Genetic Monitoring of the Endangered Columbia Basin Pygmy Rabbit Recovery

A Dissertation
Presented in Partial Fulfillment of the Requirements for the
Degree of Doctorate of Philosophy
with a
Major in Environmental Science
in the
College of Graduate Studies
University of Idaho
by
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December 2015

Authorization to Submit Dissertation

This dissertation of Stephanie DeMay, submitted for the degree of Doctorate of Philosophy with a Major in Environmental Science and titled "**Genetic Monitoring of the Endangered Columbia Basin Pygmy Rabbit Recovery**," has been reviewed in its 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

Monitoring demographic and genetic parameters of reintroduced populations of endangered species is crucial for evaluating and informing conservation strategies to maximize the chances of a successful recovery. I developed a suite of 19 microsatellite loci to enable genetic monitoring of the recovery of the Columbia Basin pygmy rabbit in central Washington, USA. I performed a pilot study evaluating degradation rates of fecal DNA and compared the advantages, disadvantages, and costs of noninvasive genetic monitoring using fecal DNA with radio telemetry, another common monitoring method. Finally, I used these molecular tools to evaluate reproduction and population genetic trends inside large field breeding enclosures, and post-release dispersal, survival, and reproduction of pygmy rabbits reintroduced to the wild. DNA degradation was influenced by sample age, DNA type, locus length, sex of the rabbit, and weather conditions. Systematic surveys to monitor the reintroduced population took place during winter to maximize success rates for genetic samples.

Tissue samples were collected from all pygmy rabbits released to the wild during the summers of 2012-2014 to generate a database of reference genotypes. From this data I characterized and evaluated breeding habits of pygmy rabbits in the enclosures. They displayed a promiscuous mating system, multiple paternity within litters was common, juvenile breeding occurred on rare occasions, and reproductive output was influenced by genetic diversity, population density, and paternal ancestry.

Each winter following releases we surveyed on and around the release area to locate active burrows and collect fecal pellets. Fecal genotypes were used to evaluate post-release dispersal, survival, and reproduction. Compared with telemetry of juvenile pygmy rabbits,

fecal DNA sampling provided information for a longer time period, although at a coarser temporal scale. Over the course of this study, 1206 pygmy rabbits were released to the wild, and we detected 44-91 surviving each year. Survival differed across years and was positively influenced by release date, release weight, and heterozygosity. Reproduction was low, with only 14 wild-born individuals detected. Three years in to the renewed reintroduction effort, this project is in its infancy. Within an adaptive management framework, this research provides information to guide future recovery actions.

Acknowledgements

This work was undertaken with the financial and moral support of many organizations and individuals. For financial support of this research I would like to thank the Association of Zoos and Aquariums, Oregon Zoo Foundation, Palouse Audubon Society, Phoenix Zoo, Riverbanks Zoo and Garden, Roger Williams Park Zoo, Washington Chapter of The Wildlife Society, Washington Department of Fish and Wildlife (WDFW), U.S. Fish and Wildlife Service (USFWS), the University of Idaho Environmental Science Program, and the University of Idaho Department of Fish and Wildlife Sciences. I would like to thank Wyatt Knickerbocker, Chelsea Sink, Kelsi Potterf, and Zachary Andrews for their assistance processing samples in the lab. Thank you to the University of Idaho students enrolled in Endangered Species Population Monitoring during spring 2015, and the countless volunteers who helped capture, process, and release rabbits, and conduct surveys in the field. Thank you to the WDFW field and office staff for your partnership and friendship over the years, and to the hardworking individuals at WDFW, USFWS, the Oregon Zoo, Washington State University, Northwest Trek Wildlife Park, and other partners who labored long and hard for the Columbia Basin pygmy rabbit long before I came on the scene. Thank you Chris Dixon for taking a special interest in me my very first day visiting the University of Idaho as a high school senior, and for being a constant encouraging presence since then, challenging me to challenge myself and do great things.

I would like to thank my advisers Dr. Janet Rachlow and Dr. Lisette Waits for taking me on and each lending their creativity, insights, and ambition to both my research, personal and professional development, and the greater goal of successfully recovering pygmy rabbits in Washington. I would have been fortunate to have had just one of you as my advisor, and am so grateful to have had both of you solidly in my corner. Many thanks go to my

committee. I thank Dr. Tim Johnson for his statistical help and amusing comments ("I'm up to my knees in poop going bad over time!"), Dr. Lisa Shipley for her insights based on a long history with this recovery effort, and Dr. Jay O'Laughlin for his endangered species policy expertise. I thank Dr. Penny Becker for going above and beyond the call of duty to help shape me into a wildlife biologist, providing opportunities and encouragement throughout our time together. I am indebted to the whole Becker clan, Penny, Warren, Kodi, and Kaya, for being my friends and family during my time in the field in the booming cultural center of Ephrata, Washington.

I would like to induct two individuals to the roommate hall of fame: Rebecca Slagg for coming to lab with me late at night to read to me while I did DNA extractions, and Joe Holbrook for intellectual and moral support, many good conversations, and many good nights of mutual procrastination. Thank you to my labmates and my friends who have gone through this journey with me, and to my parents Betsy and Dave DeMay who raised me with a sense of adventure, appreciation for the natural world, and a stubborn streak that occasionally comes in handy. Thank you to my friends Rachel, Monica, Phoebe, Joey, Chandler, and Ross for help making data entry and genotyping more bearable. Finally, thank you to my fish Sam for sticking with me through this whole PhD, for enduring several road trips across Washington to stay with me during my field seasons in addition to 3 moves to different houses just in Moscow; in such an unstable nomadic stage of life, it has been comforting to have you with me this entire time. You are a trooper.

Dedication

To Pandapple you stud.

Thank you for your dedication to the preservation of your species.

I can only hope I've had as much of a positive impact on this recovery as you have.

Rest in peace little guy, your efforts have not been in vain.

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Introduction

The pygmy rabbit (*Brachylagus idahoensis*) is the smallest rabbit in North America and is unique among rabbits in that they dig their own burrows and rely heavily on sagebrush, a plant toxic in high quantities to most mammals, for food. This species has been present within the sagebrush-steppe of the Columbia Basin in Washington State for over 100,000 years, and has been separated from the rest of the species' range for 10,000 years (Lyman 1991). Large-scale loss and fragmentation of native shrub-steppe habitats, primarily for agricultural development, likely played a primary role in the initial decline of the Columbia Basin pygmy rabbit. Once population numbers dropped below a certain threshold, a combination of other factors such as environmental events (e.g., extreme weather and fire), predation, disease, loss of genetic diversity, and inbreeding likely contributed to their further decline until only one population remained at Sagebrush Flat Wildlife Area. Emergency provisions of the federal Endangered Species Act listed the Columbia Basin pygmy rabbits as an endangered distinct population segment in 2001, with a final rule continuing the listing in 2003 (Federal Register 2001, 2003). Since the initial population decline, major strides have been taken by government and non-governmental organizations to acquire and restore shrub-steppe habitat, and the U.S. Department of Agriculture's Conservation Reserve Program has allowed many landowners in central Washington to remove their land from agricultural production and restore it to more natural conditions.

In 2001, sixteen Columbia Basin pygmy rabbits were captured to found a captive breeding program. Disease and low reproductive output, in part as a result of inbreeding, meant that a large enough number of rabbits were not produced in captivity to allow for full-scale reintroductions. To meet the recovery goal of reestablishing a free-ranging population in

Washington, the strategy was adapted in 2011 to phase out off-site captive breeding, focus on field breeding efforts, and to allow for translocation of wild rabbits from other range states (USFWS 2012). Since 2011, over 1200 pygmy rabbits of mixed ancestry have been released onto Sagebrush Flat Wildlife Area from large field breeding enclosures. The goal of this dissertation was to develop, evaluate, and apply genetic tools to evaluate the success of the reintroduction. This work has and will continue to inform changes to the conservation strategy and generate a dataset upon which to build to address longer term questions and recovery goals.

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Chapter 1

Evaluating DNA degradation rates in faecal pellets of the endangered pygmy rabbit

Published: DeMay, SM, PA Becker, CA Eidson, JL Rachlow, TR Johnson, and LP Waits (2013) Evaluating DNA degradation rates in faecal pellets of the endangered pygmy rabbit. Molecular Ecology Resources 13:654-662.

Abstract

Noninvasive genetic sampling of faecal pellets can be a valuable method for monitoring rare and cryptic wildlife populations, like the pygmy rabbit (*Brachylagus idahoensis*). To investigate this method's efficiency for pygmy rabbit monitoring, we evaluated the effect of sample age on DNA degradation in faecal pellets under summer field conditions. We placed 275 samples from known individuals in natural field conditions for 1 to 60 days and assessed DNA quality by amplifying a 294 base pair (bp) mitochondrial DNA (mtDNA) locus and 5 nuclear DNA (nDNA) microsatellite loci (111 – 221 bp). DNA degradation was influenced by sample age, DNA type, locus length, and rabbit sex. Both mtDNA and nDNA exhibited high PCR success rates (94.4%) in samples <1 day old. Success rates for microsatellite loci declined rapidly from 80.0% to 42.7% between days 5 and 7, likely due to increased environmental temperature. Success rates for mtDNA amplification remained higher than nDNA over time, with moderate success (66.7%) at 21 days. Allelic dropout rates were relatively high (17.6% at < 1 day) and increased to 100% at 60 days. False allele rates ranged from 0 to 30.0% and increased gradually over time. We recommend collecting samples as fresh as possible for individual identification during summer field conditions. Our study suggests that this method can be useful for future monitoring efforts, including occupancy surveys, individual identification, population estimation, parentage

analysis, and monitoring of genetic diversity both of a reintroduced population in central Washington and across their range.

Introduction

Noninvasive genetic sampling (NGS), specifically using faecal DNA, is a valuable tool for monitoring of wildlife populations (De Barba et al. 2010, Schwartz et al. 2007, Waits and Paetkau 2005), but is limited by DNA persistence. Longevity of faecal DNA can be affected by sample age (Murphy et al. 2007, Panasci et al. 2011, Piggott 2004, Santini et al. 2007), weather conditions/season (Lucchini et al. 2002, Murphy et al. 2007, Piggott 2004), preservation or storage method (Nsubuga et al. 2004, Panasci et al. 2001, Santini et al. 2007), and diet (Panasci et al. 2011). DNA degradation rates can differ among species and even within species in response to changes in diet or environmental conditions. Consequently, results are not necessarily transferable across species or study sites, and pilot studies are recommended for each study system to determine DNA degradation rates, genotyping error rates, and the number of replicates necessary to gather reliable genotypes (Taberlet et al. 1999). Nevertheless, general trends have emerged. The above studies have shown that fresh samples yield the highest quality DNA, mitochondrial (mt-)DNA maintains higher success rates over time than nuclear (n-) DNA, and dry and/or cold environmental conditions preserve DNA most effectively.

The pygmy rabbit (*Brachylagus idahoensis*) is a rare and cryptic sagebrush obligate in the Great Basin of the western United States. A geographically and genetically distinct population in the Columbia Basin of central Washington, USA, has been identified as a distinct population segment by the US Fish and Wildlife Service and is listed as endangered under the Endangered Species Act (Federal Register 2003). Reintroduction efforts are

underway including the release of rabbits with varying amounts of ancestry from captive-bred rabbits (Columbia Basin inter-crossed with Idaho pygmy rabbits) and translocated wild rabbits from other Great Basin states (WDFW 2011). Monitoring the reintroduced population is essential to informing future recovery efforts.

Monitoring small and endangered populations is often difficult and risky using traditional methods and NGS can be an efficient and cost-effective tool for assessing the success of reintroductions. Noninvasive genetic monitoring of Leporids using microsatellites is not widespread, but mitochondrial loci are increasingly being used to identify species. Pygmy rabbits have previously been surveyed using a species-specific mtDNA test on faecal pellets (Adams et al. 2011) and faecal DNA is also being used to monitor the range of the New England cottontail (*Sylvilagus transitionalis*), a rabbit species of conservation concern in the northeastern United States (Kovach et al. 2003, Litvaitis et al. 2006). Microsatellite loci and primer pairs have been designed for pygmy rabbits (Estes-Zumpf et al. 2008) and loci designed for other rabbit species have been amplified in pygmy rabbits (Mougel et al. 1997, Rico et al. 1994, Surridge et al. 1997, Estes-Zumpf et al. 2010). While these loci have been successful using high quality tissue DNA, they have not been tested on faecal DNA.

We designed this study to determine how NGS can best be used to monitor reintroduced pygmy rabbits. Specifically, we examined the effect of sample age, DNA type, PCR product length, and sex on DNA degradation in faecal pellets from the endangered Columbia Basin pygmy rabbit. We evaluated DNA degradation over 60 days under summer field conditions by analyzing samples for PCR success rates for mtDNA and nuclear microsatellite loci, and genotyping error rates for microsatellite loci. We expected mtDNA to perform better than nDNA and that PCR success rates would decline as genotyping error rates

increase over time. This pilot study during harsh summer conditions will inform protocols for future field surveys and genetic monitoring efforts for this species.

Methods

Sample Collection

We collected a total of 275 samples of at least four pellets per sample from 14 adult pygmy rabbits of known identity (9 males, 5 females) between June 20 and August 17, 2011. The rabbits were housed individually in outdoor soft-release enclosures on Sagebrush Flat Wildlife Area in central Washington. All pellets deposited by each rabbit in one day were gathered and placed in natural conditions within 15 cm of the base of a shrub of big sagebrush (*Artemisia tridentata*) outside the soft release enclosures. We used shrubs of similar height (60-90cm) to create similar conditions among piles, but microclimate effects could not be precisely controlled. Each pile consisted of pellets produced by the same rabbit on the same day. This was repeated for multiple days, resulting in multiple piles per rabbit. Samples were collected from these piles at the following sample ages: < 1 day (n=36), 3 days (n=23), 5 days (n=29), 7 days (n=30), 14 days (n=27), 21 days (n=30), 28 days (n=30), 42 days (n=31), and 60 days (n=39). Not every pile was sampled at each time point to avoid running out of pellets, and some were sampled multiple times at a single time point. Samples were placed in paper coin envelopes, desiccated with silica gel beads, and stored at room temperature until DNA extraction. We collected weather data (temperature, rainfall, relative humidity, and dew point) at the field site every 30 minutes during the study period using an Ambient Weather WS-2080 Wireless Home Weather System (Ambient Weather, Chandler, AZ).

DNA extraction and PCR amplification

We extracted DNA from four pellets per sample to obtain an adequate amount of DNA (Adams et al. 2011) using a Qiagen QIAamp DNA stool mini kit (Qiagen Inc., Valencia, CA.) in a laboratory dedicated to low quantity DNA processing. An extraction negative was included in each extraction batch to test for contamination (Beja-Pereira et al. 2009, Taberlet and Luikart 1999, Waits and Paetkau 2005). Samples were randomized before extraction and each extraction batch contained both older samples (>7 days old) and fresher samples (≤7 days old). The fresher samples served as an extraction positive in cases where none of the older samples yielded amplifiable DNA.

To evaluate mtDNA success rates, we amplified a 294 base pair (bp) fragment of cytochrome b using a pygmy rabbit mtDNA species ID test developed by Adams et al. (2011) to differentiate among six sympatric rabbit species. The PCR reaction concentrations were as published in Adams et al. (2011) but PCR conditions differed. The PCR profile had an initial denaturation of 95°C for 10 minutes, followed by a touchdown of 15 cycles with a 30 second denaturation at 95°C, 30 second annealing step at 63°C decreasing 0.5°C each cycle, and 60 second extension at 72°C. Following this were 30 cycles of a 30 second denaturation at 95°C, a 30 second annealing step at 55°C, and a 60 second extension at 72°C. There was a 3 minute final extension at 72°C and a 10 minute cool down at 4°C.

To test nDNA success rates, we amplified 5 nuclear microsatellite loci ranging in size from 111 to 221 bp in a single multiplex PCR reaction (*A121, A124, A133, A2*: Estes-Zumpf et al. 2008, *Sat7*: Mougel et al. 1997) using the Qiagen Multiplex PCR kit. The 7 µL PCR mix contained 0.071 µM A121, A124, A133, and A2 primer pairs, 0.129 µM Sat7 primer pair, 1x Qiagen Master Mix, 0.5x Qiagen Q-solution, and 1 µL DNA extract. The PCR profile began

with initial denaturation at 94°C for 15 minutes, followed by a touchdown of 10 cycles with a 30 second denaturation at 94°C, 90 second annealing step at 62°C decreasing 0.5°C each cycle, and 60 second extension at 72°C. Following this were 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. There was a 30 minute final extension at 60° C and a 10 minute cool down at 4°. Each PCR included a negative control to test for contamination and a positive control to confirm proper PCR conditions.

Samples were run on an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems Inc., Foster City, CA), and results were viewed in Genemapper 3.7 (Applied Biosystems) and checked visually. Samples that failed to produce a positive PCR were amplified and analyzed in a second trial to exclude random non-amplification (Murphy et al. 2007). For both mtDNA and nDNA, we considered a PCR successful if it resulted in a fragment in the expected size range with an intensity >150 fluorescent units.

Tissue samples were also collected from the same individuals to provide a reference genotype. Tissue samples were collected from the ear using a 3mm biopsy punch, stored in 95% ethanol, and extracted using a Qiagen DNEasy blood and tissue kit (Qiagen Inc., Valencia, CA.). The PCR profile was identical to the faecal DNA profile except the 57°C annealing step ran for 15 cycles rather than 30. Tissue samples were run in duplicate to ensure an accurate reference genotype.

Genotyping error rates

We used tissue samples to establish a reference genotype for each individual at the 5 microsatellite loci. Genotyping errors from faecal DNA were classified as allelic dropout (ADO) or false alleles (FA) and quantified using the methods described by Broquet and Petit

(2004). We classified errors as ADO when only one allele of a heterozygote amplified and as FA when we observed alleles not present in the reference genotype.

Data Analysis

We used mixed-effects logistic regression to model three response variables: PCR success, ADO, and FA, each allowing for a random effect of the individual rabbit and of the pile from which the sample was collected. The sample unit for each response variable was each individual locus within each sample. Fixed effects for these models included sample age (log transformed), sex and DNA type. We categorized DNA type as either mtDNA, nDNA at locus A2, or nDNA at other loci in order to test for significant differences in amplification rates between mtDNA and nDNA, and to test the effect of locus length on amplification and genotyping error rates. Locus A2 was separated out because it ranged in length from 111 to 134 bp while the other four nDNA loci overlapped and ranged from 188-221 bp in length. In the genotyping error models, we included an additional variable for sample quality, indicating whether >50% (at least 3 of 5) of the loci amplified. It is a common laboratory procedure to exclude lower quality samples with <50% amplification from further analysis. Each fixed effect was allowed to interact with sample age. We performed joint tests to evaluate the null hypotheses that each fixed effect did not significantly affect the relationship between sample age and the response variable. All analyses were conducted in SAS 9.3 (PROC GLIMMIX; SAS Institute Inc. 2010).

We used a separate model to investigate the effect of storage time on PCR success, with storage time being the period between sample collection and extraction when samples were stored on silica. All samples were deposited within a week of each other and samples were randomly grouped and extracted within 37 days of each other. Thus, the main driver of

storage time was sample age, with fresh samples being stored on silica approximately 2 months longer than the oldest samples. To eliminate the confounding effect of sample age, we tested the effect of storage time on each sample age individually, while controlling for sex and DNA type (mtDNA, nDNA locus A2, nDNA other loci), and including random effects of individual rabbit and pile.

Results

Weather conditions

Over the study period, temperatures ranged from 4.2°C to 37.5°C with an average temperature of 20.8° C. The average daily temperature fluctuation was 22.1°C. The average high temperature over the entire study was 30.7°C and the average low was 8.5°C. Relative humidity ranged from 11% to 94% with an average of 43.1% and an average daily fluctuation of 55.4%. The study site received rain on 8 days during the study period for a total of 29.1 cm, 16.8 cm of which occurred during a single 4-hour period on July 7, 2011, between weeks 2 and 3 of our trials.

PCR success

Both mtDNA and nDNA showed high success rates for amplification in fresh samples, with nDNA success declining more rapidly as sample age increased (Figure 1.1). Mitochondrial DNA was successfully amplified in 58.2% (n=275) of samples across all time points. Of these, 30.0% (n=160) amplified successfully during the second PCR trial after failing to amplify during the first. Nuclear DNA had lower overall success, amplifying 41.8% (n=1375) of the time. At least one locus amplified in 68.5% (n=275) of samples, 12.2% (n=181) of which amplified only during the second PCR replicate. PCR success of mtDNA and nDNA was equally high (94.4%) in fresh samples < 1 day old. Success remained

relatively high for mtDNA (66.7%) in samples that were 21 days old while nDNA success dropped rapidly from 80.0% at day 5 to 42.7% at day 7 and was only 29.3% at 21 days. PCR success was very low (mtDNA: 7.7%, nDNA: 4.1%) for samples after 60 days in the field.

All parameters tested in the PCR model significantly influenced success rates (Table 1.1). We observed that mtDNA showed the highest PCR success rates (58.2%), followed by nDNA at the A2 locus (48%), with the longer loci showing the lowest success (39.6-41.5%; Figure 1.2). PCR success was higher in females than males. Samples from females succeeded 62.6% (mtDNA) and 44.4% (nDNA) across all time points compared to 55.7% (mtDNA) and 40.2% (nDNA) for males. This higher average success for females was apparent despite a difference in distribution of sample ages between sexes. Female samples were skewed towards older samples, with 44.4% of samples older than 21 days, while only 31.8% of male samples were at least that old.

There was no consistent support for a negative effect of storage time on PCR success. Six sample age groups showed no detrimental effect of storage time, while three sample age groups showed a significant negative effect of storage time. Samples extracted earlier in the 60 day old ($p = 0.04$), 3 day old ($p < 0.001$), and 1 day old ($p = 0.003$) age classes performed better than samples from the same time points but extracted later. In the case of the 60 day old samples, the difference in storage times was only 2 days. Storage times for 1 day old samples ranged from 130-140 days and 3 day old samples were stored 133-139 days. Storage times for 5 day old samples overlapped much of this same range (129-137 storage days) and did not exhibit a negative effect of storage. Samples from the 5 and 7 day old age classes showed a significant trend (both $p < 0.001$) in the opposite direction, with samples stored longer performing better. The remaining 4 time points showed no significant effect of storage time.

The lack of a consistent trend in these results indicates that our findings reflect the impact of DNA degradation in the field and that they are not confounded by DNA degradation during storage.

We used weather data in a post hoc analysis to further investigate the rapid decline in nDNA PCR success between days 5 and 7. We collected 7 day old samples on June 27 (initially deposited June 21) and on June 28 (deposited June 22). Maximum temperatures reached 30.1°C on June 27 and 32.8°C on June 28; this June 28 reading was the highest recorded temperature up to that time during the study period. Of the samples collected on June 27 (n=10), 68% of nDNA loci amplified, while only 31% of nDNA loci amplified from samples collected on June 28 (n=19). It was not possible to compare samples originally deposited on June 21 and 22 and collected at earlier time points because all samples deposited on June 21 were collected on day 7. However, samples deposited on June 22 had success rates similar to the general trend (3 days: 84% success, 5 days: 80% success) before dropping to 31% on day 7. Consequently, the drop recorded on June 28 was likely not due to an intrinsic difference in sample quality. Mixed logistic regression modeling of these samples revealed a significant effect of collection date ($p<0.0001$). Controlling for sex, locus length (A2 vs. other four loci), and the random effects of rabbit and pile, the odds of a sample collected on June 27 successfully amplifying was 13.6 times higher than one collected on June 28.

Genotyping error rates

Allelic dropout rates were relatively high, occurring at 17.6% (n=91) of successfully amplified heterozygous loci for samples < 1 day old. Rates of ADO were variable, but exceeded 50% in samples ≥ 5 days old. Excluding samples for which < 50% of microsatellite loci amplified (n = 69 of 353 heterozygous loci across all sample ages), which is common in

NGS studies (Stenglein et al. 2010), significantly improved ADO rates ($p = 0.0067$) and lowered overall ADO from 49.6% (n = 353) to 41.6% (n = 284; Table 1.2). Sample quality and sample age were the only significant variables affecting ADO (Table 1.1, Figure 1.3a). Raw ADO rates were lower for females than males at every sample age < 42 days, although it was not statistically significant ($p = 0.1487$).

False allele rates were lower than ADO with 1.9% (n=157) of successfully amplified loci exhibiting FA in fresh samples < 1 day old. FA did not exceed 10% in samples <28 days old. The highest FA rate observed (30%; n=10 successfully amplified loci) was in samples that were 42 days old. Unlike ADO rates, screening low quality samples did not significantly alter FA rates ($p =0.4190$). Sample age and locus length (A2 vs. other four loci) were the only variables significantly influencing FA (Table 1.1, Figure 1.3b). The random effect for pile was removed from this model because we were unable to estimate a non-zero value for the variance.

Discussion

Our study was the first to use NGS methods to amplify nDNA of Leporids from faecal pellets and to evaluate mtDNA and nDNA degradation and error rates for faecal pellets of pygmy rabbits. The results from this pilot study will be crucial to developing genetic monitoring techniques to evaluate the reintroduction efforts for the federally endangered rabbit population in central Washington. Adams et al. (2011) developed the mtDNA species ID test used in this study and found 72% PCR success for non-winter pygmy rabbit pellet samples of unknown age (Adams et al. 2011). Noninvasive genetic sampling and mtDNA species identification is also an important tool for monitoring the distribution of threatened New England cottontails (Kovach et al. 2003, Litvaitis et al. 2006). New England cottontails

are sympatric with two other rabbit species, and Kovach et al. (2003) was able to unequivocally identify species in 133 out of 140 faecal samples collected. Individual identification using nDNA from faeces will add another dimension to the NGS Leporid monitoring methods already in use.

Factors affecting DNA degradation

Sample age significantly affected rates of both DNA amplification and genotyping error. For pygmy rabbit faecal pellets deposited in summer conditions, we found species identification based on mtDNA could be determined at least 60% of the time within 21 days of pellet deposition. In contrast, for analyses using nDNA such as individual identification and parentage analysis, our research indicated that similar success rates may only be possible with fresh samples ≤ 5 days old. Similarly, a degradation study of coyote scat collected during the summer in New Mexico reported highly degraded nDNA in 5 day old samples compared to 1 day old samples (Panasci et al. 2011). Allelic dropout rates in this study were higher than reported in comparable studies. In a review of genotyping error rates from NGS by Broquet and Petit (2004), 13 of 17 studies on faecal DNA resulted in ADO rates lower than the ADO we observed in our freshest samples (17.6%). Ten of the 17 studies reported ADO < 5%. This high incidence of ADO in DNA from our samples indicates that more replicates may be needed to verify a homozygous result, increasing the per sample cost for analysis. In contrast, FA rates in this study were low and on par with those reported in other faecal DNA studies (Broquet and Petit 2004). For older samples, in our study both ADO and FA rates were highly variable, likely because of low sample sizes due to lower amplification rates.

We hypothesize that weather conditions played a role in governing DNA degradation rates in our study. The steep drop in amplification success for nDNA between days 5 and 7, and the more surprising drop between two groups of 7 day old samples deposited 1 day apart, provides support for this hypothesis. The absence of any other notable weather irregularities suggests that temperature might have been a driving factor. This conclusion, however, is based on one comparison, and additional studies are needed to confirm this phenomenon, as there were likely other unmeasured factors involved as well. The higher quantity of mtDNA per cell might have shielded it from responding as dramatically as nDNA, as we observed moderate mtDNA amplification success for two more weeks from the same samples and no major decrease in success during this 5 – 7 day time period.

Other studies have also documented the impact of temperature on fecal DNA degradation. Nsubuga et al. (2004) reported that higher temperatures decreased the concentration of amplifiable nDNA from the faeces of African primates (wild mountain gorilla, *Gorilla beringei beringei*; western chimpanzee, *Pan troglodytes verus*) when the maximum temperature reached only 28°C. Murphy et al. (2007) identified a rapid initial decline in nDNA success of brown bear faeces, with a decrease of over 30% between days 1 and 3, and temperature significantly impacted both nDNA and mtDNA success rates. In contrast, Piggott (2004) did not observe a rapid initial decline in nDNA quality from either the brush-tailed rock-wallaby (*Petrogale penicillata*) or the red fox (*Vulpes vulpes*). In that study, amplification success of nDNA remained high and declined gradually over time until no samples successfully amplified after 3 months. These differences may have been due to weather or climate conditions; in Piggott's system, the average high temperature during

summer sampling was 25°C (Piggott 2004), which is cooler than the conditions during both our study and that of Murphy et al. (2007).

We documented significantly higher success rates and significantly lower FA rates at microsatellite locus A2 than the four longer loci. This difference was likely due to the size difference between A2 (111-134 bp) and the longer loci (188-221 bp). When DNA is degraded, shorter fragments are more likely to remain intact and are expected to have higher success rates (Broquet et al. 2007). Other studies have documented patterns similar to our study (Buchan et al. 2005, Hoffman and Amos 2005).

The effect of sex on PCR amplification and ADO rates that we documented has not been previously reported. This study was not originally designed to explore this factor, and sample sizes were skewed towards males, both in number of samples (176 male, 99 female), and number of rabbits (9 males and 5 females, with one female contributing only one sample). Because the distribution of female samples across sample ages also was skewed towards older samples, in the absence of an effect of sex, we might expect to observe lower PCR success and higher genotyping error rates in females solely due to the sample distribution. We observed the opposite trend, superior DNA quality from females, suggesting that there might be a real effect of sex. Further study is needed to confirm this pattern and explore the causal mechanism. One possible hypothesis is that success rates are higher for females because female pygmy rabbits reach a slightly larger adult size than males (Dobler and Dixon 1990) and typically excrete larger pellets. Larger faecal pellets would have more surface area to collect epithelial cells, and consequently, might result in a higher quantity of DNA. Another hypothesis is that differences in baseline levels of male and female hormones or hormone metabolites excreted in faeces may affect DNA degradation. The female rabbits used in this

study were not pregnant or lactating at the time of pellet deposition, so levels of sex hormones should not have differed markedly from non-breeding baseline levels (Scarlata et al. 2011).

Future directions

Our study was conducted during the summer; however, future pellet surveys for genetic monitoring of the reintroduced pygmy rabbit population will also occur during winter. Consequently, evaluation of DNA degradation under winter conditions is recommended, as both temperature and moisture affect DNA degradation (Lucchini et al. 2002, Maudet et al. 2004, Murphy et al. 2007, Piggott 2004). Adams et al. (2011) reported amplification success rates of 93% for mtDNA from pygmy rabbit faecal pellets of unknown ages collected during winter from the snow surface compared to 72% for samples collected at other times of the year. We would expect a similar improvement in nDNA success rates for winter samples.

Weather is not the only factor that could cause seasonal differences in DNA degradation. Panasci et al. (2011) and Murphy et al. (2003) observed an effect of diet content on reliability of genotyping coyote (*Canis latrans*) scats and brown bear scats, respectively. Pygmy rabbit diet differs seasonally with sagebrush constituting up to 99% of their winter diet and up to 51% of their summer diet (Green and Flinders 1980, Siegel Thines et al. 2004). The rest of their summer diet consists of grasses and forbs. The difference in seasonal pellet composition is further evidenced by the difference in initial pellet color between the breeding and nonbreeding seasons (Sanchez et al. 2009). Sagebrush contains relatively high concentrations of terpenes, volatile oils toxic in high amounts to most mammals (Shipley et al. 2006). Pygmy rabbits have evolved physiological capabilities to digest large amounts of sagebrush, but the effect of these volatile compounds on faecal DNA is not known. Alternately, differences in the fiber content between the rabbits' summer and winter diet and a

resulting difference in intestinal abrasion by faecal pellets could cause a seasonal difference in the quantity of DNA recoverable by NGS (Maudet et al. 2004).

Monitoring implications

This pilot study demonstrated that non-invasive genetic monitoring for pygmy rabbits is possible under summer field conditions, but that it may be challenging or impossible to identify individuals from older samples, especially during the higher temperatures of late summer. However, a positive species ID, even with poor nDNA amplification, can still inform future sampling efforts and allow focused resampling for fresher samples. To increase the success of genetic analyses, we recommend collecting samples during cool and dry times of the year. If summer surveying is necessary, early summer would be preferred to avoid the extremely high temperatures typical of mid to late summer that may yield usable nDNA for an even shorter window of time than observed in this study. We stored our samples on silica for at most 140 days and did not detect a decrease in amplification with increasing storage time. This storage method was effective for our study, but we cannot predict how DNA degradation might respond to longer storage periods.

Careful selection of microsatellite loci and primers can increase success rates as well. For the genetic monitoring of these pygmy rabbits beyond this pilot study, we will use a suite of ~20 microsatellite loci. Choosing or redesigning primer pairs with short (< 250 bp) PCR product lengths is a common practice in NGS studies (Stenglein et al. 2010, Taberlet et al. 1999). Our data supports this practice, suggesting that using shorter product lengths will yield better results. Due to our high observed ADO rates, we recommend requiring more PCR replicates to confirm a homozygote genotype. With the 41.6% ADO rate across all high quality samples (>50% amplification), 6 replicates would be needed to reduce the per sample

ADO rate to <1%. Excluding poor quality samples (<50% amplification) will reduce genotyping error rates and save both time and money in the laboratory; including poor quality samples in analyses would require 7 replicates to achieve a per sample ADO rate <1%.

As pygmy rabbits from multiple source populations are released into central Washington, it will be important to monitor the success of the reintroductions. Schwartz et al (2007) identifies two categories of genetic monitoring: using molecular markers for traditional population monitoring, and monitoring population genetic parameters. This method will allow monitoring of both types of parameters. Individual identification of the reintroduced population using faecal DNA will allow us to monitor survival and reproduction and assess any effect of ancestry on fitness. Dispersal monitoring of juvenile pygmy rabbits with traditional telemetry techniques can be limited by the short retention time of glue-on transmitters (typically less than 3 weeks; Estes-Zumpf and Rachlow 2007) and the difficulty of tracking long distance dispersal movements of juvenile pygmy rabbits (Estes-Zumpf and Rachlow 2009). Sampling faecal pellets will allow continued monitoring of the movements of individual rabbits without periodic recapture to reapply radio transmitters, and such methods will allow monitoring range expansion as more rabbits are released. These methods also can enhance detection of unknown populations in new areas, both in Washington and across the Great Basin. As more rabbits are released into and start breeding in the wild, we will be able to monitor genetic diversity and the contribution of each lineage into the persisting population. By limiting the effects of DNA degradation and genotyping error with optimal sampling and analysis methods, NGS of faecal pellets will be a reliable and effective way to survey for and monitor pygmy rabbits in Washington and across their range.

Acknowledgements

For providing funding for this research, we would like to thank the Riverbanks Zoo and Garden Conservation Support Fund, Association of Zoos and Aquariums Conservation Endowment Fund, U.S. Fish and Wildlife Service, Washington Department of Fish and Wildlife, and University of Idaho Environmental Science Department. We thank the University of Idaho Laboratory for Ecological, Evolutionary, and Conservation Genetics for laboratory use and especially Dr. Jennifer Adams for her assistance in the laboratory. We also thank the members of the Waits lab group for their helpful comments on this manuscript.

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Table 1.1. Results from models for fixed effects influencing PCR success, allelic dropout (ADO), and false allele (FA) rates of DNA from pygmy rabbit faecal pellets exposed to summer field conditions in Washington, USA. The F test statistic and p-values for the effect of sample age are reported from the type III tests of fixed effects, while the remaining values result from the joint test that each fixed effect significantly affects the relationship between (log) sample age and the response variable. Significant p-values are shown in bold.

	PCR Success		ADO		FA	
Parameter	F	P-value	F	P-value	F	P-value
SampleAge[a]	210.25	**<0.0001**	6.51	**0.0112**	5.40	**0.0205**
Sex	4.03	**0.0179**	1.92	0.1487	1.89	0.1527
DNAtype[b]	15.26	**<0.0001**	NA	NA	NA	NA
LocusLength[c]	11.43	**<0.0001**	1.15	0.3167	6.09	**0.0024**
SampleQuality[d]	NA	NA	5.10	**0.0067**	0.87	0.4190

[a] Log transformed
[b] nDNA vs. mtDNA
[c] Dummy variable for A2 locus vs. other four loci
[d] Samples in which >50% of loci successfully amplified vs. samples in which <50% of loci amplified

Table 1.2. Rates of allelic dropout and false alleles observed in DNA extracted from pygmy rabbit faecal pellets aging from < 1 to 60 days old during summer conditions in Washington, USA. Screened ADO percentages include only high quality samples that amplified successfully at ≥ 3 of 5 microsatellite loci. Screened FA percentages are not shown, as screening had no significant effect on false allele rates

	Sample Age (Days)								
	<1	3	5	7	14	21	28	42	60
ADO									
%	17.58	37.50	51.35	75.56	68.97	73.33	87.50	71.43	100.00
N	91	56	74	45	29	30	16	7	5
screened									
%	15.73	31.25	51.56	68.57	57.14	75.00	60.00	-	100.00
N	89	48	64	35	21	20	5	0	2
FA									
%	1.91	0.00	8.62	3.13	6.52	4.55	18.52	30.00	12.50
N	157	88	116	64	46	44	27	10	8

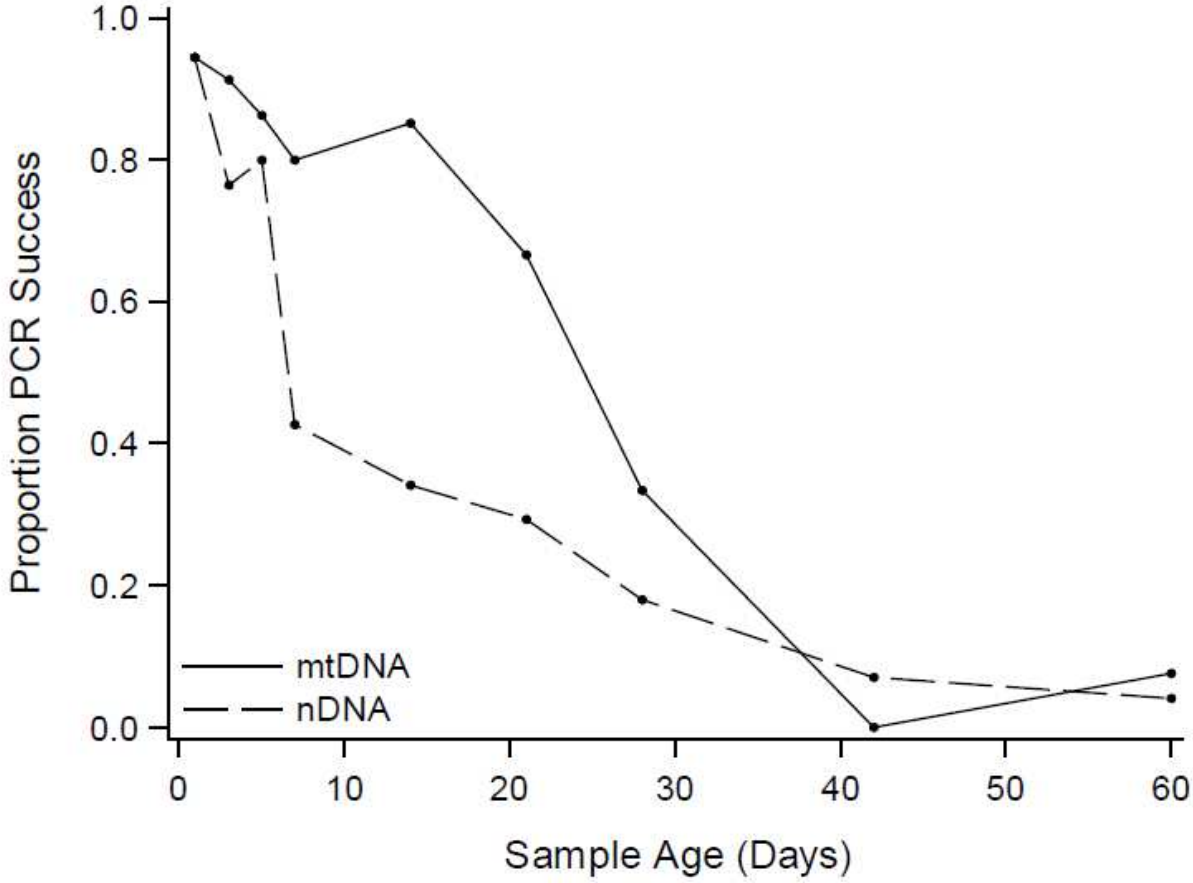

Figure 1.1. Observed PCR success rates for mtDNA and nDNA for pygmy rabbit faecal DNA samples collected during summer conditions in central Washington, USA. Success rates are averaged over both sexes and nDNA rates are averaged over all 5 loci.

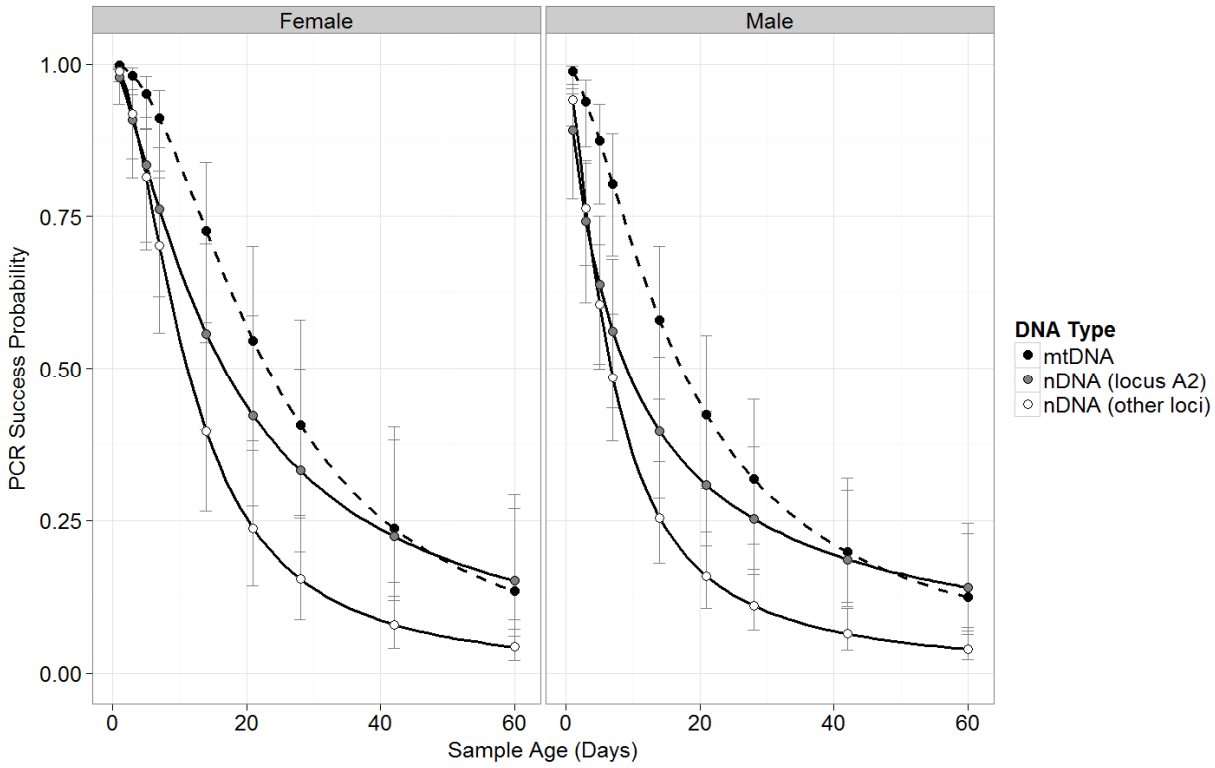

Figure 1.2. Predicted PCR success rates for pygmy rabbit faecal DNA samples deposited during summer in Washington, USA. Significant fixed effects include DNA type (mitochondrial vs. nuclear DNA), nDNA locus length, and sex. Locus A2 represents the shortest locus at 111-134 base pairs in length while the other four loci overlapped and ranged from 188-221 base pairs in length. Samples from females exhibited significantly higher PCR success than those from males.

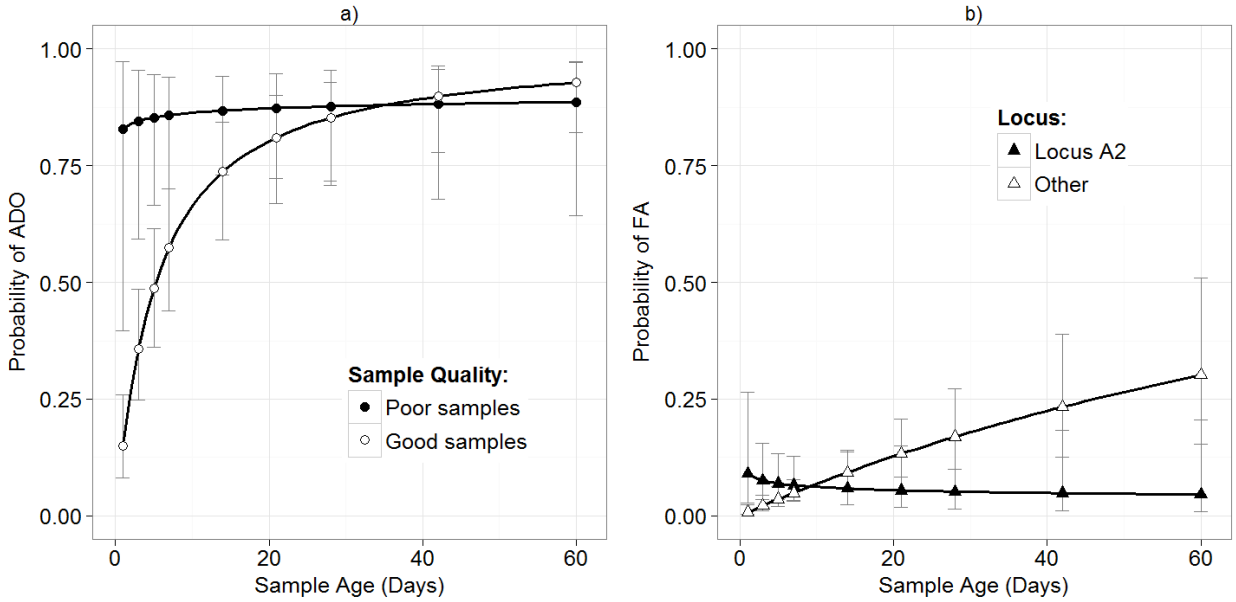

Figure 1.3. Genotyping error rates of a) allelic dropout (ADO) and b) false alleles (FA) predicted for pygmy rabbit faecal DNA left in summer field conditions in Washington, USA. Only those fixed effects significant in the full model are included (a- sample age and sample quality, b- sample age and locus length).

Chapter 2

Comparing telemetry and fecal DNA sampling methods to quantify survival and dispersal of juvenile pygmy rabbits

Published: DeMay, SM, JL Rachlow, LP Waits, and PA Becker (2015). Comparing telemetry and fecal DNA sampling methods to quantify survival and dispersal of juvenile pygmy rabbits. Wildlife Society Bulletin 13:654-662. *Featured on cover

Abstract

Age-specific life-history data are needed to understand animal ecology and inform conservation strategies. We compared telemetry and noninvasive genetic sampling (NGS) as methods for monitoring survival and dispersal of juvenile pygmy rabbits (*Brachylagus idahoensis*) reintroduced to central Washington, USA. During summer 2012, we released 104 juvenile rabbits, 85 of which were fitted with glue-on radiotransmitters and located 2–4 times/week while transmitters were retained ($\bar{x}$ = 15 days). We tracked and recovered 63 transmitters, while signals were lost from 22. Most rabbits remained near the release site, with 9 dispersing >1 km, and only 2 moving >3 km. During winter, we surveyed nearly 9 km^2 around the release site and collected 117 fecal samples for genetic analysis. Forty-two individuals were identified, 38 from the summer releases (37% survival) and 4 born in the wild from parents released in 2011. Using NGS, we identified rabbits 1) released without transmitters, 2) with undetected transmitter signals, 3) presumed dead, and 4) produced in the wild. Short-term dispersal behavior was best gathered with telemetry, but information was limited, and we were unable to estimate survival probabilities because of the short duration of transmitter retention. The information on dispersal, survival, and reproduction provided by NGS allowed us to evaluate longer term reintroduction success, but was limited by the area we were able to search. We compare the results, costs, benefits, and limitations of each

method for addressing specific monitoring objectives.

Introduction

Because juvenile demographic parameters can strongly influence the dynamics of populations, data on age-specific patterns of survival and dispersal can improve our understanding of animal ecology and inform conservation strategies. Survival of juveniles, which influences recruitment into the reproductive age classes, is influenced by many factors, including predation, disease, resource limitation, and juvenile dispersal (Caughley 1966, Krebs et al. 1986, Gaillard et al. 1998). Dispersal contributes to genetic and demographic connectivity among populations, and influences recolonization probabilities of habitat patches, which can be critical for persistence of metapopulations (Bowler and Benton 2005). High rates of mortality and natal dispersal behaviors can make monitoring juvenile animals more challenging than monitoring adults (Promislow and Harvey 1990, O'Donoghue 1992), and these difficulties should be considered when implementing monitoring strategies.

Radiotelemetry is a commonly employed technique for monitoring demographics and movements in wildlife species; however, the small size of juvenile animals often limits the weight and size of transmitters that can safely be used (Sikes and Gannon 2011). Small transmitter size can limit signal strength, battery life, and ability to incorporate additional features such as GPS technology. In addition, continued growth of juveniles can preclude standard designs for attachment of transmitters, necessitating alternative methods such as expandable collars, use of adhesives to attach transmitters, or implantation of telemetry devices (Fuller et al. 2005). For example, glue-on radiotransmitters have been used to study survival and dispersal of juveniles for a variety of small-bodied species, including snowshoe hares (*Lepus americanus;* O'Donoghue and Boutin 1995), pygmy rabbits (*Brachylagus*

idahoensis; Price et al. 2010), and lemmings (*Dicrostonyx groenlandicus;* Blackburn et al. 1998).

An alternative monitoring method is noninvasive genetic sampling (NGS), in which DNA is collected from sources that animals leave behind, such as hair or feces (Waits and Paetkau 2005, Schwartz et al. 2007, De Barba et al. 2010). Because animals do not need to be captured or observed, NGS is useful for detecting and monitoring rare and elusive species. Applications for NGS include gathering information about population size (Stenglein et al. 2010), distribution (Litvaitis et al. 2006), dispersal (Douadi et al. 2007, De Barba et al. 2010), kinship (Lucchini et al. 2002, Becker et al. 2012), genetic structure (Triant et al. 2004), and the demographic trends and genetic diversity of populations (De Barba et al. 2010). The utility of NGS is, however, limited by the low quantity and quality of the DNA compared with more invasively collected sources like blood or tissue, and steps must be taken in the sampling design, laboratory, and analysis methods to account for and minimize these impacts (Waits and Paetkau 2005, Valiere et al. 2007).

We monitored movements and survival of juvenile pygmy rabbits using both telemetry and NGS methods. The pygmy rabbit is a small-bodied, semi-fossorial lagomorph native to the sagebrush steppe of the western United States. Breeding occurs between February and July, and females typically produce multiple litters of 1 to 9 kits in a single breeding season (Elias et al. 2006, 2013). Most juveniles exhibit a distinct natal dispersal within their first 3 months, with females dispersing somewhat farther than males. Although median natal dispersal distances for males and females were 1.0 km and 2.9 km, respectively, individuals have been detected moving up to 12 km (Estes-Zumpf and Rachlow 2009). Previous studies have documented high but variable rates of survival for both adult and juvenile pygmy rabbits

across time and space (Westra 2004, Sanchez 2008, Crawford et al. 2010, Price et al. 2010).

The geographically and genetically distinct population segment of pygmy rabbits in the Columbia Basin of central Washington, USA, is listed as endangered at both the state and federal levels, and a reintroduction program is currently underway (Federal Register 2003, USFWS 2012). Monitoring dispersal and survival of the reintroduced individuals, along with reproduction and genetic parameters, is crucial for evaluating recovery efforts and modifying future conservation strategies. The goal of this study was to compare the utility of radiotelemetry and NGS approaches to monitoring survival and dispersal of reintroduced juvenile pygmy rabbits. The 2 methods were implemented over slightly different time periods, and therefore, quantitative estimates of survival and movements are not directly comparable. However, we provide cost estimates and an assessment of qualitative differences in the information obtained. We expected telemetry to provide reliable information about post-release settlement and mortality. Because of documented patterns of DNA degradation (DeMay et al. 2013), we predicted that surveying for pellets during winter following a fresh snowfall would lead to high success rates for genetic analyses. Finally, we predicted that telemetry would cost more than NGS overall and per rabbit because of the relatively high cost for radiotransmitters compared with genetic analysis. Understanding the costs and benefits of complementary monitoring approaches can help managers to choose among potential methods to design and execute monitoring protocols to support recovery of uncommon species.

Methods

Study area

This study took place at the Sagebrush Flat (SBF) Wildlife Area (approx. 15 km^2) in the Columbia Basin of central Washington, USA, where temperatures range from an average

minimum of −5.9° C in January to an average maximum of 31.6° C in July (WRCC 2013). The climate was semiarid and averaged about 20 cm of annual precipitation, over half of which was typically from snow (WDFW 2006, WRCC 2013). The landscape was dominated by 'mima mounds,' mounds of deep soils, and dense sagebrush with relatively sparse and low-growing vegetation between mounds (Tullis 1995). At SBF, pygmy rabbit burrow systems were almost exclusively located on mima mounds. The site was surrounded by state, federal, and private lands, with a land-cover mosaic of sagebrush steppe and dryland wheat fields. Predators of pygmy rabbits on SBF included badgers (*Taxidea taxus*), long-tailed weasels (*Mustela frenata*), coyotes (*Canis latrans*), and several raptor species. Two large, predator-resistant enclosures (approx. 25,000 m^2, and approx. 40,000m^2) constructed on SBF served as breeding enclosures for a semi-wild adult pygmy rabbit population (WDFW 2011). Enclosure fences were buried approximately 45 cm into the ground and featured a 'floppy top' design to protect against terrestrial predators, while protective netting over pygmy rabbit burrow systems and bird spikes installed on fence posts discouraged avian predators.

Field methods

From May to July 2012, we captured 104 juvenile pygmy rabbits (kits) born in the breeding enclosures for release onto SBF. Prior to release, we weighed and sexed kits, treated them for parasites, and sampled each for DNA with a 3-mm biopsy punch in the ear. Tissue samples for genetic analysis were stored in 95% ethanol and frozen at −20° C until laboratory analysis.

Of the 104 kits released, we fitted 85 with radiotransmitters (Holohil Systems Ltd., Carp, ON, Canada) glued onto their backs. Transmitters, ranging in weight from 1 g to 4 g, were first glued to mesh window screening with 'wings' to increase surface area for

attachment (Estes-Zumpf and Rachlow 2007). Kits fitted with 1-g, 2-g, 3-g, and 4-g transmitters weighed on average 164 g, 191 g, 254 g, and 272 g, respectively. We did not use strict weight categories to assign transmitters because of the limited availability of all transmitter sizes at any given time. A viscous livestock adhesive (Kamar Inc., Zionsville, IN) was used to attach the transmitters to the mesh, and we trimmed the mesh wings to fit to each rabbit so as not to impede movement. We used Loctite adhesive (Henkel Corp., Westlake, OH) to attach the mesh to the rabbit's back and glue fur over the top of the transmitter. Transmitters were reused on multiple rabbits throughout the release season as they were recovered.

Release sites were located between 35 m and 650 m from the breeding enclosures, and we used both soft and hard release methods. We housed soft-released juveniles singly or in pairs in 2.5-m-diameter soft-release enclosures for 7 days prior to release. After the 7 days, we breached the enclosures so rabbits could exit and return freely. In comparison, our hard-release strategy did not involve an acclimatization period in an enclosure. We provided artificial burrows, auger holes, and supplemental food at hard-release sites. We placed kits individually inside artificial burrows, plugged both entrances with burlap, and left them to acclimate to the burrow for approximately 5 minutes, after which we quietly removed the burlap. We did this to allow individuals to recover from the stress of being transported and to increase the probability that kits would stay close to their release sites. Although we used 2 release methods initially, a lack of differences in residency and survival between the hard and soft releases prompted a discontinuation of the use of soft-release pens in June of 2012 (unpublished data).

We used telemetry to track the movements of radiotagged kits on SBF. We located kits every 2 days for the first week post-release, and then at least twice per week in subsequent weeks until transmitters were recovered. To minimize disturbance of animals, we obtained precise locations only once per week, during which we either obtained visual confirmation that the rabbits were alive, or recovered transmitters. During nonvisual tracking days, we recorded if the rabbit was in the same general area as the last known location or if it had moved, but did not approach close enough for visual confirmation. We conducted one aerial telemetry flight, covering 333 km^2 on and around the release site, during the monitoring period to attempt to locate missing individuals. Unlike Estes-Zumpf and Rachlow (2007), we did not recapture kits to reglue or replace transmitters because recapture was deemed risky for recovery of this endangered population; recapture could cause stress, discourage residency, or draw the attention of predators. These methods were approved by the University of Idaho Animal Care and Use Committee (Protocol 2012-23) and are consistent with standards for use of wild mammals in research established by the American Society of Mammalogists (Sikes and Gannon 2011).

During December 2012 and January 2013, after fresh snowfall, we conducted surveys on and around the release sites to locate active burrows and collect fecal pellets for genetic analysis. We surveyed along 50-m-wide belt transects oriented north to south, prioritizing the release area and working outward as time allowed, reaching a maximum of 2.4 km from the center of the release area. We also surveyed specific drainages with dense sagebrush outside of the belt transect area, reaching a maximum of 3.6 km from the center of the release area. Rabbit tracks, active burrows, and fecal pellets were conspicuous on fresh snow during surveys. Burrows were considered active if they exhibited fresh rabbit sign including tracks,

fecal pellets on top of snow, and digging (Sanchez et al. 2009, Price and Rachlow 2011). We collected fecal pellet samples from the snow surface and ≥4 pellets/sample were collected when possible to ensure an adequate amount of DNA for analysis (Adams et al. 2011). Samples were stored in paper coin envelopes and desiccated with silica gel beads until laboratory analysis.

Laboratory methods

We acquired genotypes for all released pygmy rabbits from tissue samples and created a reference database containing genetic and demographic information for each individual in the recovery project. We halved each 3-mm tissue punch and extracted DNA from one half using Qiagen DNeasy blood and tissue kits (Qiagen Inc., Valencia, CA.), while the other half was retained for archiving or re-extraction. Each extraction batch included an extraction negative to test for contamination (Taberlet and Luikart 1999, Waits and Paetkau 2005, Beja-Pereira et al. 2009). Tissue DNA was amplified in duplicate at 22 loci in 3 PCR multiplexes: 21 polymorphic microsatellite loci and 1 Y-chromosome microsatellite used for sex identification, which agreed with sex identification based on morphological features (see Appendix A for multiplex development). These loci include 7 with primers developed for other rabbit species and cross-amplified in pygmy rabbits (7LID3, Korstanje 2003; Sat5, Sat7, Sat8, Mougel et al. 1997; Sol08, Rico et al. 1994; Sol44, Surridge et al. 1997; Y05, Putze et al. 2007), 9 developed for pygmy rabbits (A10, A121, A124, A133, A2, D103, D118, D121, D126; Estes-Zumpf et al. 2008), and 6 new loci with primers developed for this pygmy rabbit monitoring program (A113, A12, A128, A129, A140, D2; GenBank accession numbers KM871174-KM871179). Primers for loci A10, D118, and D121 were redesigned to produce shorter fragment lengths appropriate for noninvasively collected DNA (Buchan et al. 2005).

Samples were run on an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems Inc., Foster City, CA), and results were viewed in Genemapper 3.7 (Applied Biosystems Inc.) and checked visually.

We extracted DNA from the surface of ≥4 fecal pellets/sample when possible using the Qiagen QIAamp DNA stool mini kit (Qiagen Inc.) in a laboratory dedicated to low-quantity DNA samples, and an extraction negative was included in each extraction batch to test for contamination (Waits and Paetkau 2005). To determine species, we amplified a 294 base-pair fragment of cytochrome B from the mitochondrial genome in a species ID test designed to distinguish between pygmy rabbits and sympatric cottontail rabbit species (*Sylvilagus nuttallii, S. audubonii, S. floridanus*; Adams et al. 2011). For samples confirmed as pygmy rabbit pellets, we amplified 8 loci in one PCR multiplex (PyRbM1) and analyzed the results using the same protocol as tissue samples. Cottontail samples and samples that failed to yield species ID result were excluded from further analysis.

Initially, we ran 4 repetitions for each NGS sample, and completed more as needed to acquire consensus genotypes for ≥5 of the polymorphic microsatellite loci to achieve a probability of identity siblings (PIDsib; Waits et al. 2001) value of <0.01 (i.e., probability of <1% that 2 siblings would match at the genotyped loci). We required 2 repeats of each allele to confirm a heterozygous genotype and 4 repeats to confirm a homozygous genotype based on pilot work showing high allelic dropout rates in pygmy rabbit fecal DNA during summer conditions (DeMay et al. 2013). If half of the tested loci did not initially amplify in a given sample, we dropped that sample from further analysis. Fecal sample genotypes were matched with reference (tissue) genotypes using Genalex 6.5 software (Peakall and Smouse 2006, 2012). We checked matches individually, and any pairings with mismatches at 1 or 2 loci

were investigated further to determine whether human error or allelic dropout could have caused false mismatches. If the only mismatch between 2 samples appeared to be due to allelic dropout, we considered it a match.

Fecal samples that did not match a previously sampled rabbit were amplified at all loci, checked for 95% reliability using RELIOTYPE (Miller et al. 2005), added to the database as new wild-born individuals, and analyzed for parentage using a strict exclusion approach in Cervus 3.0 (Marshall et al. 1998, Kalinowski et al. 2007).

Data analysis

We used telemetry data to quantify post-release movement and mortality of individuals fitted with transmitters. To quantify distance moved, we measured the linear distance from the release site to the settlement site and to the transmitter recovery location for each monitored rabbit, assuming that the rabbit was at that location and the transmitter was not carried there by a predator. Estes-Zumpf and Rachlow (2009) reported that most juvenile pygmy rabbits initiated and completed natal dispersal within 1 week, after which they remained fairly stationary. Consequently, released pygmy rabbits were considered settled when they were tracked to one mima mound or adjacent mounds (within 100 m) for 3 or more consecutive visual locations, corresponding to ≥2 weeks on the same or adjacent mounds. When a settled pygmy rabbit was located on adjacent mounds for consecutive locations, we selected for measurement either the mound at which they were most frequently located, or if they were located at adjacent mounds with equal frequencies, the most recent location. We also noted the duration (days) of transmitter retention, which was estimated conservatively to the last visual location of the individual before transmitter recovery. To estimate immediate post-release mortality, we attempted to determine, based on condition of recovered

transmitters and surrounding evidence, whether or not transmitters were removed by predators (Crawford et al. 2010).

For NGS samples collected on snow, we measured the distance between the individual's release site and the location of the active burrow where we collected the fecal sample to quantify movement. If multiple samples were collected for the same individual, we took the average distance from release site to all active burrow systems where fecal samples from that individual were collected. For rabbits that were tracked or detected with both methods, we measured the distance between their NGS sample location and location where we recovered their radiotransmitter (or their last visual location if their transmitter signal had later been lost) to estimate the amount of movement between sampling periods (summer telemetry and winter NGS). For settled rabbits, we measured the distance between telemetry settlement site and NGS location to investigate whether settled rabbits continued to move between summer and winter.

Cost calculation

We report the total cost per method for this study, as well as the cost per animal tracked and per animal detected. For telemetry, we defined the number of animals tracked as every individual released with a transmitter. The number of animals detected was defined as all individuals from which we were able to recover a transmitter. For NGS, we defined the number of animals tracked as all released individuals, and the number of animals detected as all individuals that we identified from fecal DNA collected during winter. The number of animals we were able to track was actually higher than the number of animals released, because with NGS it was possible to detect previously unsampled individuals already present on the landscape. Finally, we report the per sample costs for laboratory supplies and labor for

each of our genetic sample types. Our calculated costs for each method exclude the costs for time and supplies required to capture and release kits, which were common to both monitoring methods.

Telemetry costs included the purchase of transmitters and supplies for transmitter attachment, personnel hours for monitoring, one telemetry flight, and vehicle fuel. We included fuel costs only when we were actively tracking rabbits; fuel for transportation to and from the study area was excluded because telemetry was combined with other field and husbandry activities at SBF. For our reported cost estimate, we assumed that each individual received a unique transmitter, while in fact we reduced costs by reusing transmitters on multiple rabbits throughout the season.

The cost estimate for NGS included supplies for collecting tissue and fecal samples, laboratory supplies and labor, and personnel costs for winter surveys. Our laboratory costs are likely lower than commercial rates because they do not include overhead, or equipment upkeep. We also did not include development and optimization of the microsatellite markers. These up-front optimization costs will vary among studies depending upon availability of microsatellite loci, genetic variation within the study species or population, and the research questions being asked. Our estimates do not include capital equipment costs like vehicles and receivers for telemetry or laboratory equipment for genetic analysis.

Results

Telemetry

Telemetry monitoring of released rabbits lasted from mid-May until mid-September when the last transmitter was recovered. Throughout the monitoring period, we tracked and recovered transmitters from 63 individuals, while signals were lost from the remaining 22

individuals. Transmitters were retained on the rabbits for an average of 15 days, although one transmitter was retained for 87 days. The condition of most recovered transmitters was ambiguous, and we were unable to classify whether or not they had been removed by predators. We recovered one transmitter that was cracked with inner wires exposed, suggesting a predator with powerful jaw strength, and one that was recovered near a gut pile typical of an avian predator; however, we documented no other strong evidence of predation. Characteristics that potentially indicated predation, such as large amounts of fur on recovered transmitters and chewed off 'wings,' were not diagnostic of predation, as evidenced by NGS detection of individuals during winter that were presumed dead during summer based on these criteria.

Most monitored rabbits remained near the release site, with 9 individuals moving >1 km and only 2 moving >3 km (max. = 7.8 km; Figure 2.1a). Using our settlement criteria, 14 rabbits settled by the time their transmitters were recovered. Settlement sites ranged from 0 m to 1.5 km from release sites, with an average distance of 204 m (Figure 2.1b). Rabbits that were detected moving longer distances (>1.5 km) did not settle before their transmitters were recovered or we lost their signals.

Noninvasive genetic sampling

During winter, we surveyed for rabbit sign around the release site and collected 117 fecal samples for genetic analysis. Excluding samples identified as cottontail (n = 6), 86% of samples yielded a successful individual identification. We identified 42 individuals, including 38 juveniles from the summer releases, indicating 37% minimum survival to winter. Released rabbits that we detected included 8 kits released without transmitters, 8 with lost transmitter signals, and 2 presumed dead based on the condition of recovered transmitters. We also

identified 4 individuals that did not match any of our released rabbits. Parentage analysis confirmed that these individuals were born in the wild from parents released 2 summers previously (parent pair nonexclusion probability for full suite of loci = 9.74×10^{-15}). Four loci exhibited high frequencies of null alleles and were either dropped from our multiplexes (A10, D121, D126) or excluded from parentage analysis (A124).

Winter NGS samples were collected an average of 833 m from release sites (range = 80 m to 2.6 km; Figure 2.1c). For rabbits that were tracked with telemetry and subsequently detected with NGS (n = 30), NGS samples were on average 522 m from the transmitter recovery location (or last telemetry location if the signal was subsequently lost) for the same rabbit (range = 1 m–1.7 km). When we considered only those rabbits that had settled during the summer, the average distance from NGS sample to settlement site fell to 445 m. This average was skewed by one rabbit moving 1.7 km from its settlement site (Figure 2.1d). Excluding this individual, all other settled rabbits (n = 5) were detected with NGS within 250 m of their summer settlement sites.

Costs

We calculated the total cost (U.S. dollars) for each method as well as costs per rabbit tracked and detected (see Appendix B for itemized costs). Telemetry costs were $21,817, or $257/rabbit tracked ($n$ = 85) and $346/rabbit detected ($n$ = 63). The cost of NGS was $12,744, or $123/rabbit tracked ($n$ = 104) and $303/rabbit detected ($n$ = 42). The per-sample costs for NGS were $13 (pellets, species ID only), $25 (pellets, species ID, and one microsatellite multiplex to match with a previously sampled individual), $56 (pellets, species ID and 3 multiplexes for a new individual), and $21 (tissue, 3 multiplexes for a new individual).

Discussion

An understanding of the benefits and limitations of alternative techniques is useful for helping wildlife ecologists and managers make informed decisions about how to effectively and efficiently meet their specific research or management goals. Our investigation of 2 monitoring methods highlights the different types of information that can be gained, as well as unique benefits and limitations of each method (Table 2.1). Telemetry provided detailed movement information immediately after release, but was limited by transmitter retention time and signal detection, and we were unable to confirm mortalities. Noninvasive genetic sampling provided detailed dispersal, survival, and reproduction information, but these data had low temporal resolution, and detections were limited to where and when we surveyed. Despite their limitations, both methods contributed to our understanding of the reintroduced population of pygmy rabbits in Washington.

Telemetry

Telemetry allowed us to investigate dispersal of released individuals during the first few weeks after release. Because transmitter attachment necessitates handling individuals, telemetry provided the added benefit of being able to assess physical condition and take biological measurements, which can be included as covariates in downstream analyses. Our ability to assess dispersal was limited by short transmitter retention time ($\bar{x}$ = 15 days), and only 16% of tagged rabbits settled while we were tracking them. Our average retention time was lower than that documented by Estes-Zumpf and Rachlow (2007), where only 10% of glue-on transmitters fell off juvenile pygmy rabbits in 14 days, similar to those for transmitters glued-on snowshoe hare leverets (approx. 14 days if not recaptured; O'Donoghue 1994) and lesser horseshoe bats (*Rhinolophus hipposideros,* 2–16 days; Bontadina et al.

2002), and greater than retention times for similar transmitters on lemmings (3–7 days; Blackburn et al. 1998).

We were unable to reliably detect mortality events because of the ambiguous condition of recovered transmitters. We did not recapture kits to reglue transmitters or swap the glue-on transmitter for a radiocollar upon reaching adult size, although we recommend doing this in certain scenarios. Doing so would have improved both our transmitter retention time and our ability to detect predation, but recapture in the wild was judged to be too risky to the recovery of this endangered population. Estes-Zumpf and Rachlow (2007, 2009) were able to identify predation events from glue-on transmitters recovered from juvenile pygmy rabbits in a nonendangered population in Idaho, USA, and were able to attribute 58% of the predation events specifically to either avian or mammalian predators. In our study, we rarely detected evidence of predation at recovered transmitters, indicating that either predators consumed or carried away the entire animal, or that most of the transmitters had simply fallen off.

Other characteristics specific to our study species and environment impacted the effectiveness of telemetry. First, the small size of juvenile pygmy rabbits necessitated the use of small transmitters with a limited battery life and weak signal strength compared with larger transmitters. Battery life was not a problem in our study because transmitters fell off of rabbits before the batteries would have failed (6 weeks to 6 months for 1-g–4-g transmitters, respectively). Challenges due to weak signal strength were amplified by the hilly topography at our study site, fossorial behavior of pygmy rabbits, and their ability to make rapid long-distance movements (Estes-Zumpf and Rachlow 2009). Small transmitters are also limited in the extra features they can include, notably mortality sensors and GPS with remote download

capabilities, which can increase the spatial and temporal resolution of telemetry data.

Noninvasive genetic sampling

Noninvasive genetic sampling proved effective for longer term monitoring goals and will allow further research into post-release survival, reproduction, and population expansion, but cannot provide information about immediate post-release movement or survival. Because genetic tags are not lost, individuals can be detected using NGS throughout their life and long-term study questions about survival, reproduction, movement, and genetic diversity can be investigated. Such long-term monitoring questions have been addressed with NGS for many species, including wolves (*Canis lupus;* Lucchini et al. 2002) and brown bears (*Ursus arctos*) in the Italian Alps (De Barba et al. 2010), and black bears (*Ursus americanus luteolus*) in the southeastern United States (Triant et al. 2004).

As with telemetry, long-distance movements provide a challenge for NGS monitoring as well. With NGS, it is only possible to detect animals where surveys are conducted. Without telemetry or other supplementary data to inform NGS survey efforts, it is easy to miss isolated pockets of individuals that have dispersed far from the core study area. Scat-detecting dogs (Wasser et al. 2004) or aerial surveys (J. L. Rachlow and J. H. Witham, University of Idaho - Moscow, unpublished data) can be used to improve detection in these cases, and are options we plan to explore in the future.

Local climate conditions can impose limitations on NGS for monitoring cryptic species in 2 ways: limiting sample detection (Halfpenny et al. 1995, Sanchez et al. 2009), and influencing DNA degradation rates. Heat and humidity can accelerate DNA degradation relative to samples deposited in cold and dry conditions (Lucchini et al. 2002, Piggott 2004, Murphy et al. 2007, DeMay et al. 2013). In our study, fecal sampling was dependent on snow.

Tracks, fecal pellets, and burrow activity detected on fresh snow guaranteed that the activity was recent, rather than weeks to months old. Additionally, pellets deposited on snow were kept cold and well-preserved, leading to our high success rates in the laboratory compared with samples deposited in warmer conditions (DeMay et al. 2013).

Cost

Our cost calculations indicated that NGS was more cost-effective (overall and per rabbit tracked and detected) than telemetry for our study. Costs per rabbit detected were similar, but the total cost per method and cost per rabbit tracked were approximately half as much for NGS than for telemetry. This difference is due in part to the number of rabbits tracked. Over 70% of the cost of telemetry went toward purchasing transmitters, so telemetry costs can increase or decrease markedly depending on how many animals are tracked. For NGS, >55% of the cost went toward personnel hours for surveying the release site and collecting fecal pellets. These costs for surveying a finite area would remain essentially fixed regardless of the number of animals 'tracked' within that area. Techniques for reducing costs include reusing recovered radiotransmitters, and soliciting volunteer help during surveys.

Juvenile pygmy rabbit dispersal and survival

Although we documented some longer distance movements, the average dispersal distance for settled rabbits was 204 m from their release site. Loss of signals from marked rabbits that moved longer distances likely biased our average downward; average natal dispersal distances for pygmy rabbits in Idaho were 1 km and 2.9 km for males and females, respectively (Estes-Zumpf and Rachlow 2009). Post-release dispersal is not the same as natal dispersal, and it is not known how the disturbance associated with relocation might alter normal natal dispersal behavior. In one study in East-central Idaho, juvenile pygmy rabbits

initiated natal dispersal at 2.5–12 weeks of age, completed their dispersal movement over about 1 week, and then did not move much subsequently (Estes-Zumpf and Rachlow 2009). In our study, released rabbits ranged from approximately 4 weeks to >12 weeks of age. If juveniles were released before the age at which they would have dispersed naturally, they could have dropped their transmitters before natal dispersal. Alternatively, rabbits released after the age at which they would have dispersed might not exhibit a marked dispersal movement.

Between summer telemetry monitoring and winter NGS sampling, rabbits continued to move. Like the telemetry data, the average distance between release sites and NGS winter sample sites was lower than natal dispersal distances documented by Estes-Zumpf and Rachlow (2009). The average NGS distance from release sites was larger than the average distance to telemetry settlements in part because our telemetry tracking was limited by transmitter loss during or prior to dispersal, and because NGS sampling occurred 4–6 months later than telemetry. Adult rabbits with established home ranges continue to shift seasonally across the landscape (Sanchez and Rachlow 2008), and we likely captured that movement of settled rabbits as well. We emphasize that these data are from 1 year of monitoring, and more in-depth analyses will follow as we apply what we have learned and continue to monitor the reintroduced population throughout its recovery.

Survival of pygmy rabbits is typically low and variable across time and space (Westra 2004, Sanchez 2008, Crawford et al. 2010, Price et al. 2010). Our NGS results indicated a minimum 37% survival rate to winter (not to be confused with annual survival). This rate is biased low because it accounts only for rabbits detected on or near the release site; we did not have the ability to detect long-distance dispersers. Results from a trial release in Idaho of

captive-reared pygmy rabbit kits indicated 32% annual survival, with significant differences in survival depending on season of release (Westra 2004). Survival of wild-born kits to the next breeding season in 2 populations in East-central Idaho was 19% (n = 58; Price et al. 2010). Continued monitoring of the Columbia Basin population will allow managers to evaluate the demographic and genetic responses to reintroduction, and continuously improve the recovery strategy for this endangered population.

Acknowledgments

We would like to thank the following funding sources: Association of Zoos and Aquariums, Oregon Zoo Foundation, Palouse Audubon Society, Phoenix Zoo, Riverbanks Zoo and Garden, Washington Chapter of The Wildlife Society, Washington Department of Fish and Wildlife, U.S. Fish and Wildlife Service, the University of Idaho Environmental Science Program, and the University of Idaho Department of Fish and Wildlife Sciences. We would also like to thank W. Knickerbocker and R. Irvin for their help processing samples in the lab, as well as W. Estes-Zumpf for sharing her previous work on development of microsatellite loci. We appreciate the constructive feedback from Dr. J. Homyack and 2 anonymous reviewers on an earlier draft of this article. Finally, we wish to thank the Waits and Rachlow lab groups for their continued feedback and support.

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Table 2.1. Comparison between telemetry using glue-on radiotransmitters and noninvasive genetic sampling (NGS) for studying survival and dispersal of juvenile small-bodied mammals.

Component	Telemetry	NGS
Study goal	Postrelease movements and survival	Long-term survival and reproduction
Handle animals?	Yes	Not necessary
	Can assess condition, treat for parasites, and collect other biological information	Can capture for collection of high-quality genetic reference sample
	Recapture for longer transmitter retention	
Weather conditions	Any	Cold and dry is best for DNA preservation
		Snow improves detection of fresh samples
Cost	$257/rabbit tracked	$123/rabbit tracked
	$346/rabbit detected	$303/rabbit detected
Method-specific limitations	Small transmitters limited in size, signal strength, battery life, and extra features	Can only detect animals where sampled and may need other data to inform sampling design
	Low signal strength can be challenging with semifossorial species, topography, rapid long-distance movements	DNA degradation
	Short transmitter retention time	

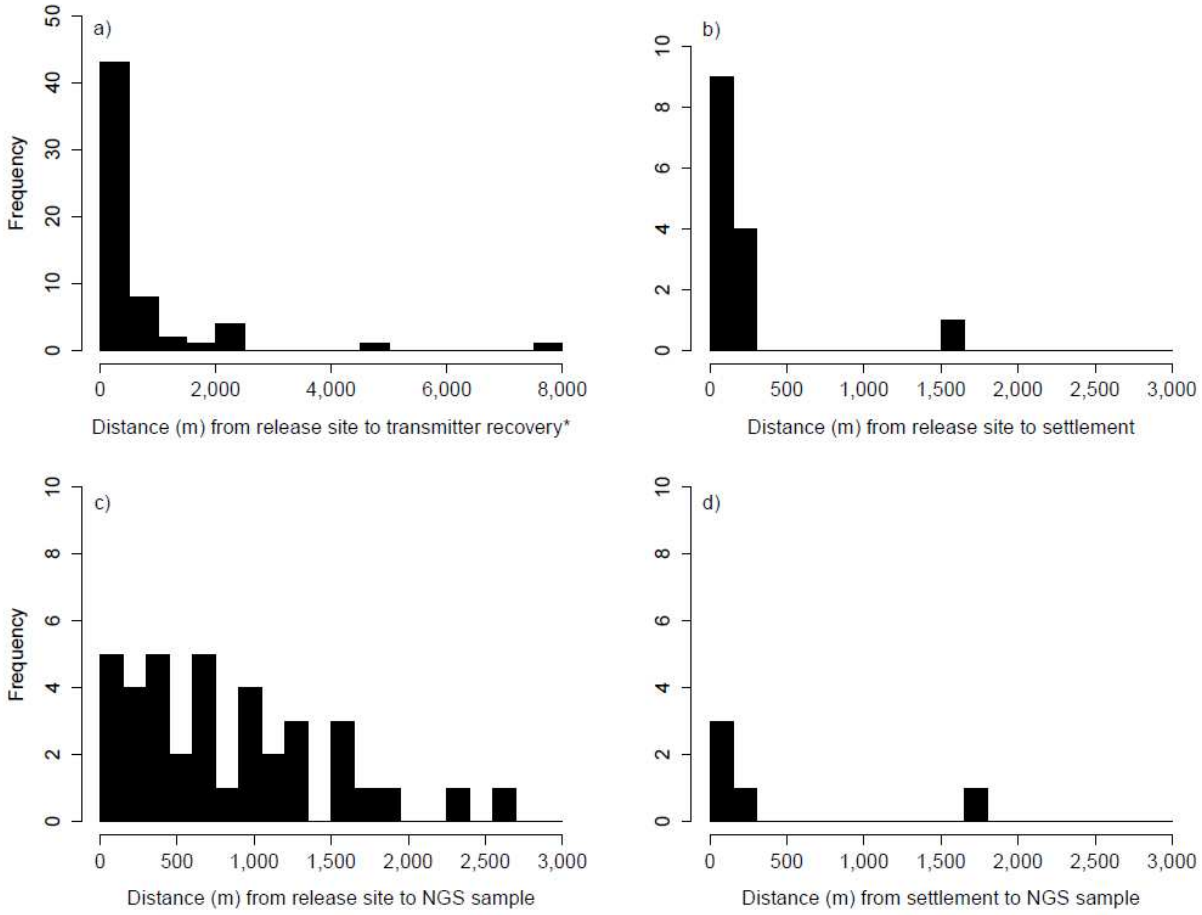

Figure 2.1. Distances moved by juvenile pygmy rabbits released on Sagebrush Flat Wildlife Area in central Washington, USA, during summer 2012 (a) from release site to radiotransmitter recovery locations for individuals tracked with telemetry during summer (n = 60; * note different axis scale); (b) from release site to settlement, defined as ≥3 successive telemetry locations at the same or adjacent mounds during summer (n = 14); (c) from summer release site to location where a noninvasive genetic sample (NGS: fecal pellets) was collected during winter surveys (n = 38); and (d) from summer settlement site to winter NGS collection

Chapter 3

Consequences for conservation: population density and genetic effects on reproduction of an endangered lagomorph

Accepted for publication: DeMay, SM, PA Becker, LP Waits, JR Rachlow (In Press). Consequences for conservation: population density and genetic effects on reproduction of an endangered lagomorph. Ecological Applications.

Abstract

Understanding reproduction and mating systems is important for managers tasked with conserving vulnerable species. Genetic tools allow biologists to investigate reproduction and mating systems with high resolution and are particularly useful for species that are otherwise difficult to study in their natural environments. We conducted parentage analyses using 19 nuclear DNA microsatellite loci to assess the influence of population density, genetic diversity, and ancestry on reproduction, and to examine the mating system of pygmy rabbits (*Brachylagus idahoensis*) bred in large naturalized enclosures for the reintroduction and recovery of the endangered distinct population in central Washington, USA. Reproductive output for females and males decreased as population density and individual homozygosity increased. We identified an interaction indicating that male reproductive output decreased as genetic diversity declined at high population densities, but there was no effect at low densities. Males with high amounts (>50%) of Washington ancestry had higher reproductive output than the other ancestry groups, while reproductive output was decreased for males with high northern Utah/Wyoming ancestry and females with high Oregon/Nevada ancestry. Females and males bred with an average of 3.8 and 3.6 mates per year, respectively, and we found no evidence of positive or negative assortative mating with regards to ancestry. Multiple paternity was confirmed in 81% of litters, and we report the first documented cases

of juvenile breeding by pygmy rabbits. This study demonstrates how variation in population density, genetic diversity, and ancestry impact fitness for an endangered species being bred for conservation. Our results advance understanding of basic life history characteristics for a cryptic species that is difficult to study in the wild, and provide lessons for managing populations of vulnerable species in captive and free-ranging populations.

Introduction

Globally, nearly 20% of vertebrate species are classified as Vulnerable, Endangered, or Critically Endangered by the International Union for Conservation of Nature (Hoffmann et al. 2010). Conservation of many of these species will depend on a combination of addressing the factors that threaten persistence of the species, and intensive management of populations in captivity and in the wild (Seddon et al. 2014). Understanding animal habitat needs and life history characteristics, including mating systems and factors influencing reproductive rates, is crucial for the conservation and management of these small and/or declining populations (Kruuk and Hill 2008, Pemberton 2008).

Mating systems fall along a complex continuum from monogamy to promiscuity (Clutton-Brock 1989) and can often be inferred from behavioral observations. With the exception of birds, monogamy is rare among vertebrates and various forms of polygamy are the norm (Emlen and Oring 1977). However, advances in molecular methods have revealed mating systems that are more complex than previously understood. Genetic mating systems have differed from observed social structures in many species including lions (*Panthera leo*; Lyke et al. 2013), marmots (*Marmota marmota*; Goossens et al. 1998), pronghorn (*Antilocapra americana*; Carling et al. 2003), lemurs (*Cheirogaleus medius*; Fietz et al. 2000), and many socially monogamous birds (Griffith et al. 2002). Genetic investigations have also

revealed multiple paternity (i.e., multiple sires for a single litter or clutch) in some species, a strategy by which females can increase their reproductive output by reducing the fitness consequences of breeding with an inferior male (Jennions and Petrie 2000, Stockley 2003, Uller and Olsson 2008).

Variation in mating and reproduction strategies among and within species can be driven by population density, mate availability, operational sex ratios, resource availability and distribution, physiology, and phylogenetic history (Emlen and Oring 1977, Clutton-Brock 1989, Reynolds 1996). Reproductive output has been shown to decline with increasing population density in many populations, as a result of direct competition for limited resources, elevated stress levels from intraspecific interactions, and/or an increased proportion of low-quality breeders at high densities (Arcese and Smith 1988, Wauters and Lens 1995, Coulson et al. 2000, Rödel et al. 2004, Dreiss et al. 2010). Individual genetic diversity can also influence reproductive rates, especially in small or declining populations experiencing high rates of inbreeding or genetic drift. Low genetic diversity of male and female breeders can lead to increased homozygosity and expression of deleterious genes, resulting in reduced pregnancy rates, low birth rates, and low juvenile survival and growth rates (Ruiz-López et al. 2012, Elias et al. 2013). Population density can interact with genetic diversity to influence reproductive output; high densities create a stressful environment that can alter the expression and fitness costs of low genetic diversity (Armbruster and Reed 2005, Fox and Reed 2011, Reed et al. 2012).

In managed populations where genetic rescue or translocations from other source populations have occurred, ancestry can influence fitness. Animals from more distant (geographically or genetically) sources might have lower fitness in the new environment than

animals from the locally adapted population, or they might have higher fitness if the local population suffers from inbreeding depression (Tallmon et al. 2004, Vergeer et al. 2004, Verhoeven et al. 2011, Funk et al. 2012). Animals from different source populations can display positive or negative assortative mating, leading to different implications for the managed population. If individuals mate selectively with animals like themselves, it can limit gene flow and increase inbreeding compared to a randomly mating population (Tregenza and Wedell 2000). Alternately, if individuals selectively choose mates more different from themselves, offspring can have increased fitness due to hybrid vigor, or decreased fitness if coadapted gene complexes are broken up (Penn and Potts 1999, Tallmon et al. 2004, Vergeer et al. 2004).

The intricacies of mating and reproduction can be challenging to study in wild populations of species that are uncommon or cryptic, exhibit little to no parental care, or experience high rates of juvenile mortality or dispersal. Genetic tools allow for an increased resolution to investigate these life history traits of such species (Andrew et al. 2013). We conducted genetic parentage analyses using microsatellite loci to study mating and reproduction in a semi-captive population of pygmy rabbits (*Brachylagus idahoensis*) in the Columbia Basin of central Washington, USA. The Columbia Basin distinct population segment (DPS) of pygmy rabbits is listed as Endangered under the United States Endangered Species Act (Federal Register 2003). The semi-captive population has been augmented with translocated pygmy rabbits from other portions of the species' range, for both genetic and demographic rescue, and the rabbits produced in the breeding enclosures are being reintroduced to the wild in Washington. Pygmy rabbits are cryptic, birth and nursing of young occur quickly at concealed natal burrows, and the species exhibits relatively little parental

care (Rachlow et al. 2005, Elias et al. 2006), making mating and reproductive habits of this species difficult to study in the wild. As a result, many aspects of their behavior and reproductive ecology have only been documented in captivity, where behaviors may differ from those of free-ranging populations.

The objectives of this study were to 1) document reproductive output of male and female pygmy rabbits, 2) estimate the influence of genetic diversity, population density, ancestry, maternal age, and multiple paternity on reproductive output, and 3) improve understanding of the mating system of the species. We predicted that yearly reproductive output for both males and females would decrease with population density as a result of increased competition for resources and social stress at high densities. We predicted an increase in reproductive output with increasing individual genetic diversity, with an interaction whereby the detrimental effects of low genetic diversity would be more strongly expressed in stressful environments (high population densities). We expected individuals with high amounts of Washington ancestry to have relatively low reproductive output because of a history of inbreeding depression in the population (Elias et al. 2013). Alternately, individuals with high Washington ancestry might show relatively high reproductive output due to local adaptations not found in individuals from other sources. We also predicted that females with litters sired by multiple males would produce more offspring (kits) than those that produced only single-sire litters because multiple paternity would allow for superior males to compete and contribute to litters even after a given female had already mated with an inferior male. This research contributes empirical insights about the demographic consequences of differences in mating strategies, genetics, and population density for an endangered

population undergoing conservation breeding and provides guidance for biologists managing populations of rare or declining species.

Methods

Study system

Pygmy rabbits are obligate burrowers that occur in sagebrush (*Artemisia spp*.) steppe habitats in the western United States (Dobler and Dixon 1990). Most of what is known about mating and reproduction by pygmy rabbits was learned from work at off-site captive breeding facilities established to recover the endangered Columbia Basin DPS (Elias et al. 2006, USFWS 2012). After initial low reproductive output and survival, pygmy rabbits from Idaho were incorporated into the captive population in 2003 to counteract inbreeding depression (Elias et al. 2013, USFWS 2012). In captivity, males and females were paired strategically for breeding to maximize the genetic diversity of the resulting offspring (WDFW 2011). Due to continued low juvenile survival and lack of sufficient space in captivity, the captive breeding program was phased out by 2012, and all captive rabbits were transferred to large naturalized breeding enclosures constructed in the species' natural habitat in their historic range in Washington. Wild pygmy rabbits translocated from other states were also placed in the enclosures to boost the total numbers and genetic diversity of rabbits available for breeding and subsequent reintroduction. This conservation strategy presented a unique opportunity to study mating and reproduction in a more natural setting than the off-site captive breeding facilities. Because enclosures were stocked with known rabbits, nearly all of the candidate adult breeders and kits were known and genetically sampled over the course of this study.

This study was conducted in the Columbia Basin of central Washington, USA, at multiple sites within the historic range of the pygmy rabbit in Grant and Douglas Counties. This region has a semi-arid climate, and the dominant native vegetation is big sagebrush (*Artemisia tridentata*) with interspersed grasses and forbs. Adult pygmy rabbits were housed in large predator-resistant enclosures for breeding (17-49 breeding adults per enclosure throughout the study), and subsequently, juveniles and adults were released to reestablish a free-ranging population. Enclosure fences about 2 m tall were buried approximately 45 cm into the ground and featured a 'floppy top' to provide protection from terrestrial predators, while protective netting over burrow systems and bird spikes installed on fence posts discouraged avian predators. Regardless, over the course of the study, 4 weasels were removed from the enclosures, one from each enclosure, and avian predations did occur in unprotected matrix between covered burrow systems. In 2012, two enclosures (2.3 and 4.4 ha) were established at one site, approximately 150 m apart at their closest point. For the 2013 breeding season, a third enclosure (2.2 ha) was added at a second site, 25 km to the north. For the 2014 breeding season, a fourth enclosure (3.8 ha) was constructed 17 km southeast of the first two. Adults held in these enclosures included individuals from the captive breeding program with mixed Washington/Idaho ancestry, wild pygmy rabbits translocated from Oregon, Nevada, Utah, and Wyoming, and their mixed-ancestry offspring. We provided artificial burrows consisting of buried 10 cm diameter plastic drainage tubing, approximately 1 m long, with the bottoms cut out to allow for rabbits to dig additional tunnels, as well as segments of drainage tubing placed above ground to provide temporary shelter for adults and kits until they could dig burrows. Supplemental commercial rabbit food (Purina Rabbit Chow-

Professional, Purina Animal Nutrition LLC, Minneapolis, Minnesota) and green alfalfa hay were provided *ad libitum*, as well as free water during the hot and dry summer months.

Field methods

Throughout spring and summer 2012-2014, kits were captured from the enclosures and released to the wild. Breeding seasons typically began in February, and kits began to emerge from natal burrows by March. Births of kits ended by late June or early July, but capture and releases of kits continued beyond the end of breeding (2012: July; 2013: August; 2014: November). Prior to release, we weighed and sexed kits, treated them for parasites using Advantage II kitten formula (BayerDVM, Shawnee Mission, KS, USA), and collected a 3-mm tissue biopsy from the ear for genetic analysis. Tissue samples were stored in 95% ethanol and frozen at -20°C until analysis. All adults placed in the enclosures were previously sampled for genetic analysis. A subset of kits, typically 10-20 per enclosure per year, shown to have relatively high Washington ancestry (analysis not detailed here) were swapped among the enclosures to simulate gene flow and were retained over winter for breeding during subsequent years. Because we were unable to capture and sample every kit produced in the enclosures, unsampled kits also were retained in the enclosures and contributed to reproduction. We collected genetic samples opportunistically for these unsampled rabbits when they were incidentally captured in later years. We also sampled all individuals found dead in the enclosures from predation or other causes. These methods were approved by the University of Idaho Animal Care and Use Committee (Protocol 2012-23), are consistent with standards for use of wild mammals in research established by the American Society of Mammalogists (Sikes and Gannon 2011), and were performed in accordance with applicable laws governing the use of endangered species.

Analysis methods

We generated microsatellite genotypes in duplicate from each tissue sample at 19 microsatellite loci, including a sex identification locus, following the methods of DeMay et al. (2015). Due to the recent mixing of multiple populations, loci were not expected to be in Hardy-Weinberg or linkage equilibrium across the entire recovery population, but separate analyses of two source populations indicated that loci were in equilibrium (DeMay et al. 2015). We assigned parentage using a strict exclusion approach using Cervus 3.0 (Marshall et al. 1998, Kalinowski et al. 2007). Parentage assignments that matched at all but 1 or 2 loci were checked manually for genotyping errors. A mismatch at a single locus representing a single stepwise mutation (offspring allele differs from parental allele by 2 base pairs for a dinucleotide microsatellite or 4 base pairs for a tetranucleotide microsatellite) was accepted as a match. Parentage assignments were used to determine the annual number of kits per parent, number of mates per parent, number of litters per female, number of fathers per litter, and the percentage of litters with multiple sires. In March 2014, a total of 20 adult males were released to the wild from two enclosures to alleviate crowding. Although many of those males were able to sire kits prior to their removal, they were not included in calculations of yearly male reproductive output because they were not available to reproduce in the enclosures during the entire breeding season.

Because kits from the same litter were captured at different times throughout the capture period, we used two previously constructed growth curves for pygmy rabbits (Estes-Zumpf and Rachlow 2009, Elias et al. 2013) to estimate the date of birth for each kit and group kits from the same mother into litters. When assigning kits to litters, we took into account a 22-25 day gestation period between litters (Elias et al. 2006) and the potential for

considerable variation in weights (≥100 g) within a litter (B. Elias, unpublished data). We excluded from litter assignment kits weighing >300 g, because growth rates begin to approach an asymptote and the resulting birth date estimates are less reliable. Data were included to estimate the number of litters per mother and effect of multiple paternity on female reproductive output if two conditions were met: at least 3 kits from a given mother were sampled and at least half of the kits from that mother were assigned to a litter. We could not sample any kits that evaded capture during the duration of the study or died and were not recovered. As a result, our count of kits and litters produced annually is conservative. The prevalence of > 300 g kits unassigned to a litter in later years also led to a conservative estimate of multiple paternity and its effect on reproductive output. We used a paired t-test to test whether there was a difference in reproductive output between a female's first and second breeding season. The ages of wild translocated rabbits were unknown, so most of the rabbits with known ages were those born in the enclosures starting in 2012. Because of this, we were unable to examine how female reproduction changed at later ages.

We performed two-sided Fisher's exact tests to test whether mating patterns differed from random mating among ancestry groups (group determination described below). In a given enclosure for each female of a given ancestry, we counted the number of matings with males with the same and different ancestry, and compared these frequencies to the number expected under random mating given the availability of males in each enclosure. We used the number of matings rather than the number of mates so that if the same male sired kits in 2 separate litters, it counted as two matings, or two times that the female selected that male. We tested for assortative mating in only 4 of the 9 enclosure-years. 2014 was excluded because of the high prevalence of kits unassigned to litters, making it ambiguous how many matings

occurred. We also excluded one enclosure in 2013 that had a high prevalence of parents and pairings with "mixed" ancestry.

We used mixed-effects Poisson regression to model the number of kits produced per female per year and modeling was performed using the R package 'lme4' (Bates et al. 2014). While we could not sample every kit produced in the enclosures, we had no evidence to suggest that emerged kits were not missing at random, so we did not model an explicit missing data process that would require multiple unsupported assumptions. We included a random intercept for each mother to account for innate differences between mothers, some of which bred in multiple years. We generated a candidate set of 22 models, with the full model including adult population density, homozygosity by loci, a density by homozygosity interaction term, ancestry, year, and enclosure. Ancestry and homozygosity were individual-level variables, while density was constant for all individuals in each enclosure each year, allowing for the analysis of population-level effects of density while controlling for the effects of year and enclosure, as well as individual-level analyses of the effects of ancestry and homozygosity. Realized values of population density formed two distinct groups (5-10 and 16-22 adults/ha), so we used a categorical variable (low or high density) in the models. Homozygosity by loci was estimated for each individual using the R package 'Rhh' (Alho et al. 2012, R Development Core Team 2014). Ancestry groups for this analysis included 1) Washington, 2) Oregon/Nevada, 3) northern Utah/Wyoming, and 4) southern Utah (Table 3.1). These categories were based on a prior analysis using Structure 2.3.4 (Pritchard et al. 2000, Falush et al. 2003, 2007, Hubisz et al. 2009) to group the recovery population into genetic clusters (unpublished data). Ancestry was determined by pedigree, and any individual with 51-100% of their ancestry from any one founding group was assigned to that group.

Individuals with no single founding population accounting for > 50% of their ancestry were classified as mixed. We did not have adequate sample sizes to further subdivide ancestry categories. Each rabbit with solely captive ancestry contained some Idaho ancestry (average 72% Washington/28% Idaho) from the genetic rescue initiated in 2003. A single female in our study contained 58% Idaho ancestry (42% Washington). Rather than creating a new category with a sample size of 1, we classified that female as mixed.

Paternal reproductive output is inherently limited by the number of females available, so values of kits produced per male are not directly comparable across enclosures and years with different numbers of breeding females. To account for this, we used mixed-effects Poisson regression to model the number of kits per father per year, with an offset of the log of the number of available breeding females so as to model the *rate* of kits produced per available breeding female. Fixed effects in the full model and candidate set were identical to those in the female reproductive output models, and models included a random intercept for individual father. We evaluated models using AICc, and model weights were generated to identify the 95% confidence set of top models (Burnham and Anderson 2002). We used the R package 'AICcmodavg' (Mazerolle 2014) to generate parameter estimates and 85% confidence intervals from the top model set based on recommendations by Arnold (2010). We used the top model for males and females to test specific hypotheses comparing different ancestry groups using the R package 'multcomp' (Hothorn et al. 2008). Rabbits from different ancestry groups were not present in equal frequencies in the enclosures because of logistical constraints. To determine if our ancestry and enclosure results were confounded by the *distribution* of ancestry within enclosures, we ran our top models with additional variables describing the percent composition of each enclosure for 4 of the 5 of the ancestry groups

(note that because the total percent is 100% it is necessary to omit one of the five percentages for model identification). Controlling for enclosure composition was not supported by AICc scores, and did not fundamentally change the results, so we proceeded with the analyses assuming that the variables were not confounded.

Results

The total numbers of pygmy rabbit kits produced in the breeding program increased each year with the addition of more enclosures and consequently more breeding adults (Table 3.2). Two females and 3 males bred in all 3 years of this study, 28 females and 37 males bred in 2 years, and 137 females and 127 males produced kits during only 1 year. The prevalence of animals that bred in only 1 or 2 years was due in large part to the majority of breeders being born or translocated to the enclosures during the course of the study, thus not being available to breed in all 3 years. Nearly all sampled adult pygmy rabbits in our study bred. In 2014, we captured 63 males and 84 females during the breeding season to microchip and treat for parasites and return to the enclosures. Of those, only 6% (4 males and 5 females) did not produce any kits that we sampled, although it is possible that they produced kits that died or evaded capture.

Annual kit production for females and males varied markedly. Female reproductive output ranged from 1-33 kits in a year (excluding females for which 0 kits were sampled), with an average of 7.5 kits per breeding female (SD = 5.16). We documented a range of 1-5 litters per mother in a year, with an average of 2.5 litters per year (SD = 0.96). Male reproductive output ranged from 1-33 kits in a year, with an average of 7.0 kits per breeding male (SD = 5.44). After adjusting for the number of available females, males produced on average 0.46 kits per female in a year (SD = 0.58), ranging from 0.02-4.13. Females produced

kits with an average of 3.8 different mates per year (SD= 1.95, max = 10), and males produced kits with an average of 3.6 mates per year (SD = 1.6, max = 8). There were 15 females of known age that reproduced at ages 1 and 2, and a paired 2-tailed t-test indicated no significant difference in the number of kits produced at the different ages (t=0.364, df=14, P = 0.72). We were unable to capture and sample every kit produced in the enclosures, which created gaps in parentage analyses as unsampled kits matured and bred. In 2012, 6% of sampled kits had one unsampled parent, and none had both parents unsampled. By 2014, 20% of kits had 1 unsampled parent, and 6% had 2 unsampled parents.

Factors influencing reproductive output

For both male and female pygmy rabbits, all of our fixed effects appeared in the 95% confidence model set (Table 3.3). Predicted rates of female reproduction were 1.9 times higher at low densities compared to high, while predicted rates of male reproduction per female were 5.9 times higher at low densities (Table 3.4). The predicted rate increase for males was larger than that for females because at low densities, there were fewer males competing to sire the increased number of kits that females produced. Parameter estimates for homozygosity by loci indicated that for any increase of 0.25 (homozygosity ranges from 0 to 1), reproductive output was predicted to decline by 25% for females and 33% for males.

Our data showed a density by homozygosity interaction in male reproductive output as predicted, but for females, addition of the interaction was not supported by a reduction in AICc scores. The two models included in the 95% confidence set for males differed only in the presence of the interaction term, and the model including the interaction received higher support, so we investigated the interaction further *post hoc*. We ran the full (top) male model separately for observations at high and low population densities. At low densities,

homozygosity did not significantly influence reproduction (β= -0.34, Z = -0.50, P = 0.62), but at high densities, reproductive output significantly declined with increasing homozygosity (β = -1.75, Z = -2.72, P = 0.007).

Reproductive output varied across time and space during this study. Adjusted male reproduction in 2014 was higher compared to the reference year of 2012, but year did not influence female reproduction. Compared to the reference enclosure (the first enclosure built), 2 enclosures had lower reproductive output, and one had higher reproductive output.

As predicted, ancestry influenced reproductive output of both sexes, but not in the same way. All models in the top model sets for males and females included ancestry. Washington ancestry did not significantly influence female reproductive output, but males with primarily Washington ancestry produced 1.6 times more offspring than those of mixed ancestry. Males with primarily northern Utah/Wyoming ancestry produced 0.48 times fewer offspring than the reference category of mixed ancestry, and females with primarily Oregon/Nevada ancestry produced 0.84 times fewer kits than mixed females. General linear hypothesis tests using specified contrasts reinforced these trends, showing a significant increase in reproduction for male rabbits with primarily Washington ancestry compared to other ancestries, and a significant reduction in reproduction for males with northern Utah/Wyoming ancestry compared to Oregon and Nevada, but no significant differences between contrasted female groups (Table 3.5). Our results, however, might have been skewed by a lower sample size for primarily Washington ancestry rabbits compared to other ancestries and by reproductive performance of one male Washington rabbit that sired 33 kits in a year, the maximum in our dataset. When analyses were run without this individual, model averaged parameter estimates indicated no significant difference between reproductive output

for primarily Washington ancestry males compared to the reference category of mixed, but general linear hypothesis testing still showed a significant increase in production for male Washington rabbits compared to all other ancestry groups (P = 0.03).

Mating system

Multiple paternity of pygmy rabbit litters was common in the breeding enclosures. Of the litters from which at least 3 kits were sampled, 81% of litters (50/62) were sired by multiple males. Litters with fewer than 3 kits sampled were not considered because there was a lower chance of detecting multiple paternity in litters with 2 sampled kits, and no chance of detecting multiple paternity when only a single kit was sampled. Detection of multiple paternity in litters with 3 sampled kits did not differ from those with > 3 sampled kits (Fisher's exact test, P = 1.00). Litters with 3-4 kits assigned (n = 42) were sired by an average of 2.2 males, and litters with 5-7 kits assigned (n = 18) were sired by an average of 2.9 males. One litter included kits sired by 5 different males, and 5 litters were sired by 4 males. Mothers that produced at least one litter with multiple paternity (n = 39) produced on average 2.4 more kits annually than those that produced only single-sire litters (n = 7), but the difference was not statistically significant (11.7 vs. 9.3 kits respectively, $t_{13.4} = -1.25$, P = 0.12). Two-sided Fisher exact tests comparing the observed distribution of "same" and "different" matings with the distribution of available males in each enclosure yielded p-values ranging from 0.27 to 1.0, providing no evidence of positive or negative assortative mating by ancestry in these populations.

An unexpected result from this study was the first documentation of juvenile breeding by pygmy rabbits. When no known adults could be confirmed genetically as parents of a given kit, we ran the parentage analysis using all rabbits for which we had genotypes, kits and

adults, as candidate parents in case some adults were mistakenly classified as kits. In 2013, we identified a female kit of the year (birth month inestimable due to 540 g weight at first capture on 15 July) that produced 4 kits in one litter by mid-May. Another female kit of the year born mid-March produced 7 kits in 2 litters in early and late June. In 2014, we identified another female juvenile breeder (birth month inestimable due to 546 g weight at first capture on 31 July) that produced 2 kits during its first summer. Sires of the resulting kits were identified in all cases. There was no possibility that juvenile breeders were adults misclassified as kits. In 2 out of 3 cases, the juvenile breeders were identified during the first year of production in their respective enclosures, when all adults placed in the enclosures were known. Further, parentage analysis identified both parents of the juvenile breeders from the known adults. In the third case, both parents of the juvenile breeder were identified and examination of the pedigree precluded the juvenile breeder from being a misclassified adult. Both of the juvenile breeders identified in 2013 went on to produce litters in 2014 as well.

Discussion

In this study, we applied genetic tools to obtain detailed reproductive information about an endangered species that is difficult to study in the wild, so that this information can be used to adaptively manage the ongoing conservation breeding program. As predicted, reproductive output for both males and females was influenced by population density, individual genetic diversity, and ancestry, suggesting that efforts to enhance breeding will require the management of numbers of individuals in breeding populations and the facilitation of gene flow in the population to promote and maintain genetic diversity over time while preserving local adaptations. Both sexes bred with multiple mates during a breeding season, and these results support the contention that this species exhibits a promiscuous mating

system. We discovered that multiple paternity was common, and that it increased reproductive output for females, supporting the practice of breeding this species in natural settings where individuals have access to multiple mates. In addition, we documented the first evidence of breeding by juvenile female pygmy rabbits that were previously believed to breed for the first time at one year of age. These data not only add to our understanding of life history for this species, but provide lessons for those tasked with conserving or managing small wildlife populations.

Reproductive output

General patterns of female reproduction in the breeding enclosures were similar to those documented during the captive breeding program. The average number of litters per female that we documented matched closely to those reported from breeding in captivity by Elias et al. (2013), although we were unable to assign every kit to a litter based on their weight. Dividing the average number of kits by the average number of litters per female in our study yielded an average of 3.0 emerged kits per litter. Litter size in captivity averaged 4.2 kits per litter, only half of which survived to emergence (Elias et al. 2013), indicating that litter size and/or neonate survival is higher in the large breeding enclosures than in captivity, where inbreeding depression and disease contributed to high juvenile mortality rates. It is important to note that for this study, the starting point for inference about reproductive output is not at birth, but at juvenile emergence from the natal burrow at 2 weeks of age, because we had no interaction with pre-emerged kits. Therefore, observed trends in reproductive output as a result of our explanatory variables are likely due to a combination of effects on birth rates and pre-emergence survival rates. While we do not have the ability to differentiate the effects of these two processes, inference about the number of kits emerged is of arguably higher

relevance for managers and conservation practitioners than the number of kits born that do not survive to emergence.

Both females and males experienced lower reproductive output as population density increased. Competition for food is an unlikely explanation for this trend because supplemental food was continually provided in the enclosures. The decline in female reproductive output with density might be related to competition among females for natal burrow sites or increased sociogenic stress taking a physiological toll on the breeding females. Scarlata et al. (2012, 2013) monitored stress hormones in captive pygmy rabbits and reported that housing conditions influenced stress levels, and high stress hormone levels suppressed reproduction. Myers and Poole (1962) observed that high population densities led to increased embryo resorption rates and decreased post-partum conception rates in female European wild rabbits (*Oryctolagus cuniculus*), and Marchlewska-Koj (1997) described multiple examples whereby crowding elevated stress levels and suppressed reproduction in several rodent species. Density was also confounded with duration of occupancy in our study; older enclosures held higher densities of rabbits than newer enclosures. Both high densities and long occupancy likely contributed to habitat degradation through similar mechanisms, namely a decrease in natural forage, an increase of invasive weeds as a result of soil disturbance and seeds coming in with alfalfa hay provided as supplemental food, and a potential increase in disease loads in the soils and animals (Wilby et al. 2001, Harrenstien et al. 2006).

In our system, pygmy rabbits were held at artificially high densities, well above the range described for wild populations. For example, Price and Rachlow (2011) documented a maximum density of 0.46 pygmy rabbits/ha in southeastern Idaho, compared with our range of 5-22 adult rabbits/ha. Consequently, these results should not be used to predict the response

to changes in density of wild populations that are below carrying capacity. However, population size is carefully managed in captive breeding programs, where biologists must weigh demographic, genetic, financial, and logistical considerations when determining how many animals to maintain (Ebenhard 1995, Snyder et al. 1996). In our study system, when population density increased by a factor of 2-3, annual reproduction by females fell by ~50%. Thus, managers of captive populations must weigh the population-level gains of having more breeders against the individual-level losses associated with higher population densities.

Decreased individual genetic diversity led to decreased reproduction by males and females. Similar results have been observed in captivity and in other species, with lower pregnancy rates, sperm quality, juvenile growth rates, and juvenile survival associated with increased homozygosity from inbreeding (Chapman et al. 2009, Ruiz-López et al. 2012, Elias et al. 2013). We identified an interaction between population density and homozygosity in male reproductive output. Only at high densities did the expected decline in reproduction with lower genetic diversity occur. Environmental stress has been shown to influence the magnitude of fitness-heterozygosity correlations in several plant, invertebrate, and vertebrate species (Armbruster and Reed 2005, Fox and Reed 2011, Reed et al. 2012). Environmental stress can alter how many deleterious alleles are expressed and increase the fitness costs of expressing certain deleterious alleles, making inbred individuals more sensitive to physiological stressors than their more genetically diverse counterparts (Reed et al. 2012). These results support the decision of managers to increase the genetic diversity of the pygmy rabbit recovery population by translocating wild rabbits from other portions of the species' range, and underscore the importance of maintaining healthy levels of genetic diversity in captive and wild populations. Given the rapid change in habitats for many free-ranging

species, managing for genetic diversity will help ensure that vulnerable populations are better able to persist in the face of new stressors.

Our data revealed differences in reproductive success among individuals with different ancestries. Males with primarily Washington ancestry reproduced at higher rates than those with other genetic backgrounds. This indicates that there may be locally adapted genes in the Washington pygmy rabbits that increase fitness in Washington relative to pygmy rabbits translocated from outside sources. Inbreeding depression was evident in captivity, where measures of reproductive success were depressed for males and females with 100% Washington ancestry; but those with 0-99% Washington ancestry had similar rates of reproductive success (Elias et al. 2013). No pygmy rabbits in our study had 100% Washington ancestry (maximum 78% for both males and females). The increased performance of Washington ancestry that we documented in males suggests that the genetic rescue was successful in decreasing harmful effects of inbreeding depression, and underscores the challenges that managers face in balancing the management for genetic diversity while preserving local adaptations of native populations.

Variation in reproductive output across different ancestries was more pronounced for males than females, suggesting that female reproduction might be limited more strongly by other environmental or individual characteristics than by heritable traits. Our study used adaptively-neutral nuclear DNA microsatellite loci, but further study using adaptive loci might illuminate functional differences among pygmy rabbit populations (Holderegger et al. 2006).

Mating system

Our results enhance our understanding of the promiscuous pygmy rabbit mating system. We observed both males and females breeding with an average of 3-4 mates per year. Pygmy rabbits are not thought to aggressively defend territories (Sanchez 2007), so variation in the number of mates might be driven in large part by mate choice and sperm quality (Andersson and Simmons 2006). We found no deviation from random mating in the enclosures, indicating that individuals were not selectively choosing mates with either the same or different ancestry.

In addition to multiple mates for different litters within a breeding season, multiple paternity within litters was common in our study. Females producing litters with multiple males produced on average 2.4 more kits per year than those producing only litters sired by a single male. The only previous study of multiple paternity in free-ranging pygmy rabbits identified multiple paternity in 2 out of 2 litters tested (Falcón et al. 2011). Multiple paternity has been documented in other lagomorph species as well; Burton (2002) identified multiple paternity in 4 of 16 snowshoe hare (*Lepus americanus*) litters.

In the captive breeding program for pygmy rabbits, females were usually paired with one male at a time, selected based on recommendations from the program's genetic management plan to maintain high levels of genetic diversity (WDFW 2011). With potential fitness benefits to multiple mating (Jennions and Petrie 2000, Stockley 2003, Uller and Olsson 2008), the numerical benefit of multiple mating should be considered as well as genetic diversity when pairing individuals for captive breeding. Although the highly-controlled captive breeding phase of the Columbia Basin pygmy rabbit recovery is over, these lessons from natural mating behaviors are important for captive breeding programs for other

species, particularly those with promiscuous mating systems or the potential for multiple paternity.

Our analyses provide the first evidence of breeding by juvenile female pygmy rabbits. Juvenile breeding has been documented in other Leporids as well (*Sylvilagus floridanus*, Negus 1959; *S. nutallii*, Powers 1971; *S. audubonii*, Sowls 1957; *S. transitionalis*, Chapman et al. 1977; *S. aquaticus*, Hunt 1959; *Oryctolagus cuniculus*, Brambell 1944). Juvenile breeding was not common in our study, with only 3 confirmed kit breeders over 3 years, but most kits were released to the wild before they could have bred in the enclosures. However, we expect juvenile breeding rates in the wild to be lower than those of enclosure-born kits, where rabbits likely experienced higher growth rates and better body condition than their wild counterparts.

Conclusion

The semi-captive breeding enclosures of the Columbia Basin pygmy rabbit recovery program provided a unique opportunity to learn about the life history of a cryptic endangered species and examine the effects of genetic diversity, population density, and ancestry on a component of fitness. Our results underscore the need to manage for genetic diversity, to take steps to reduce potential outbreeding depression as well as inbreeding depression when translocating animals for conservation, to monitor density effects, and to consider the mating system of the target species when designing conservation breeding programs. Results from this and similar studies can be used in conservation planning to simulate the effects of different management strategies on population trajectories. As threats to the persistence of species increase worldwide, managers must continually refine conservation strategies, to make efficient use of limited space and resources in captive breeding programs and

effectively manage populations of vulnerable species in their native habitat to meet conservation goals.

Acknowledgments

We would like to thank the following funding sources: Association of Zoos and Aquariums, Oregon Zoo Foundation, Palouse Audubon Society, Phoenix Zoo, Riverbanks Zoo and Garden, Roger Williams Park Zoo, Washington Chapter of The Wildlife Society, Washington Department of Fish and Wildlife, U.S. Fish and Wildlife Service, the University of Idaho Environmental Science Program, and the University of Idaho Department of Fish and Wildlife Sciences. We thank W. Knickerbocker, C. Sink, K. Potterf, and Z. Andrews for their assistance processing samples in the lab. Thank you to the hard working Washington Department of Fish and Wildlife staff and the countless volunteers who helped capture, process, and release rabbits in the field. We thank the individuals involved in the captive breeding program at Washington State University, the Oregon Zoo, and Northwest Trek Wildlife Park for their contributions to the continuation of this species and the research conducted in captivity. We wish to thank the Waits and Rachlow lab groups for their continued feedback and support, L. Shipley for helpful insights, and three reviewers for their help improving this manuscript.

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Table 3.1. Sample sizes of adult pygmy rabbits breeding in large enclosures in central Washington, USA. Individuals were assigned to an ancestry category if >50% of their pedigree-derived ancestry was composed of a single ancestry. Individuals were categorized as mixed if no single ancestry made up >50% of their genetic composition. Categories were derived from a separate analysis grouping the recovery population into genetic clusters (unpublished data).

	Washington	Northern Utah/Wyoming	Oregon/Nevada	Southern Utah	Mixed
Females	11	33	46	3	53
Males	8	45	48	8	39

Table 3.2. Sample sizes of kits, parents, and litters detected during 2012-2014 in the breeding enclosures of the Columbia Basin pygmy rabbit recovery project in central Washington, USA. Numbers of kits sampled and breeding females and males were detected by genetically sampling kits produced in the enclosures and performing parentage analyses. Kits unassigned to a litter either 1) could not be aged due to weight >300 g at first capture, 2) could not be reliably assigned to a litter based on their estimated birth date, or 3) could not be assigned a mother due to incomplete sampling of breeders. Values are given of kits with one or more unsampled parents. Further, we provide the number of litters that met our criteria for inclusion in our number of litters per female per year calculation (> 3 kits sampled from a given mother, and >50% of those kits assigned to a litter), and the number of mothers meeting the same criteria that did or did not produce any litters with multiple paternity.

Year	Kits sampled	Female breeders	Male breeders	Kits unassigned to litter	Unsampled mother only	Unsampled father only	Both parents unsampled	Litters	Mothers producing multiply-sired litters	Mothers producing singly-sired litters
2012	178	20	22	58	6	5	0	38	10	3
2013	449	47	55	252	31	31	2	61	14	2
2014	793	100	90	540	68	94	49	61	15	2

Table 3.3. Top models (95% confidence set) and intercept-only model predicting reproductive output of female and male pygmy rabbits in the recovery program for the endangered distinct population segment in the Columbia Basin of central Washington, USA, during 2012-2014. Female reproductive output was modeled using mixed-effects Poisson regression on the number of kits produced per female in a breeding season. Male reproductive output was modeled using Poisson regression on the number of kits produced per male in a breeding season, offset by the number of available breeding females. Random intercepts for individual mother and father were included in each model. Explanatory variables include population density (Dens: high or low), homozygosity by loci (HL), ancestry (Anc), year, and enclosure. AICc, ΔAICc, Akaike weight (w_i), and cumulative weights are given.

Model	n	Variables	AICc	ΔAICc	w_i	$\sum w_i$
Female reproduction	167	Dens + HL + enclosure + Anc	809.73	0.00	0.66	0.66
		Dens + HL + Dens*HL + enclosure + Anc	811.96	2.23	0.22	0.87
		Dens + HL + year + enclosure +Anc	813.58	3.85	0.10	0.97
		Intercept only	969.69	159.97	0.00	1.00
Male reproduction	167	(Full) Dens + HL + Dens*HL + year + enclosure + Anc	869.50	0.00	0.79	0.79
		Dens + HL + year + enclosure + Anc	872.13	2.63	0.21	1.00
		Intercept only	1296.70	427.20	0.00	1.00

Table 3.4. Model-averaged parameter estimates and 85% confidence intervals (CIs) from the 95% confidence set of top models predicting female and male reproductive output of Columbia Basin pygmy rabbits in the recovery program during 2012-2014. Models including the density-homozygosity interaction were not used to generate parameter estimates for the density and homozygosity main effects. The reference level for the year variable is the first year (2012), the reference level for the enclosure variable is the first enclosure that was constructed in 2011, and reference level for ancestry is mixed ancestry. Parameter estimates shown in bold have 85% CIs that do not overlap 0.

Variable	Female Estimate	85% CI		Male Estimate	85% CI	
		Lower	Upper		Lower	Upper
Intercept	2.19	2.00	2.37	-1.47	-1.83	-1.11
Density (low)	**0.65**	**0.46**	**0.85**	**1.78**	**1.31**	**2.25**
Homozygosity	**-1.17**	**-1.91**	**-0.44**	**-1.63**	**-2.44**	**-0.81**
Year (2013)	0.10	-0.10	0.31	0.22	-0.04	0.49
Year (2014)	0.20	-0.11	0.50	**0.64**	**0.27**	**1.00**
Enclosure (2)	**-0.43**	**-0.57**	**-0.28**	**-0.80**	**-1.00**	**-0.61**
Enclosure (3)	**0.21**	**0.04**	**0.38**	**0.57**	**0.33**	**0.82**
Enclosure (4)	**-0.44**	**-0.75**	**-0.12**	**-1.34**	**-1.79**	**-0.89**
Ancestry(WA)	-0.05	-0.34	0.23	**0.46**	**0.13**	**0.79**
Ancestry(northUT/WY)	0.03	-0.22	0.29	**-0.74**	**-1.01**	**-0.46**
Ancestry(OR/NV)	**-0.18**	**-0.33**	**-0.02**	-0.08	-0.29	0.12
Ancestry(southUT)	-	-	-	-0.43	-0.87	0.01

Table 3.5. Results from general linear hypothesis tests of contrasts comparing reproduction of pygmy rabbits with differing ancestry in the Columbia Basin pygmy rabbit recovery program during 2012-2014. Comparisons where the groups significantly differed are shown in bold.

	Female				Male			
Comparison	Estimate	SE	Z value	P value	Estimate	SE	Z value	P value
Mixed vs. other	-0.07	0.12	-0.55	0.92	-0.19	0.16	-1.15	0.67
WA vs. other	0.02	0.18	0.09	1.00	**0.89**	**0.21**	**4.16**	**<0.001**
northUT/WY vs. OR/NV	0.21	0.17	1.22	0.50	**-0.65**	**0.18**	**-3.69**	**0.001**
northUT/WY vs. southUT	-	-	-	-	-0.29	0.26	-1.09	0.71
southUT vs. OR/NV	-	-	-	-	0.37	0.29	1.28	0.58

Chapter 4

Genetic monitoring of an endangered species recovery: demographic and genetic trends for reintroduced pygmy rabbits

Abstract

Monitoring demographic and genetic parameters of reintroduced populations of endangered species is crucial for evaluating and informing conservation strategies to maximize the chances of a successful recovery. We used noninvasive genetic monitoring using fecal DNA to evaluate the recovery of the endangered Columbia Basin pygmy rabbit in central Washington, USA, during the initial 3 years of a renewed reintroduction effort. We quantified post-release dispersal, survival, and reproduction in the wild, and monitored the genetic diversity and composition of the released cohorts, wild surviving population, and breeding population held in large breeding enclosures. During this study, we reintroduced 1206 pygmy rabbits into the wild, and detected 176 individuals surviving on or near the release area. Juveniles dispersed an average of 961 m from their release sites, compared to 786 m for adults, and dispersal distances for juveniles decreased for rabbits released later in the year. Juvenile survival differed across years and was positively influenced by release date, release weight (an index for age), and heterozygosity. Adult survival was similarly influenced by release day, with some evidence for an effect of heterozygosity. Only 14 wild-born individuals were detected during the study. Genetic monitoring was an effective way to evaluate the demographic and genetic status of the reintroduced population within a limited study area, to inform changes to the conservation strategy and to generate a dataset to address long-term research and recovery goals.

Introduction

In 2015, the International Union for the Conservation of Nature estimated that 13.4% of birds, 26% of mammals, and 41% of amphibians were threatened with extinction in the wild (IUCN 2015). Those tasked with managing and recovering threatened species and populations rely on a diverse set of conservation tools, which can include habitat protection and restoration, predator or invasive species control, genetic rescue for reversing inbreeding depression, and reintroduction using captive-bred or translocated animals (Hoffmann *et al.* 2010; Frankham 2015; Dolman *et al.* 2015). Post-release monitoring of reintroduced populations is crucial for evaluating recovery success and identifying ways for managers to adapt and improve conservation strategies (Sarrazin & Barbault 1996; Seddon 1999; Armstrong & Seddon 2008; Sutherland *et al.* 2010; Seddon *et al.* 2014).

Genetic monitoring is a powerful tool for monitoring wildlife populations of conservation concern. Schwartz *et al.* (2007) classified the utility of genetic monitoring into two categories. The first category encompasses the use of molecular markers to identify species and individuals for traditional population monitoring (e.g., population size, demographic rates, geographic range). The second category comprises the monitoring of population genetic parameters (e.g., genetic diversity metrics, genetic structure and composition, effective population size) over time. Monitoring genetic parameters in addition to demographic parameters is particularly important for reintroduced populations, which are susceptible to loss of genetic diversity from inbreeding and genetic drift, and associated potential decreases in fitness (Tallmon *et al.* 2004; Wisely *et al.* 2008; Jamieson 2011; Keller *et al.* 2012; Frankham 2015). One or multiple source populations, from the wild or captivity, can be used to reintroduce populations, and the genetic composition of the founding and

subsequent generations can influence population viability through variation in fitness among the founding groups, maintenance of genetic diversity, and outbreeding depression (Serfass *et al.* 1998; Marr *et al.* 2002; Williams & Scribner 2010). Noninvasive genetic sampling, i.e. gathering DNA from sources that animals leave behind like scat or hair, increases the ability of researchers to collect data from rare, cryptic, or vulnerable species (Waits 2004; Waits & Paetkau 2005; Beja-Pereira *et al.* 2009). Using noninvasive genetic sampling to monitor both demographic and genetic parameters of reintroduced populations has provided valuable insights for the conservation of numerous species, including but not limited to brown bears (*Ursus arctos*; De Barba *et al.* 2010), African wild dogs (*Lycaon pictus*; Spiering *et al.* 2011), gray wolves (*Canis lupus*; Stenglein *et al.* 2010; Stansbury *et al.* 2014) red wolves (*Canis rufus*; Adams *et al.* 2007), European otters (*Lutra lutra*; Ferrando *et al.* 2008), greater bilby, (*Macrotis lagotis*; Smith *et al.* 2009), and swift fox (*Vulpes velox*; Cullingham & Moehrenschlager 2013).

We used noninvasive genetic monitoring to evaluate reintroductions of the pygmy rabbit (*Brachylagus idahoensis*) in the Columbia Basin of central Washington, USA. Habitat loss and fragmentation led to the extirpation of the pygmy rabbit, a sagebrush (*Artemisia spp.*) obligate, in Washington. Although pygmy rabbits are found in other sagebrush steppe habitats across the Great Basin of the western United States, the Washington population had been isolated from the others for approximately 10,000 years (Lyman 1991), and genetic analysis showed the Washington population to be distinct (Warheit 2001). In 2001, prior to extirpation, 16 adults were captured from the last known wild population in Washington and brought into captivity to establish a captive-breeding population for eventual reintroduction (USFWS 2012). In 2003, 4 pygmy rabbits from Idaho were added to the captive population to

counteract inbreeding depression (Elias *et al.* 2013), but juvenile survival remained low. Beginning in 2011, the captive-breeding program was phased out, and individuals from captivity were moved to large field breeding enclosures within the historic range of the pygmy rabbit in Washington (USFWS 2012). Those rabbits were augmented by 111 wild pygmy rabbits translocated from Oregon, Nevada, Utah, and Wyoming in 2011-2013. These translocations were conducted with the goal of increasing the genetic diversity and the number of individuals in the breeding population. Since 2011, we have undertaken large scale releases of mixed-ancestry offspring produced in the breeding enclosures. We collected a tissue sample from each rabbit prior to release and surveyed the release area to collect fecal pellets for genetic analysis each winter.

Our goal was to monitor the demographic and genetic status of the reintroduced population over time to inform adaptive management of the recovery program. We expected post-release dispersal patterns to mimic natal dispersal (Estes-Zumpf & Rachlow 2009), with high rates of dispersal from the release site for juveniles (kits) released at younger ages, and lower rates of dispersal for kits released at older ages and also for adults, when the natural urge to disperse may have already passed. We hypothesized that rabbits released early in the year would settle in suitable habitat closer to their release sites, causing rabbits released later in the year to disperse farther to find vacant home ranges. We predicted that survival rates to winter would increase for rabbits released later in the year because they were vulnerable to predation for a shorter amount of time before winter surveys. We predicted that older kits would have a higher probability of survival because they had more time in the breeding enclosures with *ad libitum* high-quality food and could have achieved better body condition

prior to release. Similarly, we expected adults released at heavier weights to display higher post-release survival rates than those released at lighter weights.

Pygmy rabbits released in this recovery program were of mixed ancestry from multiple source populations, including the original Columbia Basin population that was brought into captivity for propagation prior to their extirpation in the wild. We sought to evaluate the effect of Columbia Basin (CB) ancestry on fitness in the reintroduced population. Increased CB ancestry could lead to increased fitness as a result of local adaptations or decreased fitness as a consequence of the population bottleneck and inbreeding depression experienced by the wild and captive population in their recent history (Tallmon *et al.* 2004), or it could have no effect. Regardless of founding population, genetic diversity at the individual level can influence fitness parameters (e.g., birth rates, juvenile survival, disease susceptibility; Ruiz-López *et al.* 2012; Elias *et al.* 2013). We hypothesized that CB ancestry and genetic diversity would have no effect on post-release survival of released pygmy rabbits because the main cause of mortality in the wild is predation (Estes-Zumpf & Rachlow 2009; Crawford *et al.* 2010), but that reproduction would be positively influenced by CB ancestry and genetic diversity (DeMay *et al.* in press).

We monitored the genetic diversity and amount of CB ancestry in the enclosure populations, yearly released cohort, and surviving wild populations to evaluate our genetic management strategy. Preservation of a genetic signature from the original CB population was one of the goals of the species' recovery plan (USFWS 2012). We expected to see no decline in heterozygosity over the short time span of this study because we translocated new rabbits into the population through 2013, and we expected the percent of CB ancestry in the

population to increase as a result of selective retention of kits with high amounts of CB ancestry for future breeding.

Materials and Methods

Study Area

This study took place in the Columbia Basin of central Washington, USA, where temperatures range from an average minimum of −5.9° C in January to an average maximum of 31.6° C in July (WRCC 2015). The climate is semiarid and averages about 20 cm of annual precipitation, over half of which typically comes from snow (WDFW 2006, WRCC 2015). The landscape is dominated by 'mima mounds,' mounded microtopography characterized by deep soils and dense sagebrush (Tullis 1995), with relatively sparse and low-growing vegetation between mounds. Pygmy rabbits were bred in 4 large predator-resistant enclosures (2.2-4.4 ha) spread across the Columbia Basin, but all releases occurred on the Sagebrush Flat (SBF) Wildlife Area (1514 ha). The release area was surrounded by a mosaic of sagebrush steppe and dryland wheat fields on state, federal, and private lands. Predators of pygmy rabbits on-site included badgers (*Taxidea taxus*), long-tailed weasels (*Mustela frenata*), coyotes (*Canis latrans*), and several raptor species.

Field Methods

We captured kits from the breeding enclosures and released them to the wild during the 2012-2014 breeding seasons. Kits are born beginning in February or March, and remain in their natal burrow for 2 weeks, after which they emerge almost completely independent from their mothers (Elias *et al.* 2006). Releases began each year from late April to late May. Pygmy rabbits produce multiple litters in succession until the breeding season ends in late June or early July, and releases continued after this time until densities were reduced in the enclosures

to manage overwintering populations. We focused on releasing kits during 2012 and 2013, and began releasing adults at the end of the breeding season in 2014 to relieve crowding in the enclosures and make room for younger breeders. Prior to release, we weighed and sexed kits, treated them for ectoparasites with Advantage II kitten formula (BayerDVM, Shawnee Mission, KS, USA), and collected a tissue sample with a 3-mm biopsy punch in the ear. After preliminary results from 2012 and 2013, we released only rabbits weighing > 125 g in 2014; prior to 2014 weight was not considered. Tissue samples for genetic analysis were stored in 95% ethanol and frozen at −20° C until laboratory analysis. After a trial using acclimation periods in soft-release enclosures in 2012 (DeMay *et al.* 2015), the method was discontinued and all subsequent rabbits were hard-released. We provided artificial burrows, auger holes, and supplemental food at hard-release sites at the time of release. We placed kits individually inside artificial burrows, plugged both entrances with burlap, and left them to acclimate to the burrow for approximately 5 minutes, after which we quietly removed the burlap. We did this to allow individuals to recover from the stress of being transported and to increase the probability that kits would stay close to their release sites. Each year approximately 10-20 kits per enclosure demonstrated to have relatively high amounts of original CB ancestry (see laboratory and analysis methods below), were retained. These were swapped among the enclosures for future breeding to simulate gene flow and promote retention of the CB genetic signature in the breeding population.

Each winter following releases, we conducted surveys on and around the release area to locate active burrows and collect fecal pellets for genetic analysis. Surveys occurred after fresh snow when weather allowed, but the 2013-2014 and 2014-2015 winters had abnormally low amounts of snow, and surveys often proceeded on bare ground. When snow was present,

rabbit tracks, active burrows, and fecal pellets were conspicuous. We surveyed on foot along 50-m-wide belt transects oriented north to south, prioritizing the release sites and areas with active burrows in prior years and continuing outward as time and personnel resources allowed (8.9 – 14.4 km^2 each year). We also surveyed specific drainages with dense sagebrush outside of the belt transect area. Burrows were considered active if they exhibited fresh rabbit sign including tracks, fresh fecal pellets, and digging (Sanchez *et al.* 2009; Price & Rachlow 2011). We collected ≥4 pellets/sample when possible to ensure an adequate amount of DNA for analysis (Adams *et al.* 2011). Fecal samples were stored in paper envelopes and desiccated with silica gel beads until laboratory analysis.

Laboratory Methods

Tissue samples were extracted and genotyped using Qiagen DNeasy blood and tissue kits (Qiagen Inc., Valencia, CA) following the methods described in DeMay *et al.* (2015). Tissue DNA was amplified in duplicate at 19 loci in 3 PCR multiplexes (DeMay *et al.* 2015): 18 polymorphic microsatellite loci and 1 Y-chromosome microsatellite used for sex identification, which agreed with sex identification based on morphological features. Samples were run on an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems Inc., Foster City, CA), and results were viewed in Genemapper 3.7 (Applied Biosystems Inc.) and checked visually.

We extracted DNA from the surface of fecal pellets using the Qiagen QIAamp DNA stool mini kit (Qiagen Inc.) in a laboratory dedicated to low-quantity DNA samples (Waits and Paetkau 2005). To determine species during the 2012-2013 and 2013-2014 winter surveys, we amplified a 294 base-pair fragment of cytochrome B from the mitochondrial genome in a species ID test designed to distinguish between pygmy rabbits and sympatric

cottontail rabbit species (*Sylvilagus nuttallii, S. audubonii, S. floridanus*; Adams et al. 2011). For samples confirmed as pygmy rabbit pellets, we amplified 8 loci in one PCR multiplex. Cottontail samples and samples that failed to yield species ID results were excluded from further analysis. Additional testing revealed that cottontail samples did not amplify or produced out-of-bin alleles at many of our microsatellite loci, so for the 2014-2015 winter survey analysis we excluded the species ID step as cottontail samples could be successfully detected using only the nuclear microsatellites.

Initially, we ran 2-4 PCR repetitions for each fecal sample in the first multiplex of 8 loci, and completed more reps as needed to acquire consensus genotypes for ≥5 of the polymorphic microsatellite loci to achieve a probability of identity for siblings (PIDsib; Waits *et al.* 2001) value < 0.01. Samples were excluded from further analysis if half of the tested loci did not yield results in the first round of amplifications. We required 2 repeats of each allele to confirm a heterozygous genotype and 4 repeats to confirm a homozygous genotype based on pilot work (DeMay *et al.* 2013). Fecal samples that did not match a previously sampled rabbit with the initial multiplex were amplified at the remaining loci.

Analysis Methods

We genotyped all tissue samples and created a reference database containing genetic and demographic information of all known rabbits in the recovery program. We matched fecal sample genotypes with reference (tissue) genotypes using GenAlEx 6.5 software (Peakall & Smouse 2006, 2012). Any pairings with mismatches at 1 or 2 loci were checked further to determine whether human error or allelic dropout could have caused false mismatches. If the only mismatch between 2 samples appeared to be due to allelic dropout, we considered it a match. Genotypes for fecal samples not matching previously sampled rabbits were checked

for 95% reliability using RELIOTYPE (Miller *et al.* 2002), and added to the database as new wild-born individuals. Enclosure-born and wild-born rabbits were analyzed for parentage using a strict exclusion approach in Cervus 3.0 (Marshall *et al.* 1998; Kalinowski *et al.* 2007). Locus A124 was used only for identity matching, and not in downstream parentage and population genetic analyses due to a high frequency of null alleles (unpublished data).

Dispersal

For each released rabbit detected during winter surveys, we measured the straight-line distance between the release site and active winter burrow. If an individual was detected at multiple burrows during winter surveys, we took the average distance from the release site to all active winter burrows. We considered rabbits "dispersed" from the release site if the dispersal distance exceeded the average maximum diameter of an adult female home range. Lacking home range data for this population, we used the 276 m home range diameter calculated by Estes-Zumpf & Rachlow (2009) for pygmy rabbits in central Idaho. We used two-sided Fisher's exact tests and Wilcoxon rank sum tests to test if dispersal rates and distances differed significantly by sex for both juveniles and adults.

To determine whether post-release dispersal rates were comparable to natal dispersal rates for juvenile pygmy rabbits, we compared dispersal rates for juveniles released at different ages. Because ages were not known, we estimated age using a growth curve constructed using data from pygmy rabbits born and raised in captivity (Elias *et al.* 2013). We calculated the dispersal rate (% of rabbits dispersing > 276 m) for kits released at ages of 3-5 weeks, 5-7 weeks, 7-9 weeks, 9-11 weeks, 11-13 weeks, and > 13 weeks. In a study of natal dispersal by pygmy rabbits in Idaho, 95% of dispersing kits had dispersed by 12 weeks of age (Estes-Zumpf & Rachlow 2009).

Survival

Detection of released pygmy rabbits during follow-up winter surveys was a function of survival, dispersal distance (remaining within the surveyed area), and detection (collecting a fecal sample that yielded a positive identification). We assumed that rabbits missing due to non-detection (failure to locate and collect a sample or failure to obtain a positive individual ID from a sample) were missing at random with respect to our predictor variables. We performed a preliminary analysis to determine whether we could reasonably make inferences about survival given post-release dispersal patterns. We calculated the density of rabbits detected at increasing distances from release sites. In 2012, we used a single cluster of release sites, 2 clusters were used in 2013, and 6 were used in 2014. Each cluster consisted of 17-37 individual release sites (most had 25 sites), and we created 500 m-wide bands around the center point of each cluster out to 3500 m. We calculated densities of rabbits that settled within each circular band by dividing the number of rabbits released at the focal release area that were detected in each distance interval by the area surveyed within each distance interval. We repeated this for each year, and combined the results to get the average density within each distance interval. We found that densities were highest within 500 m of release sites (mean 6.8 rabbits per km^2), and dropped off rapidly, with no densities exceeding 1 rabbit per km^2 beyond 1.0 km from release sites (Figure 4.1). The rapid tapering of this curve suggested that although we could not detect rabbits that dispersed farther distances beyond the surveyed area, the number of rabbits missed due to long dispersal distances was likely to be small and inconsequential to analyses of survival.

We evaluated survival for juveniles and adults using logistic regression, with winter survey detection as the survival binary response variable. We evaluated *a priori* model sets

with AICc scores and model averaging using the R package 'multcomp' (Hothorn et al. 2008). Parameter estimates were averaged across all candidate models that included each given parameter. For adult survival, explanatory variables included release day (Julian day of the year), sex, homozygosity by loci (HL) calculated using the R package 'Rhh' (Alho *et al.* 2010), and percent CB ancestry derived from pedigree data. Our candidate set included all 16 possible combinations of these variables, all of which were valid biological hypotheses. Only adults released in 2014 were used for this analysis because < 10 adults were released prior to 2014 in this study. To minimize handling stress, released adults were not consistently weighed prior to release, so 35% of released adults (n=113) had no recorded weight at the time of release. In order to examine the effect of release weight, we compared the top adult model and the top model plus release weight, both analyzed only for the subset of individuals with recorded release weights, and compared resulting AICc, log-likelihood values, and parameter estimates.

For survival of juveniles, explanatory variables included release year (categorical, 2012-2014), release day, release weight, sex, HL, and estimated percent captive ancestry. Our candidate model set included year in each model, and all possible combinations of the remaining variables, for a total set of 34 models. To determine the effect of CB ancestry on juvenile survival, we used estimated captive ancestry rather than percent CB ancestry derived from pedigree data. This was necessary because we could not achieve complete sampling of parents in the breeding enclosures, and gaps in the pedigree became more prominent in later years (DeMay et al. in press). We estimated percent captive ancestry, an index of CB ancestry, using the Bayesian clustering software STRUCTURE 2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2003, 2007; Hubisz *et al.* 2009). Prior analyses (unpublished data) grouped the

founding pygmy rabbits into 4 genetic clusters: Captive, Oregon/Nevada, southern Utah, and northern Utah/Wyoming. To estimate captive ancestry for the released juveniles, we ran 500,000 Markov Chain Monte Carlo runs following a 100,000 run burn-in, with *k* set at 4 clusters with correlated allele frequencies. Allele frequencies for each cluster were estimated from individuals making up the 4 pre-defined founding clusters, and used to estimate the percent captive ancestry for all non-founding individuals. Captive ancestry was strongly correlated with ($r = 0.97$) but not equivalent to CB ancestry because only 35-84% (mean = 72%) of the genetic make-up each individual from captivity was CB ancestry, with the remaining coming from the Idaho genetic rescue.

Genetic Diversity Monitoring

We characterized the genetic diversity each year of the enclosure population, released cohort, and detected wild population. The enclosure population included all individuals born in a given year and all individuals detected as being parents in that year. The released cohort included all released individuals. The detected wild population included all individuals either detected surviving during winter surveys, or not detected directly, but detected the following year as parents of kits produced in the wild and thus known to be alive. We computed H_O and unbiased H_E using GenAlEx 6.5 (Peakall & Smouse 2006, 2012), allelic richness using FSTAT 2.9.3 (Goudet 1995), percent CB ancestry from pedigree data, and percent captive ancestry using STRUCTURE 2.3.4 as described above. We compared the percent CB and captive ancestry between released and surviving populations with two-sided Wilcoxon rank sum tests.

Results

As kit production increased from 2012 to 2014, we released an increasing number of pygmy rabbits to the wild, ranging from 104 kits released in 2012 to 830 kits and adults released in 2014 (Table 4.1). During winter, we surveyed 8.9 – 14.4 km^2 on and around the release area each year and detected 44-91 individuals from fecal pellet DNA (Table 4.1). The probability of identity for siblings was < 0.001 for the 8-locus individual ID multiplex, and < 1.0×10^{-9} for the full suite of loci. Excluding samples that failed to amplify at any loci, we generated genotypes for an average of 7.5 loci per individual. Considering only the first multiplex that all samples were genotyped with (the remaining two were used only for new individuals), we ran an average of 4.9 PCR repetitions per sample. Individual identification success rates, excluding confirmed cottontail samples, varied from 46% during winter 2013-2014 to 78% during winter 2014-2015. However, success rates from the former survey varied depending on whether they were collected prior to snow (37.5% success) or after it had snowed in late January (79% success). Per locus rates of genotyping error for allelic dropout ranged from 6.7-60.0% and per locus false allele rates ranged from 0-23.9%., with the highest error rates in the winter 2013-2014 survey. For parentage analyses, the combined non-exclusion probability for the full suite of loci was 8.2×10^{-14}. Most detected individuals were released within the previous year. For example, after the 2014 releases we detected 87 individuals released in 2014, and on the edges away from the core release area, 3 wild-born individuals and 1 individual surviving from 2013 releases (Figure 4.2). The total area surveyed differed each year, but a common area of 6.7 km^2 was surveyed every year. Within this common area, we detected 39 individuals, 32 individuals, and 61 individuals during each survey following the 2012-2014 releases.

We successfully identified both parents for each of the 14 wild-born pygmy rabbits detected on SBF, including 4 born in 2012 from parents released during a small-scale release from captivity in 2011. Two of these wild-born rabbits, both females, reproduced in 2013, producing second-generation wild-born kits. No additional wild-born rabbits were detected reproducing in 2014. Three individuals released in 2012 and 5 individuals released during 2013 were not detected during winter surveys, but were identified the following year as parents of wild-born kits, so were assumed to have been alive but unsampled during the winter immediately after their release. Over the course of this study, we detected only 4 individuals surviving on the release area for 2 consecutive winter surveys. We detected these individuals during their second winter at active burrows an average distance of 328 m (range 7 – 540 m) from where they were detected the prior winter.

Pellet samples were in nearly all cases collected from burrow systems associated with mima mounds or steep drainages with deep soils. Each individual was detected at an average of 1.8 different burrow systems, with a maximum of 5 (excluding the 2013-2014 survey due to lack of sufficient information). We identified 16 burrow systems where 2 individuals were detected, and 1 burrow system where 3 individuals were detected. Within these groupings, none of the individuals were siblings or parent-offspring pairs. Plotting of occupied burrow systems each year revealed 30 burrow systems that were active during 2 of the 3 surveys, and 5 burrow systems that were active during all 3 surveys.

Dispersal

Post-release dispersal behavior was similar between the sexes for juveniles. Of the 69 juvenile females and 58 juvenile males for which we measured dispersal distances, 83% of females and 90% of males dispersed >276 m from their release site (mean = 961 m), but the

difference between sexes was not statistically significant (2-sided Fisher's exact test, P = 0.31). Juvenile males dispersed, on average, slightly farther (1082 m, range 80-3546 m) than juvenile females (859 m, range 11-3009 m), although the difference was not statistically significant (2-sided Wilcoxon rank sum test, P=0.24). The distribution of dispersal distances was right-skewed with fewer rabbits making longer dispersals (median = 776 m for juveniles and 471 m for adults; Figure 4.3). In general, average dispersal distances decreased for juveniles released later in the year compared to those released earlier (Figure 4.4), with rabbits released early in the season dispersing about 1500 m, and later-released rabbits dispersing about 700 m. This trend did not hold for 2012, when dispersal distances showed no patterns associated with timing of release. Dispersal rates for kits released at varying ages ranged from 67% to 95%, but there was no trend with age, and a 2-tailed Fisher's exact test revealed no difference in dispersal rates across different age groups from 3 to >13 weeks old (P = 0.27). Similarly, we did not detect a trend in distances moved by dispersing kits released at various ages (Table 4.2).

As expected, adults dispersed less frequently than juveniles. For adults, 62% of females (8/13) and 73% of males (8/11) dispersed >276 m and mean dispersal distances did not differ by sex (2-sided Wilcoxon rank sum test, P=0.69). Adult dispersal rates were significantly lower than juvenile dispersal rates (1-sided Fisher's exact test, P = 0.03). Mean dispersal distances between release sites and winter active burrows were lower for adults (786 m) than for juveniles (961 m). Adults were released, on average, later in the year than kits when they were no longer reproductively active (73% were released in August or later). Considering only kits released in August or later, adult and juvenile mean dispersal distances were similar (786 m and 794 m, respectively).

Survival

Across all years, survival of juveniles from the releases to winter surveys averaged 12% (135/1086), and adult survival in 2014 was 21% (24/113). Like dispersal, this difference between age groups is inflated because of the difference in release timing for adults and juveniles. Comparing only rabbits released in August or later, adult survival was 28% (23/83) and juvenile survival was 23% (35/152), with no significant difference between the age groups (Fisher's exact test P = 0.43). Juvenile survival was positively influenced by release day, release weight, and genetic diversity (Figure 4.5, Table 4.3, Table 4.4). Weight and release day were moderately correlated (Pearson's r = 0.575), driven largely by the release of only larger rabbits after the breeding season ended in July. Regardless, the models were able to distinguish significant effects of both variables even when included in models simultaneously. Compared to the reference year of 2014, predicted juvenile survival was 2.0 times higher in 2013 and 13.1 times higher in 2012. Although sex and percent captive ancestry appeared in the top model set, their addition to the top model did not improve the log-likelihood, and 95% confidence intervals around model-averaged parameter estimates overlapped 0, suggesting that these were 'pretending variables' (Anderson 2008).

Survival of adults was influenced by release day. Of 44 adults released April-August, 3 were detected surviving (7%), while 21 of 45 (47%) adults released September-November were detected. Similar to the juvenile models, sex and percent CB ancestry were pretending variables despite appearing within the top models. Parameter estimates and AICc scores showed weak evidence for a positive effect of genetic diversity on adult survival. The addition of release weight to the top adult model did not meaningfully improve the likelihood, and the 95% confidence interval for the parameter estimate overlapped 0.

Genetic Diversity

Within the breeding enclosures, population-level observed and expected heterozygosity remained fairly constant both across years within an enclosure, and within a year across enclosures, with values around 0.8 (Table 4.5). One exception was Enclosure 3, first stocked with pygmy rabbits in 2013, which had lower observed and expected heterozygosity than the other enclosures. Of the 22 adults placed in that enclosure, 18 were translocated from Wyoming during spring 2013, while the remaining 4 were brought from the other enclosures. The lower heterozygosity values are likely a consequence of the enclosure representing primarily a single source population, while the other enclosures were more mixed.

Percent CB and captive ancestry fluctuated more than heterozygosity, and increased or decreased as a function of each enclosure's history (Table 4.5). In general, percent CB and captive ancestry increased as a result of selectively retaining kits with relatively high CB ancestry for future breeding in the enclosures. The increase in Enclosure 2 from 0% CB ancestry in 2012 to 13.6% CB ancestry in 2013 was ascribed to the addition of only 4 individuals (1 female and 3 males) with 100% captive ancestry (CB ancestry ranging from 56-75%), and the offspring they produced. That increase was tempered by the addition of 13 adult rabbits translocated to Enclosure 2 in 2013 from Wyoming and Oregon. Enclosure 1 experienced a decrease in CB ancestry over time. The amount detected in 2012 was inflated by a single highly productive male (100% captive, 76% CB) that sired over a third of the kits produced in that enclosure, but died before the 2013 breeding season. The further decrease between 2013 and 2014 was a result of random sampling error. We stocked Enclosure 4 primarily with rabbits from Enclosure 1. The exceptionally high %CB value from Enclosure 4

in 2014 shows that by chance, we captured rabbits with relatively high amounts of CB ancestry out of Enclosure 1, leaving behind a lower average proportion of CB ancestry in that enclosure.

We identified no difference in observed and expected heterozygosity values or allelic richness between rabbits released in a given year and those detected surviving during follow-up winter surveys. In 2012 and 2014, there was no significant difference in percent CB or captive ancestry between released and surviving rabbits (2-sided Wilcoxon rank sum tests, 2012: $P = 0.64/0.66$; 2014: $P = 0.10/0.21$ respectively for CB/captive ancestry). However, in 2013, the surviving wild population had significantly greater CB and captive ancestry than the population that was released ($P < 0.001$ for both CB and captive ancestry). In 2013, there was disproportionately low survival from Enclosure 3 with high amounts of Wyoming ancestry (5% of released rabbits detected versus 18-19% from Enclosures 1 and 2). Excluding Enclosure 3 in 2013, there was still a moderate increase in CB ancestry between released rabbits and detected surviving rabbits (11.6%/16.2% CB/captive *released* versus 18.4%/24.2% CB/captive *detected*, $P = 0.08/0.06$). The rate of rabbits detected surviving from Enclosure 3 did not remain low the following year (5% to 15% detection rates for all enclosures, 11% for Enclosure 3).

We investigated 2014 further because of particular characteristics of the release schedule that year. Releases occurred through November in 2014, and winter surveys began in late January 2015, leaving a relatively short amount of time for late-released rabbits to be subjected to selection pressures and display differences in fitness. Consequently, we performed the same test on rabbits released only from April to August 2014, a time span

comparable to the previous years, and P values decreased to 0.06/0.16 for CB/captive ancestry.

The amount of CB/captive ancestry of rabbits that we detected as breeders in the wild during the 2013 breeding season (n = 14) did not differ from the ancestry of rabbits that were released in their same cohort in 2012 (P = 0.22). We did not perform this same test on breeders in the wild the following year because the sample size was low (n = 5 breeders).

Discussion

Genetic monitoring is receiving increased focus in conservation biology and wildlife management (Schwartz *et al.* 2007), but has rarely been used for monitoring and adaptive management of small mammal reintroductions. Here, we used genetic tools to monitor the demographic and genetic recovery of the endangered Columbia Basin pygmy rabbit. We used the genetic data for monitoring post-release dispersal, survival, and reproduction, as well as population genetic parameters (genetic diversity and ancestry composition) and their effects on fitness. Released individuals tended to remain close to the release sites; both juveniles and adults dispersed average distances of < 1 km. We identified burrow systems that had been used by pygmy rabbits for multiple years, which could lead to future assessments of post-release habitat preference. Survival of released juveniles was positively influenced by release date, release weight, and heterozygosity, and adult survival was influenced by release date. We tracked the genetic composition of the enclosure and wild populations, and detected weak evidence for a positive effect of CB ancestry on survival. Our ability to collect these data effectively and efficiently using noninvasive genetic sampling of fecal pellets, which does not require capturing our vulnerable focal species in the wild, was a great advantage and will help biologists adaptively manage the recovery of this species as reintroductions continue.

Post-release dispersal

Most released individuals dispersed from their release sites, and juveniles dispersed more often than adults. Juveniles dispersed on average about 1 km, and adults dispersed slightly shorter distances on average, but this difference narrowed when only adults and juveniles released at similar times were compared. Post-release dispersal for juveniles did not closely mimic natural natal dispersal behaviors. Although dispersal rates were similar to natal dispersal documented for the species in Idaho, USA, we detected no difference in dispersal rates or distances depending on the age at which juveniles were released, contrary to our predictions based on the results of Estes-Zumpf & Rachlow (2009). Our results suggest that when kits were held in breeding enclosures past the age at which they would naturally disperse, there was not a natal dispersal "urge" that came and went, causing them to remain at their release sites. Instead, kits released at different ages displayed similar dispersal behaviors. There is therefore little support for strategically releasing rabbits at a certain age to maximize either settlement near the release area or expansion of the population's range.

Natal dispersal and post-release dispersal are fundamentally different processes. Natal dispersers leave their natal sites voluntarily, while translocated individuals are involuntarily moved to a novel environment with which they have no prior experience. Stamps and Swaisgood (2007) advised that reintroduction and translocation programs are likely to be more successful and animals more likely to accept a new habitat, when animals are released at a life stage comparable to when they would disperse naturally. We found that released juveniles were likely to settle close to their release sites, with few individuals making longer distance dispersals, and that adults dispersed similar distances as kits released at the same time. Rather than rejecting the new habitat and traveling farther distances, as suggested by

Stamps & Swaisgood (2007), the adults remained as close to their release sites as juveniles released at similar times. All released adults were released from large naturalized breeding enclosures with habitat similar to the release sites, rather than off-site captive breeding facilities. As a result of their prior experience with the type of habitat at the release sites, adults may have been better able to assess and accept the release area as suitable habitat than if they had been released directly from captivity. In a pilot release from off-site captive facilities in 2007, 9 of 20 released pygmy rabbits dispersed long distances averaging 4.2 km from their release site (Saylor et al. unpublished manuscript).

Contrary to our predictions, animals released earlier in the year dispersed farther than those released later, indicating that dispersing animals are not filling in nearby available home ranges first and requiring later-released rabbits to disperse farther to find openings. We do not know whether early-released individuals moved long distances in one dispersal event, or if they moved in smaller incremental steps throughout the time between releases and surveys. Finer scale telemetry or GPS data would be needed to determine which process is driving the observed pattern.

Our measurements of dispersal distances are likely biased low because we had no probability of detecting individuals that dispersed outside of our surveyed area. Juvenile pygmy rabbits can make long distance movements > 7 km (Estes-Zumpf & Rachlow 2009; DeMay *et al.* 2015), but the decrease in rabbit detection with increasing distance from release sites in our study supports the assumption that we located most of the surviving reintroduced individuals. More extensive monitoring would be needed to locate longer distance dispersers, to locate new patches of favored habitat, and map the expansion of the species back into its historic range. Possible techniques include radio collars placed on animals that have reached

adult size (glue-on juvenile transmitters have limited usefulness; DeMay *et al.* 2015), and aerial surveys using fixed wing aircraft or drones to locate burrow systems that are conspicuous on fresh snow (Linchant *et al.* 2015, Rachlow unpublished data).

Survival and Reproduction

Overall survival of released animals until winter surveys was 13% for adults and juveniles combined, with 4 individuals detected surviving > 1 year, and 14 detected kits born in the wild. These low survival and reproduction rates indicate that as of 2015, a self-sustaining wild population had not been established and supplemental releases will likely be necessary for the foreseeable future. With the high number of rabbits released in 2014, and resulting higher number and density of rabbits detected surviving the following winter, surveys during winter 2015-2016 will be particularly important for evaluating whether the wild population size reached some threshold where enough rabbits survived to breeding season to produce a more sustainable number of wild-born kits.

We found that survival was largely influenced by release date for both juveniles and adults. As animals were released later, there was less time between release and winter surveys when animals were exposed to predators and other sources of mortality. Crawford *et al.* (2010) documented peaks in pygmy rabbit mortality coinciding with raptor migrations through the western United States, and avian predators likely have a similar effect on the population in our study during the autumn migration (Goodrich *et al.* 2008). For juveniles, predicted survival probabilities increased as a function of release weight, an index of age. Kits that were older at release may have been able to achieve better body condition in the enclosures than those released at younger ages, giving them an advantage in the wild. These results indicate that there are no net detrimental effects on survival of holding kits in the

enclosures longer; where acclimatization to humans and a relative (although not absolute) lack of predator training might be expected to decrease survival probabilities of rabbits held for longer amounts of time prior to release.

Model results showed no consistent influence of Columbia Basin ancestry on post-release survival during the entire study. However, after the 2013 and 2014 releases, we did detect increases approaching significance in CB ancestry of surviving rabbits compared to those that were released. A previous study of the breeding population (DeMay et al. in press) provided additional evidence of fitness benefits associated with CB ancestry; males with higher CB ancestry had increased reproductive output compared to other ancestries in the breeding enclosures. These results underscore the need for managers of reintroductions to balance the fitness costs of low genetic diversity and inbreeding depression with the fitness costs of outbreeding depression, which occurs when offspring of individuals from geographically/genetically distant origins have decreased fitness compared to individuals that are locally adapted to a region (Marshall & Spalton 2000; Tallmon *et al.* 2004; Ficetola & De Bernardi 2005; Edmands 2007; Huff *et al.* 2011).

Regardless of ancestry, individual genetic diversity did influence post-release survival. This result was contrary to our prediction that mortality would be driven by predation, which we expected to be random in terms of the genetic composition of individual animals. The pattern that we detected of increased survival with increased genetic diversity could be explained by an increase in predation on individuals with low genetic diversity, perhaps caused by lower growth rates and body condition making individuals less able to successfully evade predators (Chapman *et al.* 2009; Luquet *et al.* 2011; Han *et al.* 2013; Brambilla *et al.* 2015). Alternately, the pattern could be driven by an increase in mortality of individuals with

low genetic diversity from other causes. Disease was a major cause of mortality of pygmy rabbits in captivity, exacerbated by low genetic diversity due to inbreeding in the original Columbia Basin population (USFWS 2012; Elias *et al.* 2013). More detailed known-fate monitoring of individuals would be needed to identify causes of mortality, and to distinguish between mortality and long-distance dispersal or non-detection within the surveyed area.

We detected variation in survival among years, with the highest rates in 2012 (35%) compared to 2013 and 2014 (13% and 10%, respectively). The decrease in survival after the first year might be explained by a numerical response of predators to the new food source we reintroduced to the landscape (Korpimaki & Norrdahl 1991; O'Donoghue *et al.* 1997; Sinclair *et al.* 1998; Stoddart *et al.* 2001; Gilg *et al.* 2006), but we lacked data on predator populations to confirm this. Alternately, survival of juvenile pygmy rabbits is highly variable across time and space, and the variation among years in our study could be due to stochastic environmental or demographic processes (Crawford *et al.* 2010; Price *et al.* 2010). A 3-year evaluation of the reintroduction of riparian brush rabbits (*Sylvilagus bachmani riparius*) similarly detected the highest post-release survival rates during the first year of their study, and speculated that the pattern could have been driven by predator response, habitat saturation, or differences in release methodology across years (Hamilton *et al.* 2010).

The distribution of wild-born rabbits and rabbits surviving in the wild > 1 year in the survey following 2014 releases suggests that continual releases at the same sites throughout the year might disturb already-settled rabbits, pushing them away towards areas farther from the release sites. The disturbance and high rabbit densities associated with releases also might decrease reproduction in the wild by increasing stress to breeding females (Myers & Poole 1962; Marchlewska-Koj 1997, DeMay et al. In Press). In 2015, we shifted the recovery

strategy to begin the reintroduction of a second subpopulation at a new site, with only limited supplemental releases at the first site that was the focus of this study. Future surveys of this first site will allow us to assess the impact of release-associated disturbance on settled rabbits, and determine whether a winter population of at least 91 rabbits was sufficient to allow for increased multi-year survival and breeding in the wild.

Adaptive management

Across animal reintroductions worldwide, several recurrent themes appear as lessons learned from monitoring (Table 4.7; Soorae 2011, 2003). During the course of this study, monitoring results have led to shifts in both the recovery strategy and the monitoring strategy. For example, in response to monitoring results, we created a lower weight limit for releasing rabbits, discontinued the use of soft-release enclosures, de-emphasized juvenile glue-on VHF transmitters (DeMay *et al.* 2015), and increased PCR success of samples collected on bare ground with increased surveyor training (unpublished data). Our current results show that an effective way to increase the number of individuals surviving in the wild until the breeding season in early spring is to release them closer in time to the breeding season. However, this was not a viable release strategy because hundreds of kits were produced in the enclosures during the spring and summer; they needed to be released as they were produced to avoid overcrowding the enclosures, which put stress on the enclosure habitat and natural forage and increased the potential for disease outbreaks. The same challenge presents itself when managing release weight; we documented the biggest increase in survival when release weights reached 500g, but increased survival of released individuals must be weighed against the logistical challenges of holding rabbits for a longer time period prior to release.

Maintaining high levels of genetic diversity can increase survival probabilities. Continued genetic monitoring of the released and enclosure populations will allow managers to maintain genetic diversity by facilitating gene flow among enclosures, identify if additional translocations from other states becomes necessary again to counteract inbreeding and genetic drift, and evaluate the need for gene flow among reestablished subpopulations. Although we observed only weak evidence that CB ancestry might influence survival rates during the study, there is evidence that it influences reproduction (DeMay et al. In Press), and preserving a signature of the original CB genome is a goal specified in the species' recovery plan. Long-term genetic monitoring will allow managers to track the persistence of CB ancestry as the recovery effort continues, but more loci and possibly a genomics approach will be necessary to increase our power to accurately measure CB ancestry as it becomes more diluted.

Three years into the renewed reintroduction effort for the Columbia Basin pygmy rabbit, this project was still in its infancy. Long-term demographic and genetic monitoring of this and other reintroductions has provided and will continue to provide lessons that allow managers to adaptively manage reintroductions in the short and long term to better meet conservation goals. Genetic monitoring using fecal DNA has been an effective method to gather large amounts of demographic and genetic data at a relatively coarse time scale in a noninvasive manner. In conservation biology, and endangered species reintroductions in particular, managers often have to make decisions with incomplete information. Within an adaptive management framework, we can collect data to continually evaluate these decisions and guide future recovery actions.

Acknowledgments

We would like to thank the following funding sources: Association of Zoos and Aquariums, Oregon Zoo Foundation, Palouse Audubon Society, Phoenix Zoo, Riverbanks Zoo and Garden, Roger Williams Park Zoo, Washington Chapter of The Wildlife Society, Washington Department of Fish and Wildlife, U.S. Fish and Wildlife Service, the University of Idaho Environmental Science Program, and the University of Idaho Department of Fish and Wildlife Sciences. We thank W. Knickerbocker, C. Sink, K. Potterf, and Z. Andrews for their assistance processing samples in the lab. Thank you to the hard working Washington Department of Fish and Wildlife staff, University of Idaho students enrolled in Endangered Species Population Monitoring during spring 2015, and the countless volunteers who helped capture, process, and release rabbits, and conduct surveys in the field. We wish to thank the Waits and Rachlow lab groups for their continued feedback and support, and anonymous reviewers for their help improving this manuscript.

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Table 4.1. Summary data from the release and monitoring of the reintroduction of endangered Columbia Basin pygmy rabbits in central Washington, USA, from 2012-2014. For each year, we report the number of breeding enclosures, when releases occurred, how many rabbits were released, when winter surveys occurred, the area surveyed each winter, individual identification success rates for genetic fecal samples, number of individuals detected during winter surveys, and the number of parents detected contributing to breeding in the wild.

Breeding Season	# Enclosures	Release period	# Released	Winter survey period	Area Surveyed	Fecal samples collected[a]	Individual ID success rate	Individuals detected	Contributing breeders to wild-born kits
2012	2	May – Jul	104 kits	Dec 2012 – Jan 2013	8.9 km^2	111	78%	45 *41 released 2012* *4 wild-born 2012*	1 female 1 male
2013	3	May – Aug	265 kits 7 adults	Jan – Feb 2014	10.1 km^2	274	46%	44 *3 released 2012* *34 released 2013* *7 wild-born 2013*	7 females 7 males
2014	4	Mar[b] – Nov	717 kits 113 adults	Jan – Mar 2015	14.4 km^2	264	76%	91 *1 released 2013* *87 released 2014* *3 wild-born 2014*	2 females 3 males

[a] Number of samples excluding confirmed cottontail samples
[b] In 2014, 20 adult males were released prior to kit production to ease crowding in enclosures

Table 4.2. Dispersal rates for juvenile pygmy rabbits released at different ages from 3 weeks to > 13 weeks old, with ages estimated from the growth curve for juvenile pygmy rabbits developed by Elias et al. (2013). Rabbits were considered "dispersed" if they were detected > 276 m from their release site. Average distances are shown for rabbits that dispersed >276 m.

	Age at Release					
	3-5 weeks	5-7 weeks	7-9 weeks	9-11 weeks	11-13 weeks	>13weeks
Juveniles detected	19	20	14	3	5	64
Juveniles dispersed > 276 m	16	19	13	2	3	55
Dispersal rate	84%	95%	93%	67%	60%	86%
Avg distance moved by dispersers	1308 m	1238 m	886 m	2072 m	1058 m	979 m

Table 4.3. AICc scores, ΔAICc, model weights, cumulative model weights, and log-likelihood values for the 95% confidence sets of models describing survival of juvenile and adult pygmy rabbits after reintroduction in central Washington, USA, during 2012-2014.

Model	n	Variables	AICc	ΔAICc	w_i	$\sum w_i$	Log-likelihood
Juvenile survival	1067	Year + Day + Weight + Homozygosity	722.83	0	0.34	0.34	-355.38
		Year + Day + Weight + Sex + Homozygosity	724.80	1.97	0.13	0.47	-355.35
		Year + Day + Weight + Captive Ancestry + Homozygosity	724.82	1.99	0.13	0.69	-355.36
		Year + Weight + Homozygosity	724.98	2.15	0.12	0.71	-357.46
		Year + Day + Weight + Sex + Captive Ancestry + Homozygosity	726.80	3.96	0.05	0.76	-355.33
		Year + Weight + Captive Ancestry + Homozygosity	726.99	4.16	0.04	0.80	-357.46
		Year + Weight + Sex + Homozygosity	727.00	4.17	0.04	0.84	-357.46
		Year + Day + Weight	727.06	4.23	0.04	0.89	-358.50
		Year + Day + Weight + Sex	728.41	5.58	0.02	0.91	-358.17
		Year + Day + Weight + Captive Ancestry	728.50	5.67	0.02	0.93	-358.21
		Year + Weight + Sex + Captive Ancestry + Homozygosity	729.02	6.19	0.02	0.94	-357.46
		Year + Weight	729.13	6.30	0.01	0.96	-360.55
		Intercept only	805.37	82.54	0	1	-401.68
Adult survival	100	Day	93.64	0	0.31	0.31	-44.76
		Day + Homozygosity	94.35	0.71	0.22	0.53	-44.05
		Day + Sex	95.63	1.99	0.12	0.65	-44.69
		Day + Columbia Basin Ancestry	95.75	2.11	0.11	0.75	-44.75
		Day + Sex + Homozygosity	95.96	2.32	0.10	0.85	-43.77
		Day + Columbia Basin Ancestry + Homozygosity	96.49	2.85	0.08	0.93	-44.03
		Day + Sex + Columbia Basin Ancestry	97.75	4.11	0.04	0.97	-44.67
		Intercept only	107.42	13.78	0	1	-52.69

Table 4.4. Model-averaged parameter estimates for parameters describing survival of juvenile and adult pygmy rabbits after reintroduction in central Washington, USA, during 2012-2014. Parameter estimates were averaged across all of the candidate models, with the exception of adult weight (collected for only 65% of released adults; n = 113), which was generated by adding weight to the top model according to AICc. Bold values indicate parameters with 95% confidence intervals that do not overlap zero.

Variable	Juvenile Estimate	95% CI		Adult Estimate	95% CI	
		Lower	Upper		Lower	Upper
Release Day	**0.006**	**0.000**	**0.011**	**0.020**	**0.005**	**0.034**
Release Weight	**0.004**	**0.001**	**0.006**	0.004	-0.010	0.017
Sex (male)	-0.042	-0.433	0.350	0.296	-0.785	1.378
Homozygosity	**-1.905**	**-3.516**	**-0.295**	-2.588	-6.806	1.630
Percent CB Ancestry	NA	-	-	0.000	-0.029	0.030
Percent Captive Ancestry	0.056	-0.790	0.901	NA	-	-
Year 2012	**2.597**	**2.007**	**3.188**	NA	-	-
Year 2013	**0.732**	**0.237**	**1.228**	NA	-	-

Table 4.5. Genetic diversity (expected and observed heterozygosity) and ancestry composition (percent Columbia Basin ancestry, percent estimated captive ancestry) metrics of the enclosure populations of pygmy rabbits being bred for reintroduction from 2012-2014. Enclosure populations included all individuals born in a given year, and all adults detected as being parents in a given year through parentage analyses.

	Enclosure 1					Enclosure 2					Enclosure 3					Enclosure 4				
	n	H_E	H_O	% CB	% Capt	n	H_E	H_O	% CB	% Capt	n	H_E	H_O	% CB	% Capt	n	H_E	H_O	% CB	% Capt
2012	118	0.78	0.78	24.9	39.7	91	0.78	0.76	0	3.3	-	-	-	-	-	-	-	-	-	-
2013	209	0.78	0.80	14.5	20.5	138	0.79	0.80	13.6	20.3	179	0.70	0.68	2.5	4.8	-	-	-	-	-
2014	220	0.79	0.77	10.9	14.9	334	0.80	0.81	17.3	21.6	274	0.75	0.74	7.5	11.5	133	0.78	0.77	28.2	38.9

Table 4.6. Genetic diversity (expected and observed heterozygosity, allelic richness) and ancestry composition (percent Columbia Basin ancestry, percent estimated captive ancestry) metrics of the released, wild, and wild parent populations of reintroduced pygmy rabbits from 2012-2014. Wild parents in 2013 and 2014 were released the previous year with the exception of 2 females born in the wild in 2012 that reproduced in 2013. Asterisks denote a significant difference in ancestry between pygmy rabbits released in a given year and those detected during the subsequent winter surveys.

		n	H_E	H_O	A_r	% CB	% Captive
2012	Released	104	0.80	0.76	9.10	22.5	30.2
	Wild	45	0.80	0.74	9.12	21.2	28.1
2013	Wild Parents	14	0.78	0.81	-	26.1	30.6
	Released	271	0.78	0.74	8.73	7.3*	11.3*
	Wild	44	0.80	0.79	8.85	17.8*	23.6*
2014	Wild Parents	5	0.70	0.80	-	7.4	11.4
	Released	828	0.80	0.77	9.15	13.4	19.2
	Wild	91	0.80	0.79	8.91	16.3	21.8

Table 4.7. Recurrent themes in lessons learned from monitoring reintroductions of vulnerable species of fish, invertebrates, herpetofauna, birds, and mammals worldwide (Soorae 2011, 2013).

Lessons learned from post-release monitoring
Effect of pre-release conditions: husbandry, housing, transport, behavioral training, captive vs. *in-situ* breeding
Release habitat requirements: composition and size
Optimal release strategy: timing of release during year, age of released individuals, number of individuals released
Effect of post-release supportive measures: supplemental food, artificial burrows, predator control
Importance (or lack thereof) of considering social structure of release cohorts: bonded pairs, familiar groups, sibling cohorts
Relative importance of threats to reestablishment: predation, disease, poaching, nutrition, dispersal, etc.
Influence of releases on communities (ecological and human) at reintroduction sites
Potential need for supplemental releases to maintain demographic and genetic stability
Determine effects of and mitigate inbreeding and outbreeding depression
Best monitoring approaches to evaluate success

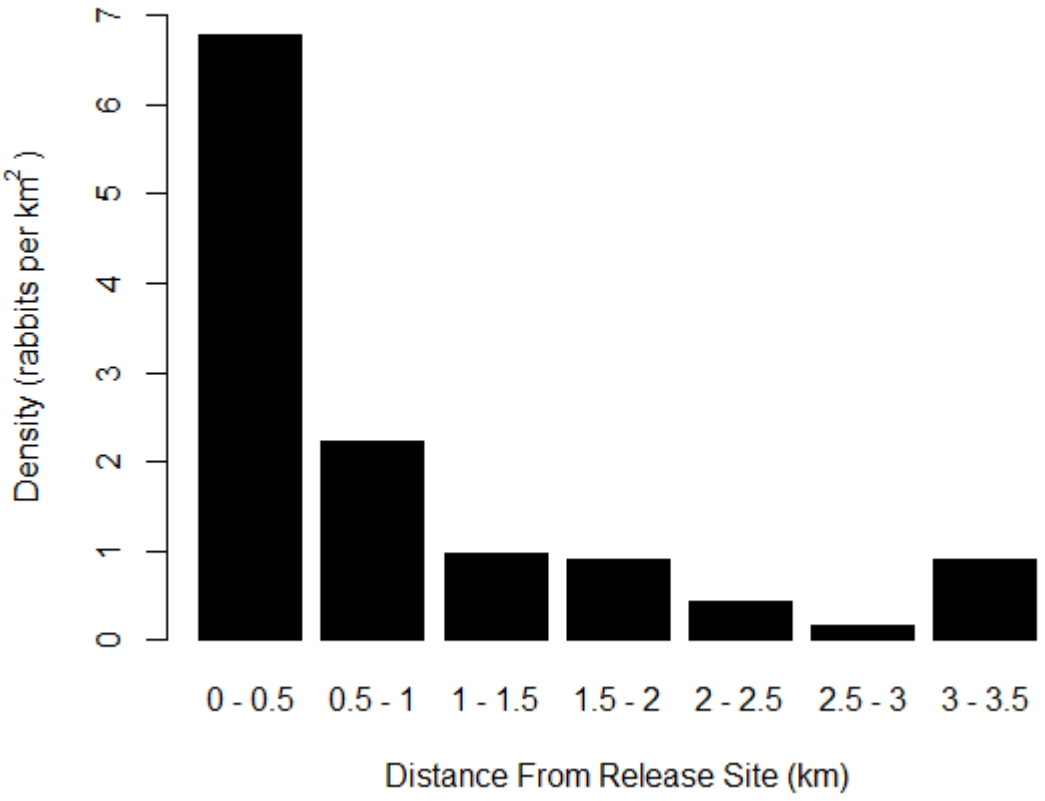

Figure 4.1. Average observed densities of pygmy rabbits detected during winter surveys at varying distances (0 – 3.5 km) from their release sites in central Washington, USA, during 2012-2014.

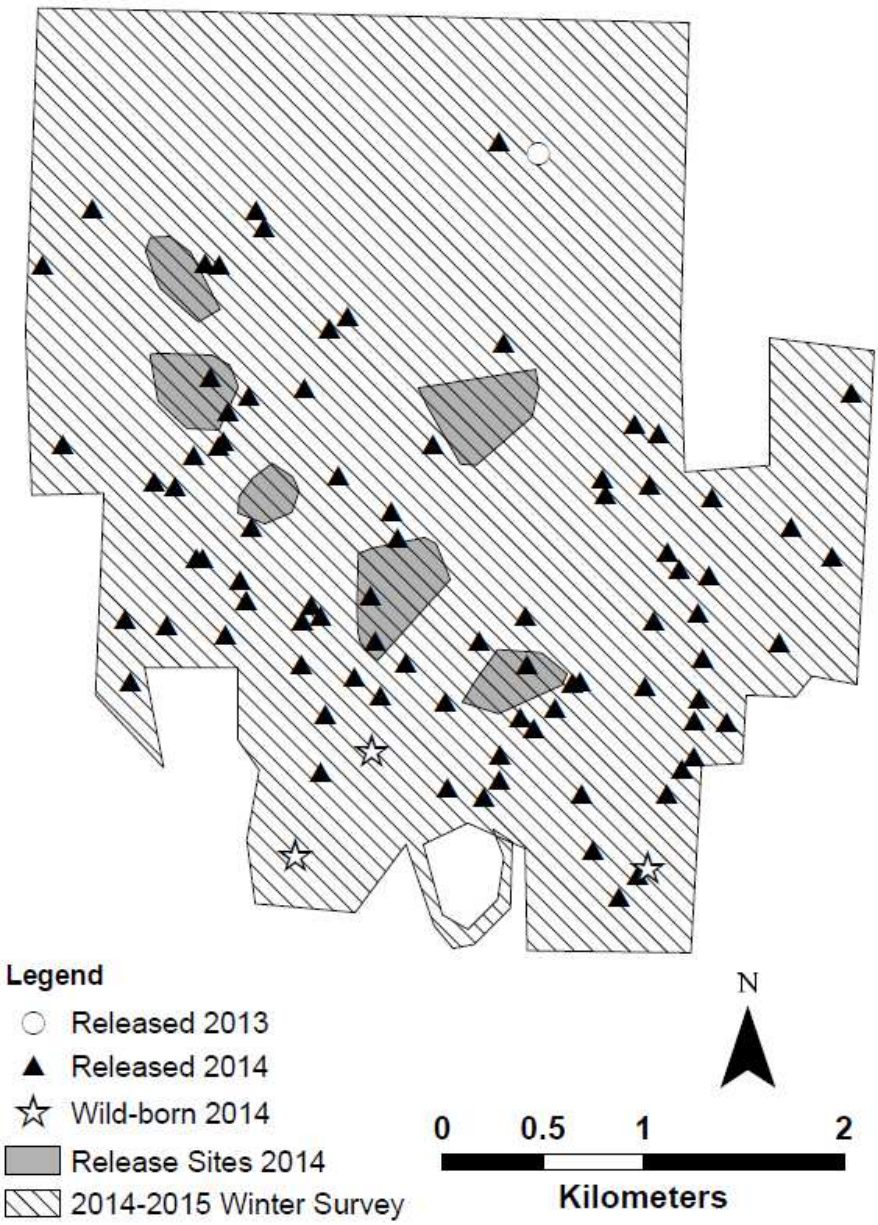

Figure 4.2. Map showing monitoring results from winter surveys conducted the winter following 2014 releases of pygmy rabbits in central Washington, USA. Dark triangles represent rabbits released in 2014, with clusters of release sites represented by gray polygons and the surveyed area represented by diagonal hatching. During this survey we also detected one rabbit surviving from releases in 2013 (open circle), and 3 rabbits born in the wild in 2014 from parents released in 2013 (open stars). Pictured is one representative location per detected rabbit, although many rabbits were detected at multiple burrow systems.

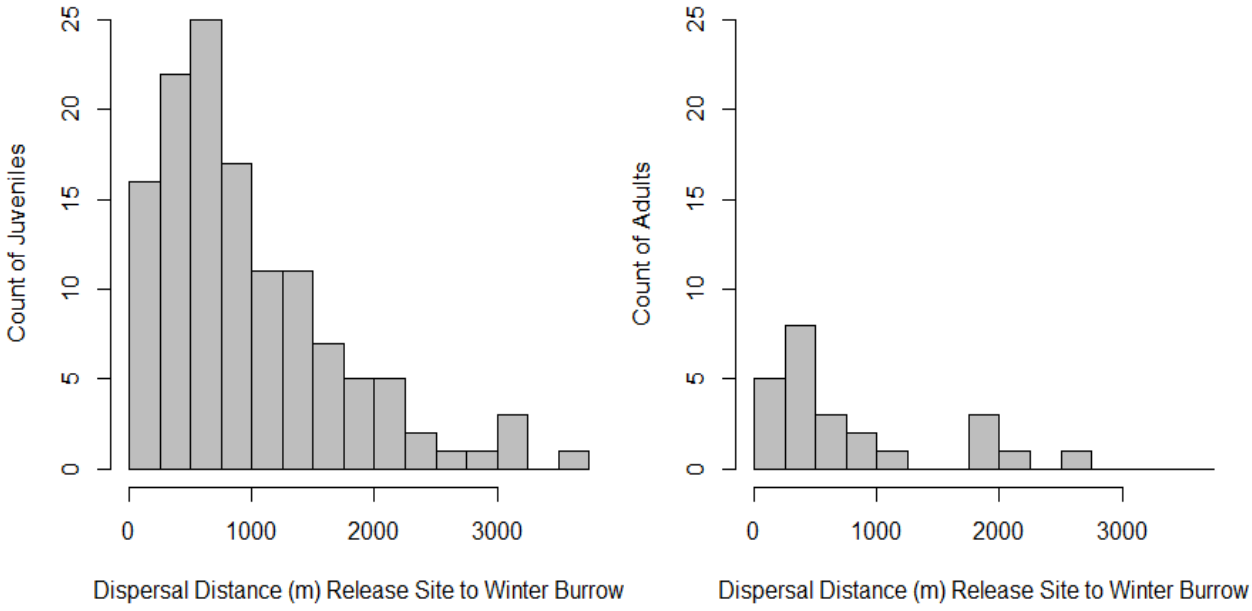

Figure 4.3. Histogram showing straight-line dispersal distances (m) between juvenile and adult pygmy rabbit release sites and active burrows subsequently detected during winter surveys on the release site in central Washington, USA.

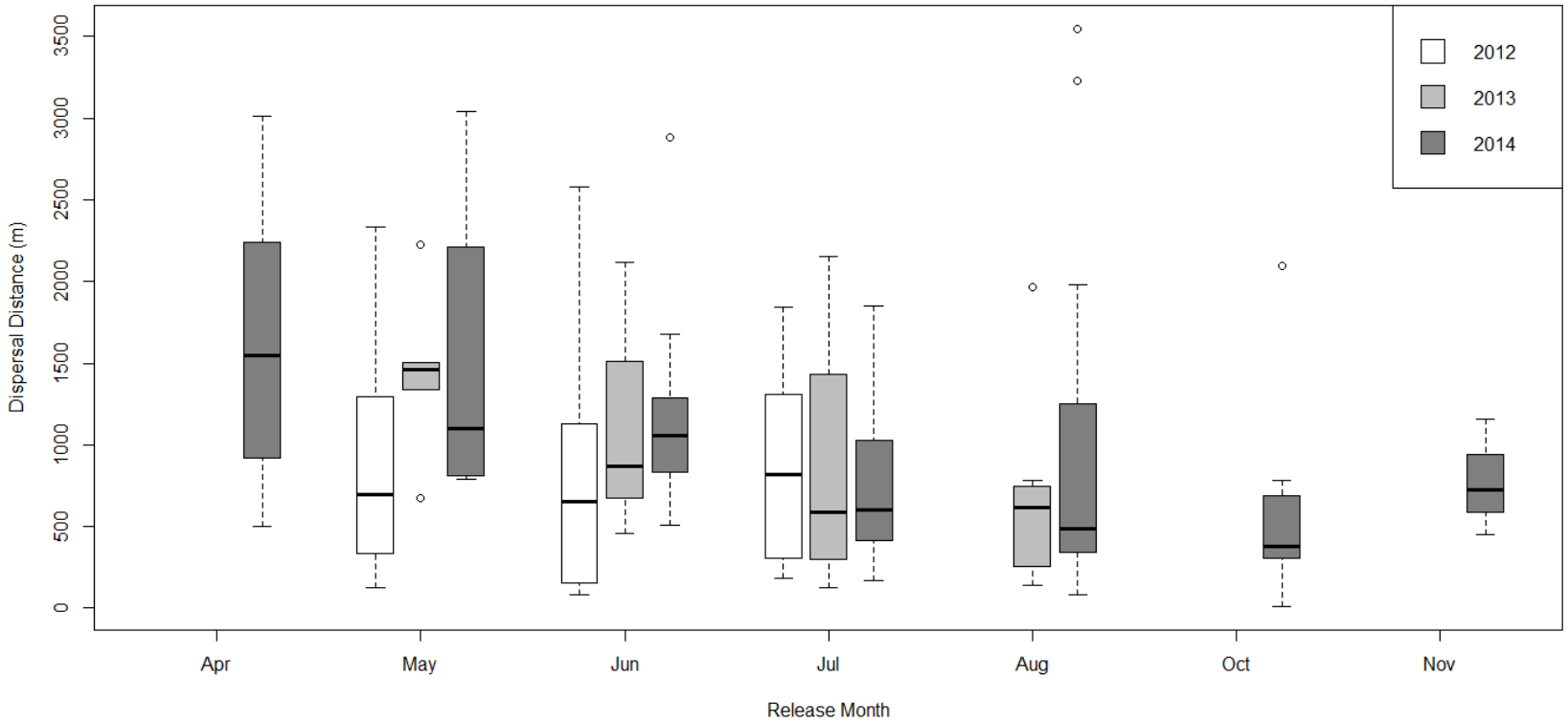

Figure 4.4. Straight-line dispersal distances between release sites and winter active burrows for juvenile pygmy rabbits released in central Washington, USA, from 2012 to 2014. Kits were released May-July in 2012 (n = 104), May-August in 2013 (n = 265), and April-November in 2014 (n = 717).

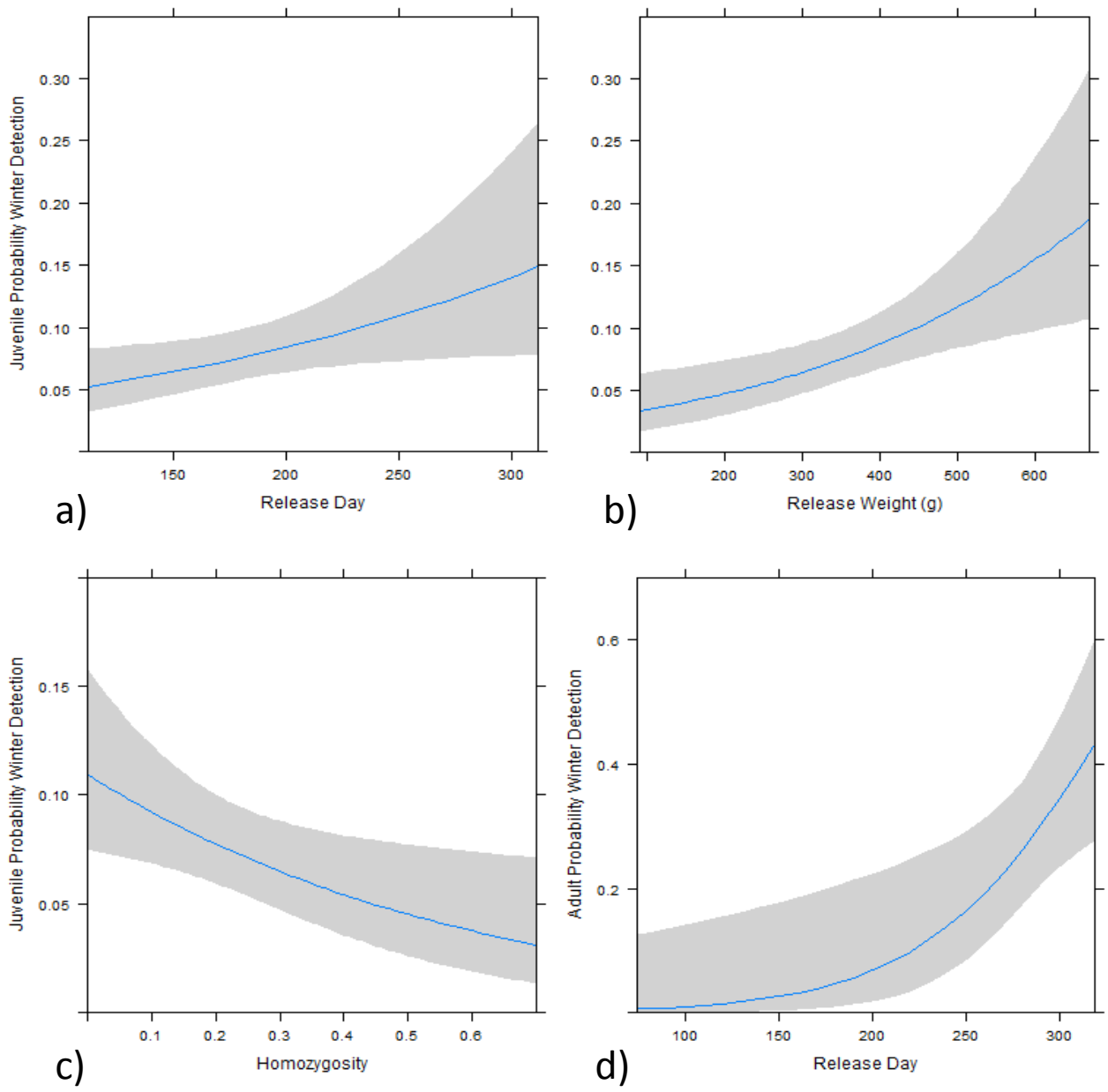

Figure 4.5. Predicted probabilities for survival (survival and subsequent detection on release site) for pygmy rabbits reintroduced in central Washington, USA, in 2014. Figures show a) juvenile survival by release weight, b) juvenile survival by release day, c) juvenile survival by homozygosity, and d) adult survival by release day. Predicted probability plots were generated from the top models for juvenile and adult survival. For the juvenile plots, the variables not being plotted were held constant at their mean for 2014 (weight = 354 g, release day = 181, corresponding to June 30, and homozygosity = 0.2113). Release day 100 corresponds to April 10, 150 to May 30, 200 to June 14, 250 to September 7, and 300 to October 27. Note different y-axis scales.

Conclusion

For this dissertation I created, evaluated, and applied molecular tools to monitor the recovery of endangered pygmy rabbits in Washington. By studying reproduction inside the breeding enclosures I provided more insight into the life history strategy of this cryptic species, and identified factors influencing reproductive rates that can be manipulated to manage populations both in controlled breeding scenarios and wild populations. By monitoring animals post-release, I described dispersal patterns, identified factors that influenced survival of juveniles and adults, and assessed whether current reproduction and survival rates could support a self-sustaining population. All of the monitoring data and inferences has been and will continue to be used by the state and federal agencies charged with managing this recovery to evaluate recovery strategies and inform future directions of managing the enclosure and wild populations. The molecular methods that I developed can and are being used to study pygmy rabbits in other parts of their range, where the species is not federally protected, but is thought to be declining in some portions of their range. Beyond pygmy rabbits, this research highlights the potential for noninvasive genetic monitoring to provide vast amounts of demographic and genetic information about cryptic and/or vulnerable species, and provides lessons about the influence of genetic diversity and composition, population density, and release strategies that can be applied to the protection and recovery of vulnerable species across taxa.

Appendix A: Microsatellite primer testing and optimization

We tested 46 microsatellite loci to include in 3 PCR multiplexes for individual identification and parentage analysis of pygmy rabbits using fecal DNA. These included loci previously developed for pygmy rabbits, loci developed for other rabbit species and previously cross-amplified in pygmy rabbits, loci developed for other rabbits but not previously tested in pygmy rabbits, and new loci cloned from a pygmy rabbit DNA library (Tables A1 and A2). The new loci cloned from a pygmy rabbit DNA library were developed as described in Estes-Zumpf et al. (2008). For each successfully amplified locus, we evaluated polymorphism, null alleles, and probability of identity between siblings (PIsibs; Waits et al. 2001), and tested for Hardy–Weinberg and linkage equilibrium using Genalex 6.5 (Peakall and Smouse 2006, 2012) and Genepop 4.2 (Raymond and Rousset 1995, Rousset 2008), respectively. Our reference collection of pygmy rabbit samples contained purebred and intercrossed rabbits from multiple geographic and genetic sources, so to evaluate Hardy–Weinberg and linkage equilibrium, as well as PIsibs, we chose the 2 groups of samples most likely to be in equilibrium (OR samples, $n = 14$; and NV samples, $n = 24$).

Tested loci were retained if the primers successfully amplified DNA, amplified fragments were <250 base-pairs in length and polymorphic, and fit into a multiplex reaction without overlapping other loci with the same dye label. Additionally, loci were excluded if null alleles were detected in >15% of samples following parentage analysis in Cervus 3.0 (Marshall et al. 1998, Kalinowski et al. 2007). An exception to this is the locus A124, which exhibited null alleles in 33% ($n = 145$) of parentage assignments in an initial exploratory analysis to identify problem loci. Despite the high frequency of null alleles, A124 was highly

polymorphic and informative for individual identification, so we retained the locus for individual identification while excluding it from parentage analyses.

After excluding loci that were monomorphic or had high frequencies of null alleles, we retained 18 loci in 3 multiplexes (19 including the sex ID locus). The first multiplex—PYRBM1—contained 7 loci (excluding the sex ID locus) and was used for individual identification of previously sampled rabbits. The remaining multiplexes—PYRBM2 and PYRBM3—contained 6 and 5 polymorphic loci, respectively, and were used for parentage analysis. The sex ID locus LeMS-Y05 was retained in 2 multiplexes for redundancy. We documented no significant deviations from Hardy–Weinberg (Table A3) or linkage equilibrium in any locus across both tested populations. The resulting PIsibs values indicate that ≥5 successfully genotyped loci in PYRBM1 are adequate to reduce the PIsibs to <1%.

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Table A1. Summary information for microsatellite loci used for genetic monitoring of the Columbia Basin pygmy rabbit. Multiplex conditions and primer concentrations are given for all loci, as well as the number of alleles and fragment lengths found in the Columbia Basin reintroduction founding population. Primer sequences and Genbank Accession numbers are provided for new primers. References for previously developed primers are provided, as well as the species the primers were developed for, if they were not developed from pygmy rabbits. Locus A124 had a high frequency of null alleles and was dropped from parentage analyses, but was still informative for individual identification.

Locus	Multiplex[a]	Concentration (μM)	No. of alleles	Allele lengths (bp)	Repeat	Primer sequences (5'-3')	Accession no.
a) New loci							
A113	PyRbM2	0.041	8	104–113	$(CA)_{15}$	F: ACATGCCGCTGTTTCTCTCAT R: TCCTTGGTAGACGGTGCTCT	KM871174
A12	PyRbM1	0.086 (fecal) 0.114 (tissue)	10	80–100	$(CA)_{14}C(CA)_2$	F: TCCTCAACTGAACATTCAGGT R: TGCCTAGAGCAGTGCAAGA	KM871175
A128	PyRbM3	0.093	10	161–180	$(AC)_{17}$	F: GAATGTCCATCTTACCTTG R: ATGGTCATCTATTGCATATG	KM871176
A129	PyRbM3	0.136	15	145–174	$(TG)_{12}(AG)_{19}$	F: ATAGCATTACTTACCCTCTGC R: GCCCTAGAATTATCCTGCCT	KM871177
A140	PyRbM1	0.050 (fecal) 0.079 (tissue)	14	123–155	$(TC)_6C_4(TC)_5(AC)_{18}$	F: GTTGCAAAGGAGAGCTCACT R: ATTGGATGCAGCCTCAGACT	KM871178
D2	PyRbM3	0.096	7	100–124	$(TAGA)_{10}$	F: CAAGAATAGTAGGAATATAG R: AATCCAGCTACAGCGATACT	KM871179
b) Redesigned primers							
D118	PyRbM2	0.083	20	150–199	$(CT)_{12}CC(CT)_2CC(CT)_2$ CCTCC$(CT)_4$	F: AATTCCTAGCTCCTGCCCAG R: ACAGGCATCCATCTGGCAAG *Redesigned from Estes-Zumpf et al. 2008	
c) Previously reported loci and primers						**Reference; species if not pygmy rabbit**	
7L1D3	PyRbM3	0.050	9	76–94	$(CA)_{10}$	Korstanje et al. 2003; *Oryctolagus cuniculus*	
A121	PyRbM2	0.057	10	194–216	$(TG)_{12}$	Estes-Zumpf et al. 2008	
A124	PyRbM1	0.279 (fecal) 0.107 (tissue)	8	209–223	$(CA)_{14}$	Estes-Zumpf et al. 2008	
A133	PyRbM2	0.031	6	195–207	$(TG)_{15}$	Estes-Zumpf et al. 2008	
A2	PyRbM2	0.114	14	111–136	$(GT)_{13}T(GT)_4GACA(GA)_{11}$	Estes-Zumpf et al. 2008	
D103	PyRbM3	0.050	8	113–138	$(CTAT)_7(TTAT)_3$…(GA)5CA(G	Estes-Zumpf et al. 2008	

					$A)_3$	
LeMS - Y05	PyRbM1, PyRbM2	0.050 (fecal) 0.064 (tissue) 0.043 (M2)	1	177	$(GT)_2(CT)_4TTC$ $T(C)_4(CT)_2TCC$ $TGT(CT)_3TT(C$ $T)_3N_{14}(AAAT)_3$	Putze et al. 2007; *Lepus europaeus*
Sat5	PyRbM2	0.089	15	202–222	$(TC)_{23}TTT(CT)_5$	Mougel et al. 1997; *Oryctolagus cuniculus*
Sat7	PyRbM1	0.043 (fecal) 0.057 (tissue)	6	188–198	$(TG)_{14}$	Mougel et al. 1997; *Oryctolagus cuniculus*
Sat8	PyRbM1	0.023 (fecal) 0.040 (tissue)	18	102–141	$(CT)_{14}(GT)_8TT($ $GT)_5$	Mougel et al. 1997; *Oryctolagus cuniculus*
Sol08	PyRbM1	0.379 (fecal) 0.136 (tissue)	9	113–133	$(TG)_{19}(N)_{15}(TG)_5$	Rico et al. 1994; *Oryctolagus cuniculus*
Sol44	PyRbM1	0.021 (fecal) 0.029 (tissue)	15	192–224	$(GT)_{17}$	Surridge et al. 1997; *Oryctolagus cuniculus*

[a] Multiplex PCR conditions:
All multiplexes: 7-µL reactions using Qiagen Multiplex PCR kit, 1× Qiagen Master Mix, 0.5× Qiagen Q-solution, and 1 µL DNA extract. All PCR programs began with a 15-min denaturation step at 94° C, followed by a touchdown, cycles at a stable annealing temperature, 30-min final extension at 60° C, and a cool-down at 4° C.
PyRbM1: 94° C—30 sec, 65° C—90 sec, 72° C—60 sec, touchdown 0.5° C per cycle to 60° C, then 21 (tissue) or 35 (fecal) cycles at 60° C.
PyRbM2: 94° C—30 sec, 62° C—90 sec, 72° C—60 sec, touchdown 0.5° C per cycle to 59° C, then 26 (tissue) or 39 (fecal) cycles at 59° C.
PyRbM3: 94° C—30 sec, 56° C—90 sec, 72° C—60 sec, touchdown 0.5° C per cycle to 50° C, then 22 (tissue) or 35 (fecal) cycles at 50° C.

Table A2. Microsatellite loci not included in final multiplexes for amplifying DNA from pygmy rabbit fecal pellets. Primer sequences are given for new or redesigned loci, and the reason for exclusion is provided for all loci.

Locus	Primer Sequences (5'-3')	Original citation; species (if not pygmy rabbit)	Reason for dropping
5L1A8	As previously reported	Korstanje et al. 2003*; Oryctolagus cuniculus*	Failed
A10	F: AATAGACTCTCCCTAGGATA R: TCTGATAATGGGATGCATGT	Redesigned from Estes-Zumpf et al. 2008	Null alleles
A106	F: TCAGTGCAGCCGCCTGTC R: GACTTCAACATATGAATCTTGG	New	Failed
A107	F: AACTTCCAGAACCCCACT R: TCGTACAGGCGTTGCTG	New	Failed
A11	F: ATTGCTTCACACCAAAACG R: GATTATGCTAGTGCTTGTTA	New	Failed
A111	F: ACCACGAGCATCGCTCT R: TTGTTTCTACCTCGACGG	New	Failed
A114	F: GGACACCACGAGCATCG R: TGCTCGTGGTGTCCCGT	New	Failed
A117	F: GTAAAAGAACTTCCAGAACC R: TACAGGCGTTGCTGAGAG	New	PCR product too long (>250 bp)
A4	F: CAACATGTGGAGCAGGAGGT R: CAGAGTTGAGCGTGTGACAG	New	Monomorphic
A8	F: TACCAGACTCCATCCCAGC R: AGTCACAGAATGGCTCAGTC	New	Monomorphic
D1	F: CTACGCTTGTTCGTGTATC R: CAGTGTGCTGCTGCATTC	New	Failed
D10	F: ATTTGACTTGTGTTTATTGTGTAT R: TCTCCAACATCGCTGACTACA	New	Failed
D111	F: ATTCAGATAAATAGATGGATGGA R: ATTATCACTGTCAATACCTGATT	New	Failed
D121	F: TGGCTCTAGCATGGGGCAG R: GGGCAATGGAAGATATATTCA	Redesigned from Estes-Zumpf et al. 2008	Null alleles

D125	F: GTACCTGAATGTGACTGAAGA R: GGTAGGTAAGGTTAGTTAGGT	New	Failed
D126	As previously reported	Estes-Zumpf et al. 2008	Difficult to score
LeMS-Y04 (sex ID)	As previously reported	Putze et al. 2007; *Lepus europaeus*	Failed
Sat12	As previously reported	Mougel et al. 1997*; Oryctolagus cuniculus*	Required very high primer concentration
Sat13	As previously reported	Mougel et al. 1997*; Oryctolagus cuniculus*	Failed
Sat16	F: TGCCTGCCAAAGTCAGTTC R: TTGCTCCAGAACATGCTTTAA	Redesigned from Mougel et al. 1997*; Oryctolagus cuniculus*	Failed
Sat2	F: AGAATTATGCAGAGAGAGGG R: TTGGGGAGTGAACTAGAAGG	Redesigned from Mougel et al. 1997*; Oryctolagus cuniculus*	Failed in multiplexes (successful alone)
Sat3	As previously reported	Mougel et al. 1997*; Oryctolagus cuniculus*	Failed
Sat4	F:AAAGTCAGAGTTACACACAGAT R: AATTGGGGAGTGAATCAGCA	Redesigned from Mougel et al. 1997*; Oryctolagus cuniculus*	Failed
Sol28	As previously reported	Rico et al. 1994*; Oryctolagus cuniculus*	Failed
Sol3	F: ACACCAATGGCTGTAAATGTT R: TACCGAGCACCAGATATTAG	Redesigned from Rico et al. 1994*; Oryctolagus cuniculus*	Failed
Sol30	As previously reported	Rico et al. 1994*; Oryctolagus cuniculus*	PCR product too long (>250 bp)
Sol33	F: GGAAGTATATAGTCTGAGATAC R: GGGCCAATAGGTACTGATC	Redesigned from Surridge et al. 1997*; Oryctolagus cuniculus*	Failed

Table A3. Expected heterozygosity (H_E), observed heterozygosity (H_O), and probability of identity between siblings (PIsibs) values for final multiplex loci, calculated using Genalex 6.5 from pygmy rabbits from Oregon and Nevada. Heterozygosity values in bold indicate loci out of HWE ($P < 0.05$).

	Oregon			Nevada		
Locus	**H_E**	**H_O**	**PIsibs**	**H_E**	**H_O**	**PIsibs**
7L1D3	0.643	0.742	0.41	0.792	0.780	0.38
A113	0.500	0.699	0.44	0.708	0.776	0.38
A12	0.833	0.795	0.37	0.708	0.779	0.38
A121	**0.643**	**0.770**	0.39	0.750	0.768	0.39
A124	**0.500**	**0.633**	0.47	0.542	0.768	0.39
A128	0.857	0.827	0.35	0.875	0.838	0.34
A129	0.929	0.890	0.31	0.875	0.892	0.31
A133	0.571	0.758	0.40	0.625	0.688	0.44
A140	0.571	0.589	0.51	0.875	0.844	0.34
A2	0.800	0.835	0.34	0.905	0.861	0.33
D103	0.692	0.648	0.47	0.652	0.659	0.47
D118	0.786	0.832	0.35	0.957	0.918	0.29
D2	0.643	0.763	0.39	0.500	0.647	0.47
Sat5	0.857	0.847	0.34	0.870	0.826	0.35
Sat7	0.429	0.709	0.43	0.667	0.747	0.40
Sat8	0.786	0.842	0.34	**0.958**	**0.911**	0.30
Sol08	0.500	0.658	0.46	0.792	0.775	0.38
Sol44	0.929	0.867	0.32	0.917	0.859	0.33

Appendix B: Itemized cost calculations

NGS	Quantity	Cost per	Total Cost
Lab costs with labor ($21 per hr labor rate)			
pellets, species ID	9	**$13.48** *$7.50 supplies* *$5.98 labor*	$ 121.36
pellets, species ID and ind ID, 1 multiplex (to match with previously sampled rabbit)	102	**$24.75** *$11.00 supplies* *$13.75 labor*	$ 2,524.07
pellets, species ID and ind ID, 3 multiplexes (new individual)	6	**$55.82** *$35.00 supplies* *$20.82 labor*	$ 334.93
tissue, 3 multiplexes (new individual)	104	**$21.34** *$13.50 supplies* *$7.84 labor*	$ 2,219.53
Staff time for surveying, 300 hours		***	$ 7,092.00
Field Supplies			
Biopsy punches	104	$2	$ 208.00
Ethanol			$ 24.00
Silica gel	6	$23	$ 138.00
Coin envelopes			$ 5.00
Freezer boxes			$ 10.00
Vials			$ 47.00
Gauze			$ 10.00
Rubbing alcohol			$ 10.00
		Total	**$ 12,743.89**

Cost per rabbit	**n**	**Cost**
Rabbits tracked	104	$ 122.54
Rabbits detected	42	$ 303.43

Telemetry	Quantity	Cost per	Total Cost
Telemetry flight			$ 2,160.00
Biologist flight hours	8	$28	$ 224.00
Transmitters	85	$180	$ 15,300.00
Glue			$ 30.00
Mesh			$ 20.00
Telemetry monitoring (2 technicians)		***	$ 3,859.00
Fuel, 40 miles per week x $0.51 per mile	11	$20.40	$ 224.40
		Total	**$ 21,817.40**

Cost per rabbit	**n**	**Cost**
Rabbits tracked	85	$ 256.68
Rabbits detected	63	$ 346.31

***Personnel Hours

NGS- Snow surveys Hours per person per day

Dates	Research Sci	Bio 1	Bio 2	Bio 4	Sci Tech	Bio 2	Research Sci	Bio 2	Bio 1
12/12/12	8	8	8	x	x	x	x	x	8
12/13/12	8	8	8	8	x	x	x	x	8
12/14/12	8	8	8	8	x	x	x	x	x
12/17/12	8	8	8	x	x	x	x	x	x
12/18/12	8	4	8	x	x	x	x	8	x
12/20/12	8	8	8	x	8	8	8	x	x
12/26/12	8	8	x	x	8	x	x	x	x
12/27/12	8	8	x	8	8	x	x	x	x
12/28/12	8	8	x	8	x	x	x	x	x
1/4/13	8	8	8	x	8	x	x	x	x
Total	80	76	56	32	32	8	8	8	x
Rate per hour	$30	$17	$21	$28	$15	$21	$30	$21	$17
Cost per person	**$2,400**	**$1,292**	**$1,176**	**$896**	**$480**	**$168**	**$240**	**$168**	**$272**
								$7,092	**Total**

Telemetry

Type of tracking day	Days	Hours per day	Total hours	
Non-visual	21	2.5	52.5	
Visual	13	4.5	58.5	
LE41 only- last rabbit	5	0.5	2.5	
			113.5 hrs	x $17 x 2 techs
				$3,859 Total

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