chooses between the two available models: the even capturability model (ECM) which assumes individuals have an equal chance of being captured and the two innate-rates model (TIRM) which models two mixtures of capturability (a lower and a higher rate) which is comparable to heterogeneity models. The individual identification represents the animal's mark, yet all captures in all sessions are included creating a capture distribution. For every true abundance in each sampling design, we simulated 100 encounter history inputs for estimating abundance and 95% confidence intervals using multi-session CMR and single-session estimators (hereafter, *capwire*). Simulations in *capwire* included one, two, and three sessions, and two and three sessions in CMR.

Abundance estimates and confidence intervals were averaged over the 100 simulations. We evaluated the performance of each sampling design and estimator by comparing the simulated abundance estimates to the true population size (percent bias), the coefficient of variation (CV), and the relative mean squared error (RMSE). The CV is commonly used to describe the precision of an abundance estimate. As a general rule, CV <10% is ideal, however, <20% indicates a precise estimate (White et al., 1982; Pollock, 1990). Thus, determining an acceptable level of precision is dependent on the question being asked (e.g., What is the harvest limit? Or how much is this population growing or shrinking

[discussed in Mowat, 2013]). CV does not, however, measure model fit, as CV could be very small with a poorly fit model. RMSE incorporates accuracy (bias) and precision (variance) and low values indicate a good balance between bias and precision. When RMSE values are >0.5, CI coverage is generally poor. Additionally we examined the 95% confidence interval (CI) coverage described as the proportion of times (out of 100 in this case) the true value was contained within the interval.

Empirical Dataset

As part of our monitoring research (Woodruff et al., In Review), we extracted and genotyped 494 and 692 fecal samples in 2013 and 2014, respectively. In 2013, we later included an additional 138 samples (hereafter, extra samples) from sessions 2 and 3 to investigate changes in abundance estimates and precision with a larger sample size. DNA analysis resulted in 474 and 476 captures of 91 and 110 individuals in 2013 and 2014, respectively. Seventy-five captures and three individuals were from extra samples in 2013. Capture-recapture analyses were performed using a CMR model (Otis et al. 1978) in Program MARK. CV was ~2% in both years indicating the potential to save time and money by reducing sampling effort (e.g., fewer sessions, fewer samples) while still producing reliable population estimates.

Abundance Estimation and Comparison of Methods

To compare sampling design and estimators, we estimated abundance using multiple approaches. First we obtained an abundance estimate using captures from all sessions using both CMR and *capwire*. We applied the same methods as described above and developed models *a priori* in Program MARK. All CMR models assumed equal capture (p) and recapture (c) probability (i.e., no effect of behavior) as initial capture is unlikely to affect

recapture in our study design. Because capture/recapture probability varied by sex and age, abundance was estimated separately for adult males, adult females, and fawns. Additionally we had five sites which were sampled only a single time. We used mean capture probability by sex from sites sampled three times (i.e., mean of males' capture probability at sites sampled three times equals capture probability for males at sites sampled once) to estimate capture probability at these sites and recapture probabilities were fixed at zero. Population estimates were summed (males, females, fawns), and we calculated 95% confidence intervals using the Delta method (Seber, 1982). To evaluate relative support for each model, we used Akaike's Information Criteria (AIC_c) corrected for small sample size. In *capwire*, the likelihood ratio indicated the TIRM model was the appropriate model in all cases, and thus we did not estimate sex and age classes separately as *capwire* inherently accounts for variation in capture probability when using the TIRM model (Miller et al., 2005). Next, we stratified the data by session and obtained population estimates for each session individually (*capwire* only), as well as combinations of sessions 1 and 2, and sessions 2 and 3, to evaluate reducing the number of sessions. Last, we compared abundance estimates using only the initially extracted samples with estimates which included the extra samples (2013 only). To evaluate the precision of each sampling design and estimator, we calculated the CV.

Cost Comparison

We evaluated the efficiency of sampling methods by calculating cost per successful sample (NGS-CR) and cost per individual monitored (minimum count) in both traditional aerial methods and NGS-CR methods. Cost per successful sample was calculated as total cost of analyzing all collected samples divided by number of successful samples. This is a more valuable measure than cost per sample because failed samples add to the cost but do not

contribute to the data. Costs included supplies for sample collection, DNA extraction and analysis, and associated labor for field and laboratory work. Because rates vary between field and laboratory personnel, labor rates were based on an average ($25.00/hour). For NGS-CR methods, the labor estimate included laboratory time and time spent collecting samples and recording them in a database. The time does not include conducting analysis for abundance and/or survival estimates. We also did not include travel time to the drinkers because management personnel visit drinkers for other management tasks with the same frequency (~ every 7 days) as our NGS-CR sampling design. The number of individuals monitored was the number of unique individuals identified in 2013 (n = 91) and 2014 (n = 110) during genotyping. For comparison, we divided cost of the biennial flight USFWS (2015) into an annual cost. This cost includes flight time and pilot salary, but does not include salary of personnel conducting the counts or salary for personnel performing analysis of sightability models. The number of individuals monitored for traditional methods was based on minimum counts during the biennial aerial count in 2012 (n = 108) and 2014 (n = 168) conducted over ~10 days.

Cost of aerial flights changes little to none with an increase in population size. NGS-CR methods, on the other hand, generally increase with an increase in population size and the need to collect more samples. Using our simulation results we determined what level of sampling effort (i.e., sample size and number of sessions) would produce a CV equivalent to that from the aerial methods (CV = ~ 21%) at a true abundance equal to the 2014 aerial survey estimate (202, 95% CI: 171–334). We also determined at what point there was a change in cost effectiveness from one method to the other. All costs are reported in US dollars ($).

5.3 Results

Simulations

We ran 126 and 83 *capwire* and CMR simulations, respectively (Table S5.1). The estimators performed differently depending on true abundance and sampling intensity (Fig. 1 and Tables 1 and S5.1). Abundance was consistently positively biased in *capwire* (average 10.5%) and negatively biased in CMR (average 5.2%), and abundance estimates between the estimators differed from 1–33%. The CV was <10% in 100% of CMR simulations and in 94% of *capwire* simulations (Tables 1 and S5.1). Three *capwire* simulations had CV>20% which all had sampling rates of ≤0.75 samples/individual/session.

Discrepancies between true and estimated abundance (bias) were larger with higher population sizes and with fewer samples per individual. Bias ranged from overestimating by 60 individuals (*capwire*: two sessions, 300 individuals, 200 samples/session) to underestimating by 25 individuals (CMR: two sessions, 300 individuals, 150 samples/session). With a single session of sampling (*capwire* only), percent bias increased with increasing true abundance. However, with true abundance of ≤150 individuals, 3 samples/individual/session produced bias of ~5%.

When sampling <2 samples/individual/session and estimating by sex (CMR only), males were consistently overestimated and females were underestimated; however, with ≥2 samples/individual/session, males were estimated correctly and females accounted for the overall underestimation. CI width ranged from 0–117% of the true abundance, and CMR had narrower CI width than *capwire* in all but one case. CMR indicated higher capture probabilities (p) for males (mean: 0.75, CI: 0.67–0.83) compared to females (mean: 0.63, CI:

0.57–0.69) but when fewer samples were collected per individual, capture probabilities were more similar between sexes.

CI coverage was poor with only 6% (n = 8) of *capwire* simulations having ≥0.90 probability of the CI containing the true abundance and the highest probability for CI coverage in CMR was 0.56. High capture probabilities led to extremely precise estimates which often missed the true abundance by 1 or 2 individuals (i.e., true abundance = 150, estimate = 149, CI: 149–149). However, 47% (n = 39) of CMR and 24% (n = 30) of *capwire* estimates were within 4% of the true abundance (e.g., 96 or 104 for true abundance of 100). Both estimators had percent bias ≤5% with at least ~1.5 samples/individual/session. With three sessions in *capwire*, capture of an individual twice in every session ensured ≥90% CI coverage in almost all cases. With two *capwire* sessions, ≥90% CI coverage was achieved only with 2.2–2.7 samples/individual/session. And for a single *capwire* session, probability of CI coverage was very poor averaging 0.16 (range: 0.00–0.73). Increasing sample size (relative to number of sessions and number of individuals) generally led to an improvement in bias and RMSE values for both estimators, but not necessarily to an improvement in CI coverage.

Empirical Data

The top model was the same for all CMR models and included equal capture (p) and recapture (c) probabilities both varying by group and time. As in simulations, the likelihood ratio indicated the TIRM model was the appropriate model in *capwire*. Across all estimates, CMR and *capwire* gave similar population estimates varying by only 1–8%, and confidence intervals substantially overlapped (Table 2). *Capwire* estimates were higher in most cases. Captures per individual (*capwire*) ranged from 2.22–5.38 and average capture probabilities in CMR were 0.42–0.71 and 0.71–0.83 for females and males, respectively (Table S5.2).

When including all three sessions, *capwire* was slightly less precise (CV ~ 3.1%) than CMR (CV range: 1.5–2.3%). With only two sessions, *capwire* abundance estimates were higher overall and had lower CV (range: 3.7–5.2%) compared to CMR (CV range: 7.8–11.1%). In 2013, single session estimates increased from 73 (CI: 62–88), to 84 (72–121), to 93 (CI: 81–107) in subsequent sessions. In 2014, estimates were 114 (CI: 103–134), 113 (CI: 100–154), and 132 (CI: 123–182) in sessions 1, 2, and 3. CV was lowest in session 3 (CV = 7.1%) in 2013 and in session 1 (CV = 6.7%) in 2014.

The extra samples (2013 only) resulted in additional captures of 37 individuals including three individuals not previously identified. One "extra" individual was detected in session 2 only and two were caught in session 3 only. Population estimates changed only slightly with the inclusion of the extra samples (Table 2). The *capwire* estimates from session 3 only decreased 9.7%, while all other estimates stayed the same or marginally increased (2–6.5%) for both methods. Most estimates had lower CV, but the CV in all sessions in *capwire* increased negligibly from 3.1% to 3.2%.

Cost Comparison

We collected 730 and 980 and analyzed 634 fecal samples and 692 fecal samples in 2013 and 2014, respectively. NGS-CR costs totaled \$18 512 and \$20 271 in 2013 and 2014 (Table 3). Cost per successful sample was approximately \$40 in both 2013 (n = 474) and 2014 (n = 502). Cost per individual monitored using NGS-CR was \$203.43 in 2013 (n = 91) and \$184.29 in 2014 (n = 110). The cost of the aerial survey is \$10 000 annually (i.e., half of the cost of the biennial count; USFWS, 2015; Table 3). Cost per individually monitored pronghorn using traditional aerial methods was \$92.59 in 2012 (n = 108) and \$59.52 in 2014 when more individuals were monitored (n = 168).

If we compare NGS-CR cost to obtain a CV of ~21% (CV of aerial estimates) using simulations, and a true abundance of 200, we required 0.75 samples/individual (confirmed consensus genotypes) in a single sampling session. Total cost for NGS-CR with this sampling design would be $5829 and cost per individual monitored (n = 121 [mean number of individuals sampled with this sampling design]) would be $47.58 (Table 4). If we match the cost of NGS-CR to the annual aerial monitoring expenditure ($10 000) for a true abundance of 200 individuals, we could obtain ~250–300 samples/individual obtained over 2 sampling sessions, or ~0.75 samples/individual/session. Cost per individual monitored (n = 160–166) would be $60.69 to 71.96. Using CMR models, CV would be < 5% and RMSE would improve over a single session and would likely be better than RMSE from aerial estimates as well. With a larger population of 250–300 individuals and expenditure of ~$10 000, it is still possible to maintain better precision (CV ~ 4%) compared to aerial methods (CV ~ 21%) by again obtaining ~250–300 samples/individual over 2 sampling sessions, or ~0.5 samples/individual/session. By collecting fewer samples, number of individuals monitored (minimum count using NGS-CR) declined. The cost of NGS became more expensive than aerial methods when improving RMSE to ≤0.5 which was possible only with population size ≤100.

5.4 Discussion

Using an abundance estimator that provides the most accurate and precise estimate is particularly important when managing endangered species. For example a highly precise over-estimate of the population would confidently give the impression that the species is more abundant and could result in the implementation of improper management actions (Noss et al., 2012). Our study illustrates the need to use the appropriate sampling design and estimator in

capture-recapture studies, and the inherent trade-off between the accuracy and precision of an abundance estimate and the effort and cost of monitoring. In this research, the estimators produced analogous estimates with overlapping confidence intervals using the empirical data and performed similarly well in terms of precision. In simulations, however, the multi-session CMR generally performed better than the single-session *capwire* estimator indicating a clear optimal estimation method. Our results also illustrate an increase in bias and reduced precision when reducing the sample size (i.e., fewer sampling sessions or fewer samples). In our empirical data, fewer individuals were identified and population estimates were generally lower with fewer samples and/or sessions. While focused on only a portion of the population (i.e., those using the drinkers) compared to the traditional aerial method, the NGS-CR method provided much greater precision (1.5%–12.3%, depending on the estimator, vs. 21%), at a lower cost, and has the potential to provide additional information on population genetic metrics and survival of different sex and age classes (Woodruff et al. In Review). Additionally, abundance estimates obtained using the NGS-CR method could easily be extrapolated to the entire population if the proportion of individuals not using the drinker were known or could be reliably estimated.

Simulations

Information garnered from simulations can be used to design an efficient sampling scheme, but a limitation of simulations is the inability to exactly mimic field situations. To ensure our simulations mirrored field conditions as closely as possible, we used estimated deposition rates based on previous work (Woodruff et al., 2015) to design our simulations. True capture probabilities were exceptionally high (0.36–0.76) in our study, and in simulations capture probabilities were 0.29–1.00. Lukacs and Burnham (2005) noted CI

coverage deteriorated with very high capture probability, although bias was negligible. Similarly, our results showed very poor CI coverage with only 7% (n = 8) of simulations having CI coverage ≥90%, but very low bias (≤4%) when capture probabilities were high (≥0.58). While *capwire* does not provide capture probability, captures per individual is a reasonable surrogate. Miller et al. (2005) suggests 2.5–3.0 captures per individual are sufficient for abundance estimates within 10–15% of true abundance. In a study using targeted sampling (fecal and hair samples) of gray wolves at rendezvous sites, Stenglein et al. (2010) found >1.7 captures per individual provided precise estimates matching those from radio telemetry data. Our simulation results matched suggestions by Miller et al. (2005), and ≥3 captures per individual resulted in bias ≤5%.

Our simulations indicated highly precise estimates due to very low CVs (mean CV *capwire* = 0.5; CMR = 0.2). However, this was deceptive, as low CVs can still have high bias (Arnason et al., 1991). For example in one simulation with a true abundance of 300 and CV of 2–3%, percent bias was >8% for both estimators, CI coverage was low (*capwire*: 0.01; CMR: 0.19), and RMSE was high (*capwire*: 8.71; CMR: 1.92; Fig. 1 and Tables 1 and S5.1). RMSE was a better representation of estimator performance, as percent bias was nearly always ≤5% for both estimators with RMSE values ≤0.5. Similar to other research (Miller et al., 2005; Conn et al., 2006; Stenglein et al., 2010; Rees et al., 2014; Roy et al., 2014), increasing the sample size and number of sessions improved both the bias and precision of the estimates.

Because *capwire* is designed for use with small population size (< 100; Miller et al., 2005), it is not surprising that as true abundance increased, the performance of *capwire* weakened. Given the input for our simulations (mean male deposition rates twice that of females), using the TIRM in *capwire* makes intuitive sense, and was supported by likelihood

ratio results. Nevertheless, nearly 100% of *capwire* estimates were high. This is common for the TIRM model when capture probabilities are equal (Miller et al. 2005); however, that was not the case in our dataset. In the presence of heterogeneity, models which assume equal capture probability (e.g., Lincoln-Peterson, ECM in *capwire*) have been shown to underestimate populations (Seber, 1982; Miller et al., 2005; Petit and Valière, 2006; Puechemaille and Petit, 2007). To evaluate bias under this model, we ran several simulations post factum using the even capturability model (data not shown). Although percent CI coverage did improve in some cases, as expected, abundance was always underestimated with these models. We also experienced situations in *capwire* when the lower CI was higher than the estimate, which is indicative of the model not capturing the distribution of the data sufficiently (M. Pennell, Personal Communication).

Empirical Data

Given the results of our simulations, we believe our empirical data estimates from *capwire* are positively biased and our CMR estimates are negatively biased. However, given our high capture probabilities (*capwire:* 2.22–5.28; CMR: 0.42–0.83, Table S5.2) we expect our estimates deviate from the true abundance by only a few individuals (Lukacs and Burnham, 2005). Capture probabilities indicate the male fraction of the population is better estimated than females. We suspect we detect nearly every pronghorn using the drinkers hence the narrow confidence intervals and high precision (Table 2). As in simulations, our results showed increased bias (positive bias) and decreased precision with reduced sample size. However, there is a limit to the benefits garnered from increased sample size. When considering increased cost, the minimal increase in precision from the addition of 138 extra samples in 2013 was not worthwhile.

In 2013, we suspect we started sampling too early in the season before all pronghorn were using the drinker (Woodruff et al., In Review), perhaps explaining the generally increasing estimates in later sessions. We began sampling slightly later in 2014, and estimates from reduced sessions mirror those from all sessions with overlapping confidence intervals lending support to the idea of sampling in fewer sessions, although we note the resulting decrease in accuracy and precision.

Other studies comparing the performance of these estimators on a variety of species have had conflicting results (American black bears (*Ursus americanus*), Robinson et al., 2009; argali (*Ovis ammon*), Harris et al., 2010; Asian elephant (*Elephas maximus*), Gray et al. 2011; Eurasian otter (*Lutra lutra*), Lampa et al., 2015). *Capwire* estimates were lower compared to CMR in some studies (Robinson et al., 2009; Harris et al., 2010), whereas in this research and others (Gray et al., 2011, 2014; Lampa et al., 2015), MARK produced lower abundance estimates than *capwire*. By design, discrete-time models ignore multiple recaptures in a single sampling interval which can result in decreased precision (Lukacs et al., 2007). Results from Robinson et al. (2009) and Harris et al. (2010) supported this notion with c*apwire* estimates being more precise than CMR. Our study and other research (Gray et al., 2011), found the opposite with CMR estimates (mean CV: 0.06) being more precise than *capwire* (mean CV: 0.10). Only when we reduced the number of sessions included did CMR have lower precision (mean CV: 0.07) compared to *capwire* (mean: 0.04). We acknowledge that collecting samples during multiple sessions and collapsing them into a continuous-occasion model is a less preferred method, but when there are not enough recaptures for CMR to effectively estimate abundance, the method proves useful (see Robinson et al., 2009). We

suggest that if data are collected on multiple sessions, a discrete-time model allows better inference to the population.

Cost Comparison

Determining the appropriate monitoring method depends on the data needed for management (e.g., abundance, survival, genetic diversity), yet resources are often limited, and effective management should employ efficient monitoring methods ensuring the costs do not outweigh the benefits (Possingham et al., 1993). An often-voiced concern of NGS-CR methods is the high cost. High costs can be associated with development of primers, and optimizing multiplexes (Schwartz and Monfort, 2008; Beja-Pereira et al., 2009), travel to sampling sites (Harris et al., 2010), collection method (e.g., scat detection dogs; Arandjelovic et al., 2015), or increased use of personnel (Poole et al., 2011). While developing a new fecal DNA study can require a substantial initial investment, costs are reduced in subsequent years. Flight costs associated with estimating abundance using sightability methods are unchanged across a wide variety of population sizes, yet costs for NGS-CR methods increase with increasing population size.

Other studies have found NGS-CR methods were more cost-effective compared to traditional methods (Solberg et al., 2006; DeBarba et al., 2010*b*). In 2014, our results show NGS-CR methods were twice as expensive overall (~$20 000 vs. $10 000) and three times ($184.29) as expensive as traditional aerial methods ($59.52) per individual monitored. Still, a direct comparison of costs is difficult given the different tasks and information acquired with each method.

Our NGS-CR simulations indicate only a marginal increase in estimator performance with more samples, and depending on desired accuracy and precision and desired expenditure,

a wide range of sampling designs is feasible. We predicted that obtaining a consensus genotype twice the number of individuals estimated to be using the drinker per session would provide the best estimates. If sampling 150 individuals at this rate, the cost per session would be $11 513 ($28.78 * 400 samples accounting for 75% genotyping success). However, with population size ≤200, a ratio of 1.3–1.5 consensus genotypes/individual/session produced RMSE values ≤0.5 in CMR with two sampling sessions. With 150 individuals, this translates to a cost savings of ~20–50%, or $3800–7600 in total cost. Additionally, if population size is <150 individuals, our results suggest a single session provides adequate estimates (RMSE <0.5) with at least 3 consensus genotypes/individual/session, saving time and money in travel to the sampling location.

Our results also indicate that at the current estimated population size (~200), the same level of precision (aerial CV ~ 21%) can be obtained using NGS-CR methods for ~$5800, or an annual cost savings of more than $4000. Other cost-saving measures include collecting and analyzing only the freshest samples (Lucchini et al., 2002) or decreasing the sampling interval with multiple sampling sessions (Marucco et al., 2009; Woodruff et al., 2015) to for higher success rates (i.e., fewer failed samples).

Compared to traditional methods, NGS-CR methods allow for assessment of an increased number of demographic parameters. For example, use of a model, such as Pollock's Robust Design (Pollock, 1982; Pollock et al., 1990; Kendall et al., 1997) generates abundance and survival estimates with a single analysis and thus little extra cost incurred. Currently in this population, traditional methods are not used to generate survival estimates, which could provide crucial knowledge for management. Survival estimates can be determined using NGS-CR methods (Woodruff et al., In Review) for little to no extra cost or through analysis

of otherwise marked (e.g., radio collared) individuals. This would incur additional costs through capture operations as well as personnel time and salary for monitoring and data analysis.

Traditional survey methods may be undesirable due to concerns for human safety, impacts to wildlife and other natural resources, and logistical complexity. Additionally, traditional methods lack the ability to provide information on genetic diversity, relatedness, and genetic structure which can provide valuable information on risk of inbreeding depression and population connectivity. This type of genetic monitoring would incur additional analysis time and labor cost, but depending on questions being asked could be conducted annually (e.g., for parentage analysis; see DeBarba et al. 2010*a*), but more typically only once per generation (see Schwartz et al., 2007 and Stetz et al., 2011 for recommendations on designing a genetic monitoring program).

Conclusions

This system presents a challenge for researchers given very high capture probabilities leading to results which indicate high levels of precision, yet the estimates still exhibit bias. To our knowledge, our capture probabilities (0.36–0.76) are up to twice as high as other published capture probabilities for ungulates (0.38 [SE = 0.047], Poole et al., 2014) and some of the highest reported in any capture-recapture study. In turn, evaluating performance of NGS-CR methods in this system provides important information for its continued use. Our research provides a guideline for designing a practical and cost-effective NGS-CR monitoring strategy to obtain acceptable levels of accuracy and precision. As this population continues to grow, this method will require the collection of more samples. If increasing the number of visits to sampling locations is more expensive, more samples could be collected in fewer

sessions. Although fewer sampling sessions or fewer samples do not provide the same precision, a minimum population size estimate is generated when using the CMR estimator, and managers may deem this level of accuracy acceptable. We do note, however, that collecting samples in multiple sessions could increase the number of unique individuals identified, as drinker visitation may not be consistent between individuals across time. In the future, when capture probabilities are high, we recommend ensuring 1.5–2 consensus genotypes/individual/session in at least two sessions and the use of a discrete-time model, such as CMR in Program MARK, to maintain RMSE ≤0.5. This method can easily be adapted for use in areas where animals congregate, such as wintering areas, roosting sites, or along migration routes. Additionally, this method could be integrated with an occupancy approach to inexpensively document population expansion to new geographic areas. However, researchers should be aware that capture probabilities are rarely this high. In other systems, substantially more effort would likely be needed to obtain this level of precision.

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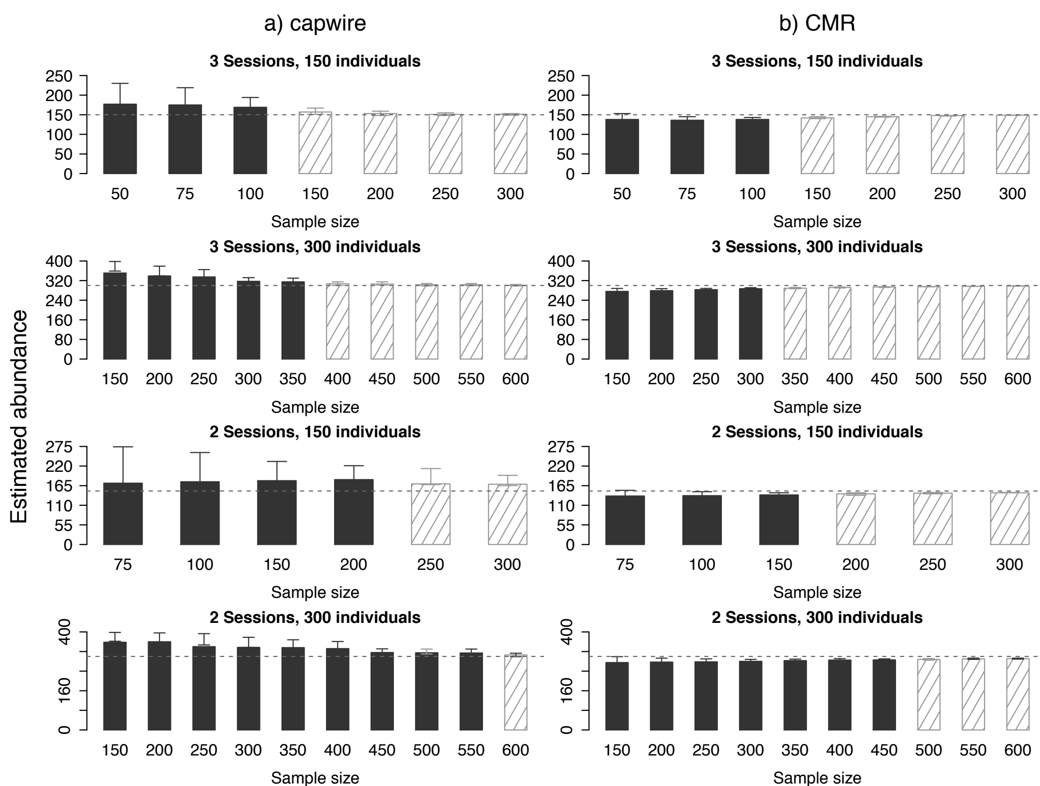

Fig 5.1. Abundance estimates from simulations for Sonoran pronghorn with true abundance 150 and 300 individuals in two and three sessions for both a) single session models in *capwire* and b) multi session closed capture (CMR) models. Solid color indicates relative mean squared error (RMSE) >0.5, and hashed represents RMSE ≤0.5. Not all results are shown, but trends were the same in all simulations. See Supporting information Table S5.1 for complete results.

Table 5.1. Results from some of the simulations performed with *capwire* (Cap) and discrete-time closed capture (CMR) models using true abundance (TA) of 150 and 300 individuals for 2 and 3 sampling sessions (Sess) and varying number of samples per session (SPS). Coefficient of variation (CV), relative mean squared error (RMSE) and percent confidence interval (CI) coverage is shown for each estimator. Note that with 300 individuals and 150 samples, *capwire* produced a lower CI that was higher than the population estimate (N). See Supporting information Table S5.1 for complete results.

Sess	TA	SPS	N (95% CI)		CV		CI		RMSE	
			Cap	CMR	Cap	CMR	Cap	CMR	Cap	CMR
2	300	150	358 (362–398)	275 (252–299)	0.03	0.04	0.00	0.48	11.38	2.03
		300	337 (338–378)	280 (271–288)	0.03	0.01	0.01	0.14	4.42	1.01
		600	306 (299–313)	291 (289–293)	0.01	0.004	0.62	0.03	0.13	0.25
3	300	150	351 (359–398)	276 (264–288)	0.01	0.02	0.01	0.19	8.71	1.92
		300	317 (310–332)	287 (283–291)	0.02	0.007	0.05	0.03	0.98	0.58
		600	301 (298–304)	298 (298–298)	0.01	8.9e-07	0.94	0.08	0.004	0.02
2	150	75	181(172–238)	136 (110–152)	0.09	0.06	0.14	0.50	6.28	13.34
		150	169 (164–194)	139 (133–145)	0.04	0.02	0.10	0.03	2.31	0.80
		300	153 (148–159)	145 (144–146)	0.02	0.003	0.74	0.15	0.07	0.14
		450	149 (149–152)	149 (149–149)	0.004	2.9e-07	0.98	0.50	7.7e-04	0.007
3	150	75	175 (169–219)	136 (128–145)	0.07	0.03	0.25	0.29	4.17	1.24
		150	157 (151–167)	142 (140–145)	0.03	0.009	0.45	0.08	0.33	0.39
		300	151 (149–153)	149 (149–149)	0.01	7.3e-07	0.97	0.36	0.01	0.009

Table 5.2. Abundance estimate comparison from *capwire* and closed capture (CMR) capture-recapture analysis of Sonoran pronghorn from noninvasively collected fecal DNA samples. Number of individuals identified (IID) and percent coefficient of variation (CV) shown for comparison. Sessions 2 and 3 (separately and combined) also include individuals from locations sampled only a single time.

		2013			2013 and extra			2014		
Estimator		IID	N (95% CI)	CV	IID	N (95% CI)	CV	IID	N (95% CI)	CV
capwire	1[a]	51	73 (62–88)	9.1	51	73 (62–88)	9.1	83	114 (103–134)	6.7
	2	54	84 (74–121)	14.3	58	84 (73–100)	8.2	73	113 (100–154)	12.2
	3	68	93 (81–107)	7.1	70	84 (76–95)	5.8	77	132 (123–182)	11.4
capwire	1 & 2	67	83 (70–87)	5.2	68	85 (71–88)	5.1	100	120 (106–125)	4.0
CMR	1 & 2	67	77 (63–93)	10.1	68	79 (64–98)	11.1	100	117 (100–138)	8.4
capwire	2 & 3	80	97 (84–100)	4.2	84	97 (84–98)	3.7	97	120 (106–128)	4.7
CMR	2 & 3	80	93 (77–112)	9.6	84	99 (82–119)	9.5	97	111 (95–129)	7.8
capwire	ALL	88	100 (88–103)	3.1	91	103 (91–104)	3.2	110	126 (111–127)	3.2
CMR	ALL	88	98 (93–102)	2.3	91	104 (100–108)	2.0	110	117 (114–121)	1.5

[a] There were no extra samples in session 1

Table 5.3. Average cost (in US dollars) per sampling method in 2012, 2013, and 2014 for Sonoran pronghorn abundance estimation in Arizona, USA. The aerial count is conducted biennially (2012 and 2014) and represents annual cost. NGS-CR methods were conducted in 2013 and 2014. NGS-CR totals represent an annual cost. Neither cost estimate includes time spent generating populations estimates in respective software.

Method	Samples collected (analyzed)	# Individuals monitored	Item/Task	($) Cost	Cost per individual monitored
Traditional					
2012	NA	108	Total	10 000	138.89
2014	NA	168	Total	10 000	89.29
NGS-CR					
2013 and 2014			Sample collection	0.15[a]	
2013 and 2014			DNA extraction	5.65	
2013 and 2014			Species ID PCR	0.55[b]	
2013 and 2014			Individual ID PCR	9.45[c]	
2013 and 2014			Salary	12.98[d]	
2013	730 (634)	91	Total	18 512.09	204.43
2014	980 (692)	110	Total	20 271.59	184.29

[a] Includes envelope to store sample, tape, pens, silica desiccant

[b] Included for all samples for comparison to other studies, yet only samples not achieving consensus genotypes were run in Species ID

[c] Includes 6 repetitions (average number needed to obtain genotype) of microsatellite multiplex and corresponding analysis on ABI

[d] Includes time for sample collection and recording sample in database, DNA extraction and analysis, PCR set up and analysis for species and individual ID. It does not include salary associated with travel time to and from sampling location because personnel are already traveling to sampling locations.

Table 5.4. A comparison of costs (USD) and bias and precision (CV and RMSE) using noninvasive genetic sampling capture-recapture methods vs. aerial sightability methods. Aerial sightability numbers based on 2014. Cost per sample (USD $) is $28.78 (see Table 3 for what is included in cost), and total cost includes cost of successful (achieving consensus genotypes) and failed samples. A multi-session closed model was used except where noted. Number of samples (SS) is number of consensus genotypes and represents 75% of number of samples collected (SC) to account for DNA degradation. Ind. is mean number of individuals with consensus genotypes in 100 simulations per sampling design and is equivalent to minimum count.

Abun[1]	SC[2]	SS	Ind.	Total $	Cost/ind	CV (%)	RMSE
Unk[2]	----	----	168	10 000	59.52	21	----
200[3]	200	150	121	5829	47.58	21	6.48
200	350	263	166	10 074	60.69	5	2.19
200	400	300	160	11 513	71.96	3	1.21
250	350	263	153	10 074	65.85	1.7	1.38
250	400	300	192	11 513	59.97	1.3	0.75

[1]True abundance

[2]Population estimate was 202, 95% CI 171–334

[3]*capwire* in a single session

5.6 Supporting information

Table S5.1. Simulations in a) capwire and b) CMR for true abundance (Abun) 100–300. # Sessions (Sess) is total number of sampling sessions, # samples per session (SPS) assuming all samples achieve a consensus genotype, and total is total number of samples needed with 75% PCR success. LCI and UCI are lower and upper 95% confidence intervals and SE is standard error. Other measures include confidence interval coverage (CI cov), coefficient of variation (CV), and relative mean squared error (RMSE).

Sess	Abun	SPS	Total	N hat	LCI	UCI	SE	CV	CI cov	RMSE
3	300	150	450	351	359.14	398.12	9.94324	0.028	0.01	8.71
3	300	200	600	339	339.44	378.59	9.98769	0.029	0.00	5.05
3	300	250	750	335	330.01	364.52	8.80376	0.026	0.02	4.06
3	300	300	900	317	310.13	332.32	5.66173	0.018	0.05	0.98
3	300	350	1050	315	309.77	329.83	5.11767	0.016	0.04	0.71
3	300	400	1200	307	300.34	315.11	3.76901	0.012	0.32	0.18
3	300	450	1350	306	302.05	314.80	3.25191	0.011	0.33	0.14
3	300	500	1500	303	297.58	307.82	2.61224	0.009	0.82	0.03
3	300	550	1650	303	298.96	307.54	2.19050	0.007	0.73	0.03
3	300	600	1800	301	297.93	304.20	1.59943	0.005	0.94	0.00
3	250	83	249	300	298.82	370.18	18.2035	0.061	0.02	9.92
3	250	125	375	296	290.00	354.00	16.3265	0.055	0.00	8.46
3	250	200	600	279	273.00	306.00	8.41837	0.030	0.03	3.36
3	250	250	750	264	257.00	278.00	5.35714	0.020	0.15	0.78
3	250	300	900	262	257.00	276.00	4.84694	0.018	0.09	0.58
3	250	350	1050	255	249.00	262.00	3.31633	0.013	0.58	0.10
3	250	400	1200	254	250.00	262.00	3.06122	0.012	0.49	0.06
3	250	450	1350	252	248.00	256.00	2.04082	0.008	0.83	0.02
3	250	500	1500	251	248.00	253.00	1.27551	0.005	0.95	0.00
3	200	67	201	237	235.16	303.76	17.4982	0.074	0.07	6.95
3	200	100	300	231	228.00	283.00	14.0306	0.061	0.02	4.81
3	200	150	450	222	216.00	246.00	7.65306	0.034	0.03	2.42
3	200	200	600	211	204.00	223.00	4.84694	0.023	0.27	0.61
3	200	250	750	209	202.00	222.00	5.10204	0.024	0.42	0.41
3	200	300	900	204	200.00	211.00	2.80612	0.014	0.56	0.08
3	200	350	1050	201	198.00	205.00	1.78571	0.009	0.90	0.01
3	200	400	1200	201	199.00	203.00	1.02041	0.005	0.92	0.01
3	150	50	150	177	169.00	230.00	15.5612	0.088	0.17	4.86
3	150	75	225	175	169.00	219.00	12.7551	0.073	0.25	4.17

3	150	100	300	169	164.00	194.00	7.65306	0.045	0.06	2.41
3	150	150	450	157	151.00	167.00	4.08163	0.026	0.45	0.33
3	150	200	600	153	148.00	159.00	2.80612	0.018	0.75	0.06
3	150	250	750	151	148.00	155.00	1.78571	0.012	0.88	0.01
3	150	300	900	151	149.00	153.00	1.02041	0.007	0.97	0.01
3	100	50	150	117	110.00	146.00	9.18367	0.078	0.26	2.89
3	100	75	225	111	106.00	127.00	5.35714	0.048	0.20	1.21
3	100	100	300	106	101.00	114.00	3.31633	0.031	0.50	0.36
3	100	150	450	102	99.00	106.00	1.78571	0.018	0.83	0.04
3	100	200	600	100	99.00	102.00	0.76531	0.008	0.87	0.00
2	300	150	300	358	362.12	398.11	9.18170	0.026	0.00	11.38
2	300	200	400	360	356.30	395.56	10.0162	0.028	0.03	12.12
2	300	250	500	340	346.54	393.49	11.9765	0.035	0.00	5.42
2	300	300	600	337	337.48	377.51	10.2125	0.030	0.01	4.60
2	300	350	700	336	332.04	368.04	9.18431	0.027	0.00	4.42
2	300	400	800	332	318.95	361.13	10.7617	0.032	0.07	3.45
2	300	450	900	316	309.08	330.91	5.56811	0.018	0.13	0.88
2	300	500	1000	315	309.89	330.39	5.23017	0.017	0.09	0.77
2	300	550	1100	314	309.88	330.48	3.61295	0.012	0.11	0.69
2	300	600	1200	306	299.05	313.21	3.61295	0.012	0.62	0.13
2	250	83	166	291	283.77	376.69	23.7054	0.082	0.23	6.57
2	250	125	250	285	262.00	376.00	29.0816	0.102	0.47	4.90
2	250	200	400	295	290.00	376.00	21.9387	0.074	0.10	8.10
2	250	250	500	279	276.10	312.06	9.17423	0.033	0.02	3.27
2	250	300	600	279	273.97	304.92	7.89515	0.028	0.02	3.31
2	250	350	700	264	256.39	289.70	8.49617	0.032	0.17	0.79
2	250	400	800	263	256.91	275.74	4.80191	0.018	0.08	0.65
2	250	450	900	262	257.40	276.75	4.93386	0.019	0.10	0.61
2	250	500	1000	256	249.23	263.21	3.56601	0.014	0.64	0.15
2	250	550	1100	251	248.96	252.67	0.94579	0.004	0.97	0.00
2	200	67	134	237	235.16	303.76	17.4982	0.074	0.08	6.95
2	200	100	200	229	209.00	334.00	31.8877	0.139	0.47	4.21
2	200	150	300	231	225.00	318.00	23.7244	0.103	0.22	4.81
2	200	200	400	224	221.01	252.86	8.12666	0.036	0.03	2.96
2	200	250	500	223	217.39	245.77	7.23980	0.032	0.08	2.59
2	200	300	600	210	204.09	223.23	4.88278	0.023	0.23	0.53
2	200	350	700	210	204.49	221.33	4.29515	0.020	0.19	0.49
2	200	400	800	204	198.00	211.00	3.31633	0.016	0.74	0.08
2	200	450	900	204	200.16	209.78	2.45427	0.012	0.53	0.06

2	200	500	1000	200	199.55	200.64	0.27819	0.001	0.91	0.00
2	150	75	150	181	172.38	237.73	16.6697	0.092	0.14	6.28
2	150	100	200	181	173.83	221.94	12.2735	0.068	0.10	6.54
2	150	150	300	169	164.36	193.55	7.44726	0.044	0.10	2.31
2	150	200	400	164	154.29	181.18	6.86027	0.042	0.38	1.33
2	150	250	500	158	151.86	167.16	3.90179	0.025	0.37	0.42
2	150	300	600	153	148.17	159.06	2.77864	0.018	0.74	0.07
2	150	350	700	153	149.75	158.27	2.17181	0.014	0.59	0.06
2	150	400	800	151	148.50	153.77	1.34407	0.009	0.93	0.01
2	150	450	900	150	148.85	152.04	0.81343	0.005	0.84	0.00
2	150	500	1000	150	149.39	151.72	0.59394	0.004	0.98	0.00
2	100	50	100	119	99.00	216.00	29.8469	0.251	0.63	3.61
2	100	100	200	121	113.00	163.00	12.7551	0.105	0.24	4.41
2	100	150	300	118	109.00	148.00	9.94898	0.084	0.26	3.24
2	100	200	400	102	97.94	106.61	2.21333	0.022	0.83	0.03
2	100	250	500	101	98.38	103.58	1.32557	0.013	0.93	0.00
2	100	300	600	104	99.00	113.00	3.57143	0.034	0.68	0.16
1	300	150	150	343	320.99	397.27	19.4580	0.057	0.33	6.16
1	300	200	200	350	344.80	397.22	13.3742	0.038	0.13	8.33
1	300	250	250	349	348.00	397.00	12.5000	0.036	0.07	8.00
1	300	300	300	360	363.00	399.00	9.18367	0.026	0.01	12.00
1	300	350	350	364	363.00	398.00	8.92857	0.025	0.01	13.65
1	300	400	400	359	355.00	397.00	10.7142	0.030	0.03	11.60
1	300	450	450	347	349.00	397.00	12.2449	0.035	0.02	7.36
1	300	500	500	339	346.00	392.00	11.7346	0.035	0.00	5.07
1	300	550	550	341	342.00	385.00	10.9693	0.032	0.00	5.60
1	300	600	600	340	339.71	379.16	10.0636	0.030	0.00	5.31
1	250	125	125	286	263.00	378.00	29.3367	0.103	0.39	5.18
1	250	200	200	298	298.00	379.00	20.6632	0.069	0.02	9.22
1	250	250	250	300	300.00	372.00	18.3673	0.061	0.01	10.00
1	250	300	300	303	300.00	364.00	16.3265	0.054	0.03	11.24
1	250	350	350	297	291.00	349.00	14.7959	0.050	0.04	8.84
1	250	400	400	285	291.00	352.00	15.5612	0.055	0.02	4.90
1	250	450	450	282	283.00	327.00	11.2244	0.040	0.01	4.10
1	250	500	500	282	279.00	318.00	9.94898	0.035	0.02	4.10
1	250	600	600	279	274.00	308.00	8.67347	0.031	0.02	3.36
1	250	750	750	264	257.00	278.00	5.35714	0.020	0.21	0.78
1	200	100	100	226	205.00	330.00	31.8877	0.141	0.49	3.38

1	200	150	150	236	227.00	324.00	24.7449	0.211	0.18	6.48
1	200	200	200	239	232.00	305.00	18.6224	0.162	0.09	7.61
1	200	250	250	240	235.00	289.00	13.7755	0.124	0.02	8.00
1	200	300	300	230	226.00	281.00	14.0306	0.134	0.08	4.50
1	200	350	350	226	225.00	271.00	11.7346	0.052	0.01	3.38
1	200	400	400	225	222.00	255.00	8.41837	0.042	0.05	3.13
1	200	500	500	223	216.00	245.00	7.39796	0.033	0.11	2.65
1	200	600	600	212	205.00	225.00	5.10204	0.033	0.18	0.72
1	150	75	75	176	150.00	281.00	33.4183	0.190	0.61	4.51
1	150	150	150	176	170.44	230.89	15.4193	0.098	0.35	4.51
1	150	200	200	179	170.35	217.94	12.1386	0.068	0.12	5.67
1	150	250	250	167	165.00	212.00	11.9898	0.072	0.08	1.93
1	150	300	300	170	165.00	193.00	7.14286	0.042	0.11	2.67
1	150	350	350	167	162.00	187.00	6.37755	0.038	0.10	1.93
1	150	400	400	166	157.00	186.00	7.39796	0.045	0.29	1.71
1	150	450	450	158	151.00	169.00	4.59184	0.029	0.36	0.43
1	100	50	50	111	91.00	197.00	27.0408	0.244	0.73	1.21
1	100	100	100	119	111.00	159.00	12.2449	0.103	0.24	3.61
1	100	150	150	117	110.00	148.00	9.69388	0.083	0.30	2.89
1	100	200	200	115	111.00	136.00	6.37755	0.055	0.11	2.25
1	100	250	250	111	103.00	125.00	5.61224	0.051	0.38	1.21
1	100	300	300	105	100.00	114.00	3.57143	0.034	0.51	0.25

b)

Sess	Abun	SPS	Total	N hat	LCI	UCI	SE	CV	CI cov	RMSE
3	300	150	450	276	264.06	287.95	6.09572	0.022	0.19	1.920
3	300	200	600	279	271.44	286.69	3.88853	0.014	0.07	1.461
3	300	250	750	283	277.55	287.91	2.64060	0.009	0.03	0.994
3	300	300	900	287	283.14	290.52	1.88342	0.007	0.03	0.578
3	300	350	1050	289	286.56	291.65	1.29739	0.004	0.02	0.396
3	300	400	1200	292	290.14	293.85	0.94745	0.003	0.03	0.214
3	300	450	1350	294	292.37	294.85	0.63380	0.002	0.02	0.138
3	300	500	1500	295	295.16	295.66	0.12651	0.000	0.02	0.070
3	300	550	1650	297	296.91	296.92	0.00086	0.000	0.03	0.033
3	300	600	1800	298	297.77	297.77	0.00027	0.000	0.08	0.017
3	250	83	249	229	210.44	248.44	9.69254	0.042	0.4	1.691
3	250	125	375	232	221.00	243.00	5.61224	0.024	0.29	1.296
3	250	200	600	235	229.76	239.96	2.60299	0.011	0.07	0.917
3	250	250	750	239	235.22	241.79	1.67596	0.007	0.06	0.528
3	250	300	900	241	239.14	243.56	1.12863	0.005	0.04	0.299

3	250	350	1050	244	242.29	245.23	0.74958	0.003	0.05	0.156
3	250	400	1200	246	245.31	245.97	0.16896	0.001	0.06	0.077
3	250	450	1350	247	246.93	246.93	0.00047	0.000	0.03	0.037
3	250	500	1500	248	248.35	248.35	0.00021	0.000	0.23	0.011
3	200	67	201	180	164.00	196.47	8.28497	0.046	0.39	1.953
3	200	100	300	185	175.20	194.97	5.04321	0.027	0.32	1.112
3	200	150	450	187	182.06	192.39	2.63475	0.014	0.08	0.816
3	200	200	600	191	187.85	193.84	1.52808	0.008	0.1	0.419
3	200	250	750	193	191.55	195.03	0.88704	0.005	0.06	0.225
3	200	300	900	195	195.02	196.02	0.25690	0.001	0.00	0.102
3	200	350	1050	197	197.18	197.19	0.00039	0.000	0.12	0.039
3	200	400	1200	199	198.61	198.61	0.00018	0.000	0.24	0.010
3	150	50	150	138	123.26	153.42	7.69365	0.056	0.53	0.906
3	150	75	225	136	128.12	144.64	4.21436	0.031	0.29	1.237
3	150	100	300	138	133.02	143.47	2.66593	0.019	0.13	0.921
3	150	150	450	142	139.87	144.76	1.24678	0.009	0.08	0.394
3	150	200	600	145	144.08	146.10	0.51591	0.004	0.07	0.161
3	150	250	750	148	147.48	147.48	0.00023	0.000	0.09	0.041
3	150	300	900	149	148.85	148.85	0.00011	0.000	0.36	0.009
3	100	50	150	91	84.16	97.62	3.43272	0.038	0.36	0.830
3	100	75	225	93	89.01	96.13	1.81533	0.020	0.19	0.552
3	100	100	300	95	92.95	97.12	1.06257	0.011	0.22	0.246
3	100	150	450	97	97.43	97.51	0.02061	0.000	0.08	0.064
3	100	200	600	99	99.20	99.20	0.00007	0.000	0.46	0.007
2	300	150	300	275	251.86	298.84	11.98688	0.044	0.48	2.025
2	300	200	400	277	260.39	292.82	8.27331	0.030	0.33	1.824
2	300	250	500	278	266.72	289.90	5.91335	0.021	0.21	1.568
2	300	300	600	280	271.20	288.46	4.40154	0.016	0.14	1.356
2	300	350	700	283	275.77	289.39	3.47547	0.012	0.14	1.011
2	300	400	800	285	280.13	290.69	2.69399	0.009	0.08	0.709
2	300	450	900	286	282.19	290.21	2.04478	0.007	0.07	0.635
2	300	500	1000	288	284.89	291.22	1.61590	0.006	0.03	0.476
2	300	550	1100	290	287.60	292.73	1.30770	0.005	0.05	0.322
2	300	600	1200	291	289.38	293.45	1.03602	0.004	0.03	0.249
2	250	83	166	230	193.79	266.40	18.52152	0.080	0.49	1.585
2	250	125	250	226	204.97	246.28	10.53704	0.047	0.33	2.376
2	250	200	400	232	220.97	243.76	5.81432	0.025	0.36	1.244
2	250	250	500	231	223.66	239.21	3.96774	0.017	0.13	1.379

2	250	300	600	236	230.40	242.23	3.01704	0.013	0.19	0.749
2	250	350	700	237	232.77	241.28	2.17157	0.009	0.07	0.673
2	250	400	800	239	236.00	242.35	1.61924	0.007	0.08	0.468
2	250	450	900	241	239.07	243.79	1.20305	0.005	0.06	0.294
2	250	500	1000	243	241.04	244.47	0.87512	0.004	0	0.210
2	200	67	134	182	150.18	213.86	16.24498	0.089	0.56	1.617
2	200	100	200	179	160.93	197.22	9.25756	0.052	0.4	2.189
2	200	150	300	184	173.38	195.54	5.65130	0.031	0.42	1.208
2	200	200	400	185	178.14	191.81	3.48766	0.019	0.2	1.129
2	200	250	500	189	183.75	193.48	2.48124	0.013	0.14	0.648
2	200	300	600	191	187.66	194.19	1.66581	0.009	0.12	0.412
2	200	350	700	192	190.11	194.40	1.09567	0.006	0.1	0.300
2	200	400	800	194	192.73	195.56	0.72154	0.004	0.09	0.174
2	200	450	900	195	194.83	195.84	0.25628	0.001	0.1	0.108
2	150	75	150	136	119.24	152.37	8.45365	0.062	0.5	1.343
2	150	100	200	137	125.80	147.93	5.64650	0.041	0.54	1.150
2	150	150	300	139	132.96	145.18	3.11833	0.022	0.3	0.797
2	150	200	400	142	138.27	145.49	1.84110	0.013	0.27	0.440
2	150	250	500	144	141.57	145.71	1.05506	0.007	0.23	0.272
2	150	300	600	145	144.61	146.40	0.45583	0.003	0.15	0.137
2	150	350	700	147	146.76	146.96	0.05029	0.000	0.14	0.063
2	150	400	800	148	148.27	148.27	0.00022	0.000	0.22	0.020
2	150	450	900	149	148.61	148.61	0.00008	0.000	0.25	0.013
2	150	500	1000	149	149.31	149.31	0.00004	0.000	0.5	0.007
2	100	50	100	90	76.72	103.27	6.77363	0.075	0.44	1.001
2	100	100	200	91	86.77	96.15	2.39324	0.026	0.31	0.729
2	100	150	300	94	92.20	96.30	1.04730	0.011	0.14	0.331
2	100	200	400	97	96.43	97.07	0.16349	0.002	0.22	0.109
2	100	250	500	98	98.39	98.44	0.01384	0.000	0.2	0.026
2	100	300	600	99	99.21	99.21	0.00005	0.000	0.5	0.006

Table S5.2. Comparison of the actual number of unique individuals (Min. #) identified in 2013, 2013 with additional samples (2013+) and 2014. There were no extra samples in Session 1. Sessions 2 and 3 (separately and combined) include individuals from single session sites because this is comparing estimates of reduced sessions and single sessions would always be included. Caps/ind represents captures per individual in c*apwire* and *p* (m: male, f: female) is average capture probability in closed capture models (CMR).

	Year	2013			2013+			2014		
Estimator	Sess.	Min. #	Caps/ind	*p*	Min. #	Caps/ind	*p*	Min. #	Caps/ind	*p*
capwire	1	51	2.49	--	51	2.50	--	83	2.60	--
	2	54	2.22	--	58	2.49	--	73	2.24	--
	3	68	2.59	--	70	3.30	--	77	2.00	--
capwire	1 & 2	67	3.54	--	68	3.70	--	100	3.68	--
CMR	1 & 2	67	--	m: 0.83	68	--	m: 0.82	100	--	m: 0.76
				f: 0.44			f: 0.42			f: 0.64
capwire	2 & 3	80	3.58	--	84	4.31	--	96	3.29	--
CMR	2 & 3	80	--	m: 0.80	84	--	m: 0.76	96	--	m: 0.76
				f: 0.71			f: 0.69			f: 0.67
capwire	ALL	88	5.38	--	91	5.21	--	110	4.63	--
CMR	ALL	88	--	m: 0.72	91	--	m: 0.71	110	--	m: 0.73
				f: 0.49			f: 0.55			f: 0.61

APPENDIX

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