A Rabbit's Tale: Genetic monitoring, genomic diversity, and habitat selection in the endangered Columbia Basin pygmy rabbit (*Brachylagus idahoensis*)

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Authorization to Submit Dissertation

This dissertation of Stacey Ann Nerkowski, submitted for the degree of Doctor of Philosophy with a Major in Natural Resources and titled "**A Rabbit's Tale: Genetic monitoring, genomic diversity, and habitat selection in the endangered Columbia Basin pygmy rabbit (*Brachylagus idahoensis*)**," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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Abstract

Loss and fragmentation of habitat led to the near extirpation of the disjunct pygmy rabbit (*Brachylagus idahoensis*) population in the Columbia Basin of Washington state. In 2003, the Columbia Basin pygmy rabbits was listed as an endangered distinct population segment under the United Stated Endangered Species Act. In 2001, 16 Columbia Basin pygmy rabbits were taken from the last remaining population in Sagebrush Flat wildlife area and used to start a captive breeding program. To counteract the high levels of inbreeding among the rabbits, a genetic rescue was performed by adding 4? pygmy rabbits from Idaho. In 2011, with the main goal of reintroduction of rabbits back to the state of Washington, the captive breeding program transitioned to on-site breeding, where genetic and demographic rescue was performed by introducing an additional 100ish pygmy rabbits from regional populations across the species range. Since the first translocations in 2012, over 1900 mixed-ancestry rabbits have been translocated into the Sagebrush Flat wildlife area. Two additional populations were established later in Beezley Hills Preserve (2017) and Chester Butte wildlife area (2018).

Monitoring of these translocated populations of endangered species rabbits is crucial for evaluating and informing conservation strategies to maximize the chances of a successful recovery. We used noninvasive invasive genetic sampling to evaluate demographic and population genetic parameters on three reintroduced populations of pygmy rabbits over 8 years (2012-2020). For each population, we evaluated spatial distribution, apparent survival rates, post-release dispersal distance, genetic diversity, reproduction, and the persistence of Columbia Basin ancestry. For five groups of pygmy rabbits maintained in large breeding enclosures within native habitat, we estimated genetic diversity and Columbia Basin ancestry from 2012-2020. Over the course of this study, 1479 rabbits (Sagebrush Flat), 461 rabbits (Beezley Hills), and 38 rabbits (Chester Butter) were reintroduced by a cooperation between state and federal agencies. Through winter and summer monitoring surveys, we identified 168 released rabbits and 420 wild-born rabbits in Sagebrush Flat, 13 released rabbits and 2 wild-born in Beezley Hills, and 16 released rabbits in Chester Butte. Survival differed across years and was positively influenced by release date, release weight, and heterozygosity (Chapter 1).

To better understand the mixed-ancestry rabbits within Washington, we needed to evaluate the genomic diversity across the species' range. We used restriction site-associated DNA sequencing (RADseq) approach on 123 rabbit samples, including pure Columbia Basin pygmy rabbits, to identify single nucleotide polymorphisms (SNPs) and determine population genetic structure across the pygmy rabbit range, assess the distinctiveness of the Washington population, and test for genomic signatures of adaptive divergence among populations. Using 12,084 SNPs, we identified four distinct

genetic groups: (1) Washington, (2) Great Basin (California, Nevada, Idaho, Montana), (3) northern Utah/Wyoming and (4) southern Utah. The Washington population was most divergent compared to the other genetic groups, reinforcing its federal protected status as a distinct population segment. Identifying genetic markers for ancestry from the multiple pygmy rabbit populations will help monitor variation in the admixed Washington population and assess the consequences of genetic rescue efforts (Chapter 2).

Through winter monitoring surveys performed between 2012 and 2020 on the wild population at Sagebrush Flat wildlife area, we observed a shift in spatial distribution of pygmy rabbit burrows from native shrub-steppe habitat (Sagebrush Flat) to Conservation Reserve Program (CRP) habitat that had been revegetated with native shrub-steppe flora in the mid-1990s. We compared vegetative and soil characteristics to test hypotheses about factors driving pygmy rabbit habitat selection. We identified that shrub canopy cover, living canopy cover, and composition of the canopy (living sagebrush) were higher in occupied sites and sagebrush was more nutritious in CRP habitat. These findings can help guide management strategies and provide the necessary tools to identify suitable habitat for future release efforts for the endangered Columbia Basin pygmy rabbit and have demonstrated the value of habitat restoration efforts like CRP (Chapter 3).

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Dedication

To my parents. Your love and support are what got me here.

To Liane, you are a true friend.

To my fluffy friend, Koda. Continue to be you and bring smiles to me and everyone else.

Table of Contents

List of Tables

List of Figures

Chapter 1: Long term noninvasive genetic monitoring guides recovery of the endangered Columbia Basin pygmy rabbits (Brachylagus idahoensis).

Abstract

Loss and fragmentation of habitat from agricultural conversion has led to the near extirpation of the pygmy rabbit population in the Columbia Basin (CB) of Washington, USA. Recovery efforts began in 2002 and included captive breeding, translocations, and reintroduction into native habitat. We used noninvasive invasive genetic sampling to evaluate demographic and population genetic parameters on three translocated populations of pygmy rabbits (SBF, BH, CHB) over 8 years (2012-2020). For each population, we evaluated spatial distribution, apparent survival rates, post-release dispersal distance, genetic diversity, reproduction, and the persistence of CB ancestry. For five populations of pygmy rabbits maintained in large enclosures within native habitat, we estimated genetic diversity and CB ancestry from 2012-2020. Over the course of this study, 1479 rabbits (SBF), 461 rabbits (BH), and 38 rabbits (CHB) were reintroduced by a cooperation between state and federal agencies. Through winter and summer monitoring surveys, we identified 168 released rabbits and 420 wild-born rabbits in SBF, 13 released rabbits and 2 wild-born in BH, and 16 released rabbits in CHB. Observed heterozygosity (Ho) values ranged from 0.62-0.84 (SBF), 0.59-0.80 (BH), and 0.73-0.77 (CHB). Allelic richness (AR) ranged from 4.67-5.35 (SBF), 3.71-5.41 (BH), and 3.69-4.65 (CHB). Effective population (Ne) within SBF varied from 12.3 (2012) to 44.3 (2017). CB ancestry persisted in all three wild populations, ranging from 14.85%-27.46%. CB ancestry persisted in 99% of wild-born juveniles identified in SBF. Post-release dispersal at SBF averaged 988 m for juveniles and 783 m for adults but did not differ significantly by sex or age. Dispersal distances of rabbits detected in a 2nd year were greater for males (804 m) than females (351 m). Apparent survival of juvenile rabbits differed across years (1 - 39%) and was positively associated with release date, release weight, and genetic diversity. Survival of adults (0 - 43%) was positively influenced by release day, with some evidence that genetic diversity positively influenced adult apparent survival. Rabbits were directly released into the wild at SBF (hard release), whereas rabbits released into CHB and BH were placed in release pens until the pens were breached in winter (soft releases). Dispersal distances were significantly shorter for soft release methods (BH – 178 m, CHB – 286 m) compared to hard releases (SBF - 988 m), and apparent survival increased 22 - 32% with soft releases. Survival of juveniles deployed to release pens was positively influenced by release day. Our findings provide critical

information on the success of the reintroduction efforts and provide information for future conservation and management efforts.

Introduction

The loss of biodiversity is one of the most important environmental problems facing the world today (Pimm et al. 2014). Rapid human population growth, environmental change, and habitat fragmentation all pose ever-greater threats to biodiversity and highlight the need for increasingly aggressive conservation efforts (Hedrick et al. 2014). Conservation biologists use an interdisciplinary tool set to develop management plans and evaluate the sustainability of species and populations. Key tools for biodiversity monitoring use methodological approaches that rely on genetic tools for evaluating change (Stetz et al. 2011). Genetic monitoring studies have been used to address many conservation issues, including population abundance (Blouin et al. 1996; Koskinen 2003; Maes et al. 2006), population assignments and population structure (Cooper et al. 2010; Khrustaleva et al. 2017), parentage analysis (DeMay et al. 2017), and population bottlenecks (Osborne et al. 2016). Noninvasive genetic sampling has become a common method for sample collection in many genetic monitoring studies. Noninvasive genetic sampling allows researchers to monitor populations through the collection of feces, hair, saliva, feathers, or any other biological material left behind by an animal (Taberlet et al. 1999; Waits and Paetkau 2005), without capturing, disturbing, or even observing individuals (Taberlet et al. 1999; Beja-Pereira et al. 2009).

Endangered species and isolated populations typically face genetically related threats such as loss of genetic variation and inbreeding that can ultimately lower the fitness of the individual and population (Tallmon et al. 2004). Genetic rescue has the potential to be one of the most powerful means to conserve small and declining populations, yet it remains controversial and is rarely applied (Mills and Allendorf 1996; Edmands 2007; Frankham et al. 2011; Whiteley et al. 2015). A major concern with genetic recues is that gene flow can decrease fitness through outbreeding depression, potentially increasing the risk of extinction (Edmands 2007). Genetic rescue has increased genetic variation and resulted in population recovery for a variety of terrestrial wildlife species including Mexican wolves (*Canis lupus baileyi*; Fredrickson et al. 2007), Florida panthers (*Puma concolor coryi*; Pimm et al. 2006), greater prairie chicken (*Tympanuchus cupido*; Mussmann et al. 2017), arctic fox (*Vulpes lagopus*; Hasselgren et al. 2018), and bighorn sheep (*Ovis canadensis*; Miller et al. 2012). Monitoring for potential negative consequences of genetic rescue is crucial in assessing the outcome of the genetic rescue of the population (Robinson et al. 2020).

Here we present an 8-year study that uses traditional tissue sampling and noninvasive fecal DNA sampling to monitor the world's smallest rabbit, the pygmy rabbit (*Brachylagus idahoensis*).

Pygmy rabbit populations are found mostly across the Great Basin of the western United States including the states of Wyoming, Utah, Nevada, Oregon, California, Montana, Colorado, and Idaho. A small, disjunct population occurs within the Columbia Basin (CB) of central Washington (Figure 1.1). The CB population in Washington has been spatially and genetically isolated for at least 10,000 years but present in the area for nearly 100,000 years (Lyman 1991; Warheit 2001). The CB pygmy rabbits were considered a distinct population segment, the smallest division of a species warranted protection under the Endangered Species Act, and were state listed (Washington) in 1993, and federally emergency listed (Endangered Species Act) in 2001, with a final ruling in 2003 (WDFW 1995, 2003; U.S. Fish and Wildlife Service 2003; Becker et al. 2011). At the time of federal listing, the population included fewer than 30 individuals in the wild, and the geographic distribution in Washington was reduced from 6 populations in five counties in the 1990s to a single population at Sagebrush Flat (SBF) in Douglas County (WDFW 1995, 2003; U.S. Fish and Wildlife Service 2003; Becker et al. 2011).

In an attempt to save the population from extinction, the last remaining 16 individuals were captured and brought into captivity in 2001, to establish a captive breeding population to support future reintroduction efforts (Becker et al. 2011). Decreased reproductive success in captivity and low genetic diversity suggested that the CB population was experiencing inbreeding depression (Warheit 2001; Elias et al. 2013). To counteract potential inbreeding depression and provide a genetic rescue, four Idaho pygmy rabbits were introduced into the captive breeding program in 2003 (Becker et al. 2011; U.S. Fish and Wildlife Service 2012). Breeding was carefully managed to prevent inundating the captive CB population with Idaho genetic variation and to preserve unique CB ancestry while maintaining genetic health (Elias et al. 2013).

With the main goal of the CB Recovery Program to establish a sustainable wild population, the captive breeding program ended in 2011 and transitioned to semi-wild onsite breeding enclosures (U.S. Fish and Wildlife Service 2012). To provide further genetic rescue and the necessary numbers needed for release, 111 wild pygmy rabbits were translocated from Oregon, Nevada, Utah, and Wyoming, and were kept in the same large enclosures to encourage interbreeding. Since the first releases in 2012 onto the Sagebrush Flat wildlife area (SBF) in Washington (Figure 1.1), a total of 1,947 pygmy rabbits have been released (1782 juveniles and 165 adults) (Hayes 2018). Monitoring of these mixed ancestries, released individuals and reproduction in the wild is crucial to the overall goal of a sustainable population. Additionally, in summer 2018, two new populations were established in the Beezley Hills (BH) and Chester Butte (CHB) recovery areas (Figure 1.1).

DeMay et al. (2016) used microsatellite loci to perform parentage analyses to assess the influence of ancestry, population density, and genetic diversity on reproduction and mating system within the breeding enclosures. As population densities increased, male reproductive output decreased as genetic diversity declined. Males with >50% Columbia Basin ancestry had higher reproductive output whereas males of northern Utah/Wyoming ancestry had lower reproductive output. Female reproductive output decreased with Nevada/Oregon ancestry (DeMay et al. 2016). This information indicates that ancestry plays a role in reproductive fitness in wild/released populations and should be monitored in the wild and enclosure populations.

Noninvasive genetic sampling of fecal pellets has become a valuable method for monitoring the reintroduced CB pygmy rabbit populations (DeMay et al. 2013, 2017). The goal of our genetic monitoring project was to combine data from 2012- 2020 to (1) assess habitat occupancy and spatial distribution of wild populations, (2) estimate dispersal distances of released rabbits, (3) assess demographics of the wild population from 2017-2020 when no releases occurred, (4) estimate genetic diversity and persistence of CB ancestry of wild and enclosure populations, (5) assess apparent survival of released rabbits in the SBF population and determine which genetic and/or demographic factors influence survival, and (6) compare apparent survival rates and dispersal distances in hard vs. soft release efforts (Table 1.1).

First, we predicted that burrow establishment would be closer to release sites within the SBF area, as was seen in the 2012-2014 cohorts (DeMay et al. 2017), with minimal occupancy on the edges of SBF. Secondly, we predicted that dispersal distances would differ between adults and juveniles but would not differ between sexes. DeMay et al. (2017) documented that median dispersal distance differed between released juveniles and adults (770m and 471m, respectively) in the 2012-2014 cohorts, and juveniles released later in the year dispersed shorter distances. Therefore, we expected to find similar results in 2015 and 2016 cohorts that were released into SBF. For our third and fourth goal, we monitored demographic factors, genetic diversity and determined genetic estimates of CB ancestry in the enclosure populations, release cohorts, and wild populations, effectively guiding management strategies. We predicted that CB ancestry will be maintained in enclosure and wild populations since juveniles with higher CB ancestry were retained as breeders. We expected a decrease in heterozygosity, over the 8 years in wild and in enclosure populations, because of the limited number of founders and $N_e < 100$. Preserving the adaptive differences in this distinct population segment by persistence of CB ancestry is a main goal of the species recovery plan (U.S. Fish and Wildlife Service 2012).

For our fifth goal, we predicted juvenile apparent survival rates in the SBF population would be positively influenced by year, release weight, release day, and homozygosity, and adult apparent survival would be positively influenced by release day and heterozygosity (DeMay et al. 2017; Scott et al. 2020). We expected that apparent survival rates 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 also expected that older juveniles would have a higher probability of survival because they had more time in the breeding enclosures with high-quality food and protection from predators and could achieve better body condition prior to being released compared to those released at younger ages (Rödel et al. 2004). Finally, we predicted that survival rates would be higher and dispersal rates lower in the soft release pens in BH and CHB than the hard releases performed in SBF.

Methods and Materials

Study Area

The study areas for this project are SBF (1514 ha), Dormaier (DM; 146 ha), Chester Butte (893 ha) in Douglas County in central Washington, and Beezley Hills (83 ha) in Grant County in central Washington (Figure 1.1). All study sites were located on the Columbia Plateau Province (Crab Creek sub-basin). SBF, CHB and DM were three of four geographically separate units of the larger Sagebrush Flat Wildlife Area (SFWA) managed by Washington Department of Fish and Wildlife (WDFW) and the only ones containing pygmy rabbits. The SFWA was managed specifically for endangered and threatened pygmy rabbits, sage grouse (*Centrocercus urophasianus*), and sharp-tailed grouse (*Tympanuchus phasianellus*; WDFW 2006). BH was a combination of private land and land owned/managed by The Nature Conservancy (Washington chapter) (Hayes 2018). These sites were characterized by dense sagebrush (*Artemisia sp*.), deep soils, and mounded micro-topography (Tullis 1995; WDFW 2006). SBF is the only site characterized by mima mounds, which are natural mounds composed of loose, unstratified sediment that is overthickened with sagebrush and other grasses and forbs. All sites were surrounded by state, federal, and private lands, with a land cover mosaic of sagebrush steppe and wheat fields. The SBF Unit was also surrounded by Conservation Reserve Program (CRP) lands, which are agricultural fields that were revegetated with sagebrush-steppe flora in the mid-1990s (WDFW 2006) (Figures 1.2,1.3). Predators of pygmy rabbits within SFWA and BH include badgers (*Taxidea taxus*), long-tailed weasels (*Mustela frenata*), coyotes (*Canis latrans*), short-eared owls (*Asio flammeus*), and several other raptor species. Temperatures (30-year average) ranged from an average minimum of -6°C in December to an average maximum of 31.2°C in July (Western Regional Climate Center 2020). This semi-arid environment averages about 20.3 cm of annual

precipitation, over half of which is from snow (WDFW 2006; Western Regional Climate Center 2020).

Two types of breeding enclosures were used. Four large, predator-resistant enclosures (2.2-4.4 ha) were located at BH (1), DM (1), and SBF (2). All on-site breeding occurred at these enclosures from 2011-2017. Because of the decrease in reproductive success and habitat degradation within these large enclosures in which thousands of pygmy rabbits had bred, managers began phasing them out in 2017. In June 2017, the Sutherland Canyon fire destroyed the BH enclosure and damaged a large portion of the surrounding habitat used for releases. At this time, new smaller mobile breeding enclosures were designed and implemented. These 1.21-ha circular enclosures were semi-predator-resistant and could easily be moved every 2-3 years to prevent habitat degradation and would house no more than 10 adults (Hayes 2018). The first of the mobile breeding enclosure was implemented at BH (2017). From 2018-2019, onsite breeding was conducted only at the large DM enclosure and the mobile breeding enclosure at BH.

Field Methods

Juveniles were captured from breeding enclosures and released to the wild or kept for breeding during the 2012-2019 breeding seasons. Individuals were captured using Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin) set at burrow entrances or known rabbit trails and covered with burlap to minimize stress on the individual. In large enclosures, juveniles were captured from open-ended artificial burrows (buried 10-cm diameter drainage tubes, approximately 1-m long) using a modified version of a plumber snake with a tennis ball on one end to easily push juveniles into a pillowcase on the opposite end. Starting in 2017, net panels were designed to capture juveniles where rabbits were herded toward a wall of net panels, enabling researchers to flush rabbits to an area. Captures and releases occurred from late April to late September.

Initially, release efforts concentrated on juveniles with minimal trapping efforts on adults (Table 1.2). In 2014, to minimize the overcrowding within the larger enclosures, we released a larger number of adults. From 2012-2014, all released individuals were translocated to SBF. In 2015, we released 153 individuals (149 juveniles and 4 adults) into SBF, but to establish a second population, we released 420 individuals (369 juveniles and 51 adults) into the BH recovery area. In 2016, we released all individuals into SBF. Due to a wildfire that burned 119km2 of sagebrush steppe habitat in June 2017 that destroyed the BH enclosure (Sutherland Canyon fire), no rabbits were released in 2017. In a second attempt by managers to establish a population at BH, and a new reintroduced population in the CHB recovery area, all rabbits from 2018-2020 were released to one of these areas. No further augmentation to the SBF population occurred after 2016. We released rabbits into the BH and CHB

release areas followed a soft release protocol. Rabbits were placed into 0.40-ha circular pens with temporary fencing made of chicken wire. These release pens were left in place until the end of winter, but rabbits were known to move in and out of the release pens across this time span. Depending on snowfall, the release pens may have been breached before the end of winter, which enabled us control of the movement of pygmy rabbits in the newly established recovery areas since most of the surrounding land was privately owned.

All juveniles and adults trapped in the enclosures were weighed, sexed, and treated for parasites with Advantage II kitten formula (BayerDVM, Shawnee Mission, Kansas). We collected a 2-mm skin biopsy from the ear, that was stored in 95% ethanol, and frozen at -20°C until laboratory analysis could be performed. Juveniles that were retained as breeders typically contained high levels of CB ancestry ($\bar{X}$=29.08%±13.86%). All individuals retained for breeding were microchipped (Avid Identification Systems, Inc., Norco, California). Individuals were also swapped among the enclosures to increase genetic diversity of future breeding.

Individuals released at SBF followed mostly hard-release methods where rabbits were released at mima mounds across 2-6 release areas (17-37 release sites per area) as described in DeMay et al. (2017). Artificial burrows, auger holes and supplemental food were provided at release sites. We placed rabbits into artificial burrows (up to 2 rabbits per release site, 1 per burrow, on a given release day) in which burlap was used to plug each end for approximately 5 minutes and the burlap was then removed quietly. This minimized the stress on the animal after translocation (DeMay et al. 2017). Augmentation in the SBF population ended in 2016; beginning in summer 2017, juveniles were placed in temporary release pens (0.40 ha) to increase survivorship and limit dispersal distances. These release pens were considered a soft release protocol, allowing for acclimatization to the new habitat, in which the pens were breached during winter months. No more than 10 juveniles were placed into a release pen.

Because of the limited number of individuals in the enclosures, in summer (2018-2019), we trapped and translocated wild-born juveniles in the SBF population to breeding enclosures and release pens in the BH and CHB release areas. Wild trapping protocols were the same as the enclosure trapping protocols described above. All wild adult rabbits caught were weighed, sexed, and a genetic sample was obtained through a 3-mm ear biopsy. Adults were then immediately released back into the burrow they were trapped from, and traps were removed from the burrow system. All methods were approved by the University of Idaho Animal Care and Use Committee (Protocol 2012-23, 2017-25, and 2020-13), were consistent with the standard for use of wild mammals in research established by the American Society of Mammologist (Sikes and the Animal Care and Use Committee of the

American Society of Mammologists 2016) and were performed in accordance with applicable laws governing the use of endangered species.

We conducted winter surveys each year following releases to locate active burrows and collect fecal pellets for genetic analysis. Ideally, surveys were conducted under fresh snow conditions, but in years with relatively low snowfall, some surveys were performed with no to minimal snow on the ground. We performed surveys of 35-50-m wide belt transects by foot, prioritizing release sites and areas with active burrows from previous years, and then expanding outward. When snow was present, we followed rabbit tracks and trails to active burrows. From 2012-2017, all winter surveys were conducted at SBF. From 2018-2020, winter surveys were conducted at SBF, BH and CHB release areas. The area surveyed each year (8.9 - 23.6 km2) depended on the availability of WDFW personnel, volunteers, and accessibility to survey areas (Table 1.2, 1.3). Total area surveyed was calculated by the global positioning system (GPS) track files from each surveyor or if track files were unavailable, the overall area was calculated by a polygon in ArcMap (ESRI, Redlands, California). At each active burrow, the GPS coordinates, number of entrances, activity level, and visual confirmation of a rabbit were recorded. A minimum of three fecal pellets were collected to ensure adequate amount of DNA for genetic analysis (Adams et al. 2011). Fecal pellets were collected from a single, distinct pile of pellets to increase the probability that the sample represented a single individual. Fecal samples were stored in paper envelopes, desiccated with silica gel beads, and kept a room temperature (~23°C) until laboratory analysis could be performed.

Beginning in 2018, we initiated summer monitoring in SBF and in 2019 for BH and CHB (Table 1.2, 1.3). Priority was given to areas near release pens or active burrows from the previous winter. At each active burrow, we used the same protocol described above for winter monitoring. Since juveniles and adults are present during the summer, multiple fecal samples were often collected from the same burrow system. Juvenile pellets were identified as pellets ≤2.5 mm in diameter, where adult pellets were typically 4-5mm in diameter. Fecal pellets were stored as described above.

Laboratory Methods

DNA was extracted from tissue samples collected from rabbits using Qiagen DNeasy blood and tissue kits (Qiagen Inc., Valencia, California) following the methods described in DeMay et al. (2015). We amplified extracted DNA in duplicate across 19 microsatellite loci (18 autosomal loci and 1 Y-chromosome locus) within 3 polymerase chain reaction (PCR) multiplexes (DeMay et al. 2015). Samples were run on an Applied Biosystems 3130xl Genetic Analyzer (Applied Biosystems Inc., Foster City, California), and results were analyzed in Genemapper 5 (Applied Biosystems Inc.) and

confirmed visually. Any unknown adult sample was compared to previously known individuals to determine if there was a match or if the individual was a new rabbit.

DNA of fecal pellets collected during winter and summer monitoring was extracted for DNA using the Qiagen QIAmp DNA Stool Mini Juvenile (Qiagen Inc., Valencia, CA) in a laboratory dedicated to low quantity-DNA samples (Waits and Paetkau 2005). We performed species ID tests using a 294-bp fragment of the mitochondrial DNA cytochrome b gene following the protocols described in Adams et al. (2011). The species ID test was designed to distinguish between pygmy rabbits and sympatric cottontail species (*Sylvilagus nuttallii, S. audobonii, S.floridanus*). For the 2012-2013 and 2013-2014 surveys, all samples underwent a species ID test but after further testing, it was determined that cottontail samples did not amplify or produced out-of-bin alleles at various microsatellite loci (DeMay et al. 2017), which could successfully exclude these individuals without performing a species identification (ID) test. During 2014-2018, only samples that failed the microsatellite analysis were run on the species ID panel. In 2018, we reinstituted the species ID panel on all samples before analysis on the microsatellite panels due to declining numbers of pygmy rabbits. Any sample that failed to amplify on the species ID test or amplified as cottontail was excluded from the remainder of the analyses.

We initially amplified all samples that were confirmed pygmy rabbit in duplicate, on the first PCR multiplex consisting of 8 loci (A12, A124, A140, Sat7, Sat8, Sol08, Sol44, sex locus-Y05) following the protocols described in DeMay et al. (2015). Genotypes at these loci were then compared to the genotypes of known individuals to determine if there was a match but also to screen out low-quality samples. Pellets had to amplify at ≥ 5 of the loci (excluding the sex loci) in the first to move on to the second multiplex consisting of 7 loci (A113, A121, A133, A2, D118, Sat5, and sex-locus Y05), and ≥4 loci were required (excluding the sex locus) to meet P(ID)sibs < 0.01 and verify a match from 2012-2017 (Waits et al. 2001; Waits and Paetkau 2005). In 2018, we used the 2nd and 3rd PCR multiplexes (5 autosomal loci – A128, A129, D103, D2, and 7LID3) in combination rather than the 1st PCR multiplex to increase statistical power in distinguishing individuals as the degree of relatedness among individuals increased. A minimum of 8 loci was required to meet P(ID)sibs < 0.01 and verify a match using multiplex 2 and 3 from 2018-2020. We ran pellet samples a minimum of four times and up to eight times to produce a consensus genotype. Two repeats of each allele were required to confirm a heterozygous genotype and 3 repeats to confirm a homozygous genotype (DeMay et al. 2013). Using the 12 loci, consensus genotypes were compared to one another to determine matching genotypes at multiple locations and matching to genotypes of previously released rabbits. Fecal

samples that did not match a known rabbit were considered new wild-born individuals and amplified for all remaining loci.

Analytical Methods

All tissue and fecal genotypes were added to a reference database, which also included morphological and demographic parameters on released and enclosure-born individuals. Fecal sample genotypes and unknown adult tissue genotypes were matched using GenAlEx 6.51 (Peakall and Smouse 2006, 2012). Matchings that contained 1 or 2 mismatches were further analyzed for human error or allelic dropout that resulted in the mismatches. We used locus A124 for individual identity and we removed it from all downstream analysis due to the high frequency of null alleles (DeMay et al. 2017). All parentage and population genetic analyses were conducted with the remaining 17 loci.

We analyzed all samples, enclosure and wild, for parentage using a strict exclusion approach in Cervus 3.0.7 (Marshall et al. 1998; Kalinowski et al. 2007). Parentage assignments that mismatched at 1-2 loci were once again examined for genotyping error, where a mismatch at a single locus, representing a single stepwise mutation, was accepted as a match. We used the program STRUCTURE to assess ancestry based on the predefined groups identified (CB, Oregon/Idaho/Nevada, northern Utah/Wyoming, and southern Utah), genetic estimates for all individuals in this study, including wild-born individuals, released individuals and enclosure individuals. STRUCTURE was run ten times with for K = 4 under an assumption of admixture, correlated allele frequencies and the LOCPRIOR model (prior information on the identified populations), with 100,000 cycles of burn-in (BURNIN = 100 000) and 500,000 Markov chain Monte Carlo samples (NUMREPS = 500 000). We estimated allele frequencies for each genetic cluster from individuals known by pedigree or capture records for each of the four predefined clusters and were used to estimate the CB ancestry for all non-founding individuals. Based on the variation observed in the CB estimates for individuals from other states assigned to predefined clusters other than the CB (range 0-4.89%), only individuals with estimates of ≥5% CB ancestry were identified as containing CB ancestry (Table 1.4-1.7).

We characterized genetic diversity and CB ancestry estimates for each enclosure or release pen per year and winter survey years (Tables 1.4-1.7). We defined the enclosure population as all trapped juveniles born in an enclosure, all individuals detected as parents for the given year (through the method described above), and all trapped adults that may not have been detected as parents for the given year. The wild population was defined as all new wild-born individuals, released individuals and previously detected individuals sampled within a single year. We evaluated allelic richness (AR) using the R program *hierfstat* (Goudet 2005) and rarefied to a sample size of 5. Observed

heterozygosity (Ho) and unbiased expected heterozygosity (He) were calculated using GenAlEx 6.5.1 (Peakall and Smouse 2006, 2012). He and AR were calculated only for sample sizes ≥5. Year to year and initial year to final year comparisons of Ho, CB, and AR were evaluated using Welch two-sample t-test in R (R Core Team 2020). Comparison of sex ratios from year to year were analyzed with two-sided Fisher's exact test in R. We determined Ne for each winter survey year using the linkage disequilibrium model with random mating, minor allele frequency equal to 0.05, and 95% intervals in the parametric model, in the program NeEstimator V2.1 (Do et al. 2014) using the co-ancestry method (Nomura 2008) for the SBF populations only. We reported Ne estimates for sample sizes ≥7 because smaller sample sizes produced infinite estimates. Density estimates were based on the minimum count of rabbits identified each survey period/potential habitat (ha) within each recovery area.

To assess dispersal distances, we measured straight-line dispersal from release area to location of burrow during winter/summer surveys. 2nd year detection dispersal distances were also measured as straight-line dispersal distances from the first year's burrow location to the second year's burrow location. Differences in age-biased and sex-biased dispersal were assessed for statistical significance using 2-sided Wilcoxon rank sum tests.

Apparent Survival Models

Apparent survival was defined as the detection of a released pygmy rabbit from fecal DNA collected during winter and/or summer surveys. Wild born rabbits were not included in the apparent survival models because their life stage was unknown. In the SBF recovery area, we used logistic regression to assess juvenile and adult apparent survival, with winter/summer detection as the explanatory variable as previously described (DeMay et al. 2017). A priori model sets were evaluated using Akaike's Information Criteria corrected for small sample size (AICc), log-likelihood values, and model average parameter estimates with 85% confidence intervals (Arnold 2010) using R package, *AICcmodavg* (Mazerolle 2020). We averaged parameter estimates across all the candidate models that included each given parameter. DeMay et al. (2017) only evaluated adults released in 2014 because of the small number of adults released in 2012-2013 but our models set also included adults released in 2015 and 2016.

For apparent survival of adults, we included the explanatory variables release day, sex, release weight, homozygosity by loci (HL) calculated using the R package *GENHET* (Coulon 2010), and genetic estimate of CB ancestry derived from the protocols described above. Our candidate model set included all 30 possible combinations of the explanatory variables and the null model (Table 1.9). Typically, before release, all juveniles were trapped and weighed, but in the case of released adults, weights were not always taken at time of release. The top model without release weight as an

explanatory variable was compared to the top model including release weight for those individuals that had a recorded weight at time of release.

For apparent survival of juveniles, we included each combination of the explanatory variables with release year (categorical variable, 2012-2016). We used 2014 as a reference year, as was done in DeMay et al. (2017) due to the large sample size. Our candidate model set included year ($p < 0.0001$) in each model and all possible remaining combinations, for a total of 32 models (Table 1.8). For apparent survival in release pens, the same explanatory variables were used as the juvenile model in SBF (Table 1.10). The top model for release pen survival was compared to the top juvenile model to test for a difference in survival rates between the hard-release and soft-release approaches.

Results

Sagebrush Flat Population

Survey Efforts and Spatial Distribution

Surveying efforts of the SBF area ranged from 5.89 – 24.28 km^2, with an average of 12.70 km^2 across the eight years of surveys (Table 1.2). During 2012-2014, a common 6.7-km^2 area was surveyed each year because burrows predominantly occurred in this area, but in 2015 (Figure 1.2a-c), most pygmy rabbit burrows shifted into the CRP area to the east and south (Figure 1.2d). Winter 2016-2017 had the greatest survey coverage (24.28 km^2) because SBF and CRP fields were both surveyed. A decrease in areas surveyed occurred in 2017-2020 because efforts were mainly placed on the CRP fields and only areas of SBF bordering CRP or areas known to have rabbits were surveyed in SBF.

The SBF population fluctuated in its population numbers during 2012-2020. Since 2012, the minimum count of rabbits identified in winter surveys ranged from 8 to 158 (Table 1.2, 1.4). During 2012-2016, the main augmentation to the SBF population occurred through reintroductions from the enclosure populations with the number of released individuals ranging from 104 to 717 juveniles and 1 to 113 adults (Table 1.2), but during 2017-2020, no rabbits were augmented into the SBF area. During 2012-2014, the number of juveniles and adults released into SBF increased because of the increased productivity within each of the enclosures. In 2014, to minimize the negative habitat effects resulting from many pygmy rabbits in the enclosure, most adults and juveniles were released into the SBF area (Table 1.2). This resulted in significantly fewer released individuals in SBF in 2015-2016. In 2017, the loss of the BH enclosure due to the Sutherland Canyon fire, greatly reduced the overall numbers in the enclosure populations, resulting in no further releases into SBF.

During 2012-2014, rabbits were spatially distributed, with most across SBF and fewer than 10% of all active burrows identified found on CRP (Figure 1.2a-c). In winter 2014-2015, more active burrows (~28%) were located on the eastern and south-eastern border between SBF and CRP (Figure 1.2c). In winter 2015-2016, ~20% of burrows located were in the SBF area and the remaining burrows (~80%) were found in CRP (Figure 1.2d). By winter 2016-2017, over 75% of all active burrows were located in CRP, 18.5% of burrows were located on private land to the west (Figure 1.2e) and 6.5 % in SBF. Samples presumed to be pygmy rabbit, based on size, were also collected from Sheep's Canyon (approximately 16.1 km southeast of SBF), but the samples did not amplify on any genetic tests. The spatial distribution of rabbits exhibited in 2016 was also observed in winter 2017-18 (Figure 1.3a), but with a decrease in the number of burrows on private land to the west. By winter 2018-2019, less than 6% of all burrows identified were located in SBF (Figure 1.3c), and by winter 2019-2020, all active burrows were located in CRP (Figure 1.3d). The summer 2018 survey also exhibited a similar spatial distribution as the winter 2018-2019 surveys, where burrows within SBF were limited to two small pockets in the west and north-east corner and the remaining burrows were found in the CRP to the east and south (Figure 1.3b).

Post-Release Dispersal

Juveniles dispersed slightly farther than adults (mean dispersal for juveniles = 988m and 783m for adults (Figure 1.4a), but the difference was not significant ($p = 0.10$). The 61 juvenile male rabbits with recorded dispersal from their release site averaged 1139 m, ranging from 80 to 3546 m. Juvenile female rabbits (n=72) dispersed a shorter average distance (859 m, range 11 - 3009 m) from their release sites (Figure 1.4b). The distribution of dispersal distance was right skewed for both male and female juveniles, with fewer rabbits making longer dispersals (median for males = 869 m and 746 m for females). Post-release dispersal distance for juvenile rabbits did not differ between sexes ($p = 0.13$). Thirty-eight percent of released juveniles (n=50) dispersed ≥ 1 km, with no difference between male and female juveniles. Likewise, adult dispersal distance did not vary with sex ($p = 0.61$). Female adults (n=15) with recorded dispersal distances ranged from 69 to 2078 m and male adult rabbits (n=12) ranged of 236 - 2559 m (Figure 1.4c). The average dispersal distance was likewise right skewed for both male and female adult rabbits (median for males = 501 m, and 464 m for females). Twenty-six percent of adult rabbits (n=7) dispersed ≥1 km, in which five were female and two were male. Wild rabbits dispersed significantly shorter distances ($p < 0.0003$) from first year of detection to subsequent year's detection compared to released rabbits (Figure 4).

Dispersal of 2nd year detection wild-born rabbits, (n=22), averaged 495.3 m (range: 48 -2025 m). Dispersal distances significantly differed between sexes ($p = 0.02$). Wild-born male rabbits (n=7)

dispersed farther than females, averaging 804 m (range: 129 – 2025 m; Figure 1.4d). Female rabbits (n=15) dispersed on average 351 m (range: 48 - 1781m). The female rabbit that dispersed the farthest (1781 m) moved from the edge of SBF, east into CRP, and the male rabbit that dispersed the farthest (2025 m) moved from the eastern edge of CRP to the southwest CRP fields. Most of the rabbits dispersed less than the right-skewed average for both sexes (median for males = 633 m and females = 187 m).

Species Identification, Minimum Count, Sex Ratios, and Rabbits/Burrows

Successful species ID amplification ranged from 78-97% and was first implemented consistently starting in winter 2018-2019. Most of the pellets collected each year were identified as pygmy rabbit with very few cottontail pellets collected (0 - 51%, $\bar{X} = 10\%$) except in the winter 2019-2020 survey effort where 51% of pellets collected were identified as cottontail. Of the pellets that were identified as pygmy rabbit, individual identity was successfully determined 20-83% of the time ($\bar{X} = 60 \pm 20\%$). Years with lower success rates typically resulted from collection with minimal to no snow present and/or rain on snow events with much freeze thawing. During 2012-2014, very few wild-born rabbits (3 - 16% of detected rabbits) were identified, and most individuals detected were released that year (77 - 96% of detected rabbits) (Table 1.2, 1.4). Beginning in 2015, a higher number of wild-born rabbits (89 - 100%) were detected with a smaller proportion of released individuals detected (6 - 8%). Only 1% (n=25) of released or wild-born individuals were detected a second year, and wild-born rabbits were more likely (5%) to be detected a second year than released rabbits (4%). Only one wild-born individual, identified in 2016 (0.1%), was identified in three consecutive winter surveys (2016-2018). Initially (2013-2015) the individuals detected a 2nd year were released individuals but as the number wild-born individuals increased, detection of 2nd year individuals were of wild-born descent (2016-2019). The highest detection of 2nd year wild-born individuals was in winter 2018-19 (14 individuals – 10% of rabbits detected that winter).

In summer 2018, a monitoring approach was used which allowed us to identify the age class of the rabbit (adult or juvenile) based on the pellet size. Two wild-born rabbits from the winter 2017-2018 monitoring season were identified and 49 new wild-born adult rabbits that were not identified during winter 2017-2018 surveys, and three wild-born juveniles. Most of the rabbits identified in the winter 2018-2019 surveys were new wild-born rabbits (123 rabbits), but 14 of the 15 recaptured individuals (93%) were from the summer 2018 monitoring. The winter 2019-2020 survey indicated a significant decline in the population with the minimum count of rabbits at eight individuals. Most of the individuals detected were new wild-born rabbits (63%) whereas the other rabbits (38%) were detected in the previous survey year or during the summer 2018 monitoring (Table 1.2). Across all

monitoring years, the sex ratio of all detected rabbits maintained an approximate 1:1 relationship (49.5% males and 50.5% females). The male to female (M:F) ratio varied by year, where the number of males detected in the surveys initially showed lower number of males compared to females during 2012-2015 (range 1:1-1:1.6), but with no differences from year to year or between sexes (p = 0.76 - 1.00; Table 1.4). The number of males significantly decreased in the summer 2018 survey (1:1.8) from winter 2017-2018 (p = 0.007) but returned to male dominant by winter 2018-2019, producing the largest M:F sex ratio difference (1.9:1).

During 2012-2020, the number of rabbits per active burrow system averaged 0.74 ± 0.22 rabbits/burrow with a range of 0.33-1.00 (Table 1.4). The winter 2019-2020 survey produced the lowest number of rabbits/burrow system (0.33 rabbits/burrow), and the 2015-2016 survey produced the highest (1.00 rabbits/burrow) where every burrow found represented a new individual. Sixty-seven percent of the years (6/9) fell above the mean, and 89% (8/9) were above 0.56 rabbits/burrow. Rabbits/burrow decreased during 2012-2014 but increased in 2015 (1.00) as rabbits shifted to CRP (Table 1.4). Density estimates varied year to year, ranging from 0.004 (2019-2020) to 0.09 (2017-2018), averaging 0.04 ± 0.03 for the SBF/CRP recovery area (Table 1.4).

Genetic Diversity and CB Ancestry

Diversity did not significantly vary between the initial diversity in winter 2012-2013 to the end of the study (winter 2019-2020; p = 0.08-0.10). Genetic diversity across the SBF population has remained relatively consistent, across the years, for H_o and H_e ($\overline{H_o} = 0.74 \pm 0.07$, range 0.62-0.84, and $\overline{H_e} = 0.79 \pm 0.03$, range 0.72-0.82) (Table 1.4) but there was a significant increase in 2017 to 0.75 ($p < 0.001$). During the summer 2018 surveys, we saw a significant decline in H_o to 0.76, ($p = 0.02$) compared to winter 2017-2018 (Table 1.4). The samples that were collected during this survey effort included adults and juveniles that were closely related, likely causing the decrease in the H_o. By winter 2018-2019, the H_o decreased ($p < 0.001$) compared to winter 2017-2018 to its lowest (0.62) and remained consistently into winter 2019-2020 survey period (Table 1.4). Although there was variability from year to year in H_o, the decrease in H_o over time (2012 compared to 2019) was only marginally significant (p = 0.05). AR (5.10 ± 0.21) varied minimally throughout the survey periods from 2012-2020, ranging from 4.67-5.35 with no significant differences from year to year (p = 0.16-0.96).

As expected, we documented a decrease in Columbian Basin ancestry over time that was influenced, in part, by translocations of individuals from other populations. CB ancestry varied from 2012-2019, averaging $18.20 \pm 10.89\%$ (Table 1.4). From 2012-2016, there were no significant differences in CB ancestry (p-values > 0.05). In winter 2017-2018, CB ancestry significantly declined

(p = 0.01) in the identified individuals, resulting in averaged CB estimates of 15.31%. CB ancestry increased significantly (p = 0.01) by summer 2018 (18.48%) and was maintained each subsequent year. In 2012, only 48.89% of individuals detected in winter surveys had estimates of CB ancestry ≥5% because many of the individuals released were obtained from populations in other states and placed in the on-site breeding enclosures. By 2013, there was an increase to 88.64% of individuals with detectable CB ancestry but then a decline in 2014 to 71.43%. During 2015-2020, all individuals detected in winter and summer monitoring surveys contained ≥ 5% CB ancestry. All individuals that were wild-born from 2012-2020 contained detectable Columbia Basin ancestry, except for two individuals (Table 1.4). During 2012-2019, the predominant ancestry in identified rabbits was from the Nevada/Oregon/Idaho genetic group (61.04 ± 14.93%) and the Wyoming/N. Utah ancestry was also represented (18.79 ± 13.00%). The S. Utah ancestry had nearly been removed from the SBF population during 2012-2019 (1.98 ± 7.18%). Initially, in 2012, S. Utah ancestry estimates averaged 10.05% but from 2013-2019, estimates ranged from 0.33-3.63%. N_e increased from 2012-2014, ranging from 15.4-30.4 (Table 1.4). In winter 2015-2016, N_e decreased to 19.3, and the minimum count of rabbits that year was also at its second lowest (n=18). The lower and upper bound of the 95% confidence interval fell within or was near the confidence intervals for the previous years. N_e of the SBF population appears to peak and stay somewhat consistent in 2016 and 2017, with values ranging from 40.7 - 44.3 individuals and overlapping confidence intervals (2016: 35.0 - 47.9, and 2017: 40.6 - 48.5). A decline in the overall N_e was observed in 2018 (both summer and winter survey estimates staying consistent between 26.9 - 27.6 individuals) and then declined even further in 2019 to 12.3 individuals.

Beezley Hills Population

Survey Efforts and Species Identification

The first attempt to re-establish the BH population occurred during summer 2015 but immediately after the release of rabbits, surveys identified numerous pygmy rabbit corpses, and it was later determined that nearly all the rabbits released contained lethal to sub-lethal levels of the parasite, coccidia (*Eimeria brachylagia*). Additionally, these sick rabbits were released during a drought year (Gallie 2016). That following winter (2015-2016), informal transect and helicopter surveys were performed but no rabbits or active burrows were identified (Table 1.3). Additional surveys were conducted in summer 2016, but still no rabbits were detected (Gallie 2016). In winter 2017-2018, a small survey effort (0.21 km^2) was conducted because rabbits that were stocked into the new mobile breeding enclosure at BH had escaped. Five escaped individuals were identified during this survey

period (Tables 1.3,1.5), but there was no evidence of individuals (or their descendants) from earlier releases (2015).

Formal reestablishment of the BH recovery area was attempted again in summer 2018. Winter and summer survey efforts were initiated after their release in which 0.69 - 1.21 km^2 were surveyed around the release pens (Table 1.3). Three individuals were detected in winter 2018-2019 in BH. Two of the individuals (67%) were captively bred and released in 2018 whereas the other rabbit (33%) was a wild juvenile translocated from the SBF population in 2018. During summer 2019 monitoring, seven individuals were identified; two (29%) were enclosure born juveniles that were released that summer, three juveniles (43%) were from the mobile breeding enclosure at BH but had escaped, and two wild-born rabbits (29%) were identified. During the winter 2019-2020 surveys in BH, five individuals were identified; four were released enclosure born rabbits from summer 2019, and the other was a wild juvenile translocated from the SBF population in summer 2019 (Tables 1.3, 1.5). Species identification success rates ranged from 80-93% ($\bar{X} = 86 \pm 7\%$) with very few cottontail pellets collected (0-2 samples per survey period). Species ID success rates varied in the winter surveys (2018: 80%, and 2019: 97%), and were high during the summer 2019 survey (85%). Individual identity success rates varied from 67 - 92% ($\bar{X} = 81 \pm 11\%$) where 3-7 individuals were identified (Tables 1.3, 1.5). Success rates varied across the winter surveys (2017: 75%, 2018: 88%, and 2019: 92%) and had the lowest success during the summer 2019 survey (67%; Table 1.3).

Post-Release Dispersal and Demographics

Pygmy rabbits identified during summer and winter monitoring were located at burrows within release pens or near the mobile breeding enclosure (49 - 530 m) and any one of the release pens (14 – 503 m; Figure 1.5). In BH, 14 rabbits were assessed for dispersal distance if they were identified during summer or winter monitoring outside of a release pen. The average dispersal distance for BH rabbits was $178 \pm 135\ m$ (range: 14 - 454) from the pen in which it was released (Figure 1.4g), but dispersal distances did not differ significantly between sexes ($p > 0.05$; Figure 1.4e). The number of identified rabbits per active burrow varied from 0.42 - 0.70 rabbits per burrow system ($\bar{X} = 0.51 \pm 0.13$ rabbits/burrow). The fewest rabbits/burrow (0.42) occurred in winter 2017-2018 when rabbits were first establishing burrows in the area and peaked in summer 2019 (0.70). Rabbit density in BH varied between 0.04-0.09, averaging 0.06 ± 0.02. The highest observed value in rabbit density occurred during summer monitoring (0.09). The M:F sex ratio has varied from year to year in BH with no significant differences detected from year to year due to the small sample size (Table 1.5).

Genetic Diversity and CB Ancestry

Genetic diversity across the BH population varied from 0.57-0.80 across the years, for H_o ($\overline{H_o} = 0.73 \pm 0.11$; Table 1.5). During 2017-summer 2019, H_o ranged from 0.75-0.80, with no significant differences ($p > 0.05$) until a decline in winter 2019-2020 (0.59) from the summer 2019 (0.80; $p = 0.002$). The decrease was not significantly different from diversity in the previous winter ($p = 0.07$), but was different from the initial levels of heterozygosity identified in winter 2017-2018 ($p = 0.01$). AR varied throughout the survey periods during 2017-2019, ranging from 3.71-5.41 ($\bar{X} = 4.31 \pm 0.54$). There was a significant decrease (3.82, $p = 0.003$) in summer 2019, where AR showed no significant difference into winter 2019-2020 (3.71, $p = 0.73$). AR levels during winter 2017-18 were comparable to SBF values with no significant differences for any given year at SBF ($p > 0.05$). AR values observed during summer 2019 and winter 2019-2020 were significantly lower compared to any year at SBF ($p < 0.0001$). Due to the small sample size, N_e estimates could not be accurately estimated for most years for BH, except for during summer 2019, where N_e was estimated to be 9.3 individuals (Table 1.5). From 2017-2019, CB ancestry did not differ significantly among years ($p = 0.86 - 0.99$) with CB ancestry ranging from 22.87-27.46% ($\bar{X} = 24.46 \pm 7.98\%$) (Table 1.5). All individuals that have been detected and released into BH have retained $\geq$ 5% of CB ancestry. The predominant ancestry in identified rabbits was Nevada/Oregon/Idaho ($\bar{X} = 62.35 \pm 9.90\%$, range 40.18%-74.75%) and the Wyoming/N. Utah ancestry was still represented across the years ($\bar{X} = 10.69 \pm 8.08\%$, 1.72-31.84%). The S. Utah ancestry had nearly disappeared from the BH population ($\bar{X} = 2.51 \pm 1.22\%$, range 1.16-7.00%).

Release Pens

Juveniles released into the BH recovery area were placed into one of two temporary release pens starting in summer 2017, but unfortunately, all juveniles were killed in the 2017 fire. BH release pens contained 5-7 individuals each (Table 1.6). Five rabbits were kept in each release pen in summer 2018, where each consisted of three females and two males. In summer 2019, each BH release pen contained seven individuals, three females and four males in BH-1, and 4 females and three males in BH-2. Each year, release pens contained enclosure born individuals and juveniles translocated from the wild SBF population. Within BH release pens from 2017-2020, average H_o level ranged from 0.65-0.72, with no significant differences across years. Initially, the pens had higher levels of H_o (0.77, 0.78) but decreased in summer 2019 (0.70, 0.61), although the differences are not significant ($p = 0.06$; Table 1.6). This occurred because the majority of juveniles produced within the mobile breeding enclosures were half siblings or full siblings, since only a single male survived through

winter. CB ancestry was maintained in release pens across years, and between pens, averaging $20.51 \pm 6.36\%$ (Table 1.6).

Chester Butte Population

Survey Efforts and Species Identification

The CHB recovery area was established in summer 2018 with the release of 17 juveniles into temporary release pens, and then augmented with an additional 21 juveniles in summer 2019 (Table 1.3, 1.6). From 2018-2020, 1.07-2.43km^2 of habitat surrounding the temporary release pens was monitored (Table 1.3), increasing the area of each monitoring survey after the initial release (winter 2018-2019: 1.07 km^2, summer 2019: 1.53 km^2, and winter 2019-2020: 2.43 km^2). Species identification success rates ranged from 81-93% ($\bar{X} = 91 \pm 9\%$) with very few cottontail pellets collected (0-2 samples per survey period). Winter species ID success rates (2018: 95%, 2019: 97%) were higher than summer rates (80%). Individual identity success rates varied from 67-92% ($\bar{X} = 86 \pm 6\%$) where 5-10 individuals were identified (Table 1.3). Individual identity success rates were higher during summer monitoring (93%), compared to winter (2018: 84%, 2019: 81%). All rabbits that were identified during winter surveys were rabbits that had been released into release pens that year. There was no evidence of wild-born rabbits in CHB.

During winter 2018-2019 surveys, six rabbits were identified in which one individual (16.7%) was an enclosure born rabbit and the remaining five rabbits (83.3%) were juveniles translocated from SBF. During summer 2019 surveys, five of the 21 (23.8%) juveniles that had been released into pens were identified outside of release pens, all were enclosure born rabbits. In winter 2018-2019, 10 rabbits were identified either in the released pens or in the wild. Nine of the ten rabbits (90%) were enclosure born rabbits and one rabbit (10%) was a juvenile translocated from the wild SBF population.

Post-Release Dispersal and Demographics

Pygmy rabbits that were identified during summer and winter monitoring were burrowing within close proximity to one of the release pens (1 – 558 m) (Figure 1.6). In CHB, 11 rabbits were assessed for dispersal distance from the release pen. The average dispersal distance for CHB rabbits was $286 \pm 196\ m$ (range: 37 - 748m) from the pen in which it was released (Figure 1.4g). There were no significant differences in dispersal distances between sexes (p = 0.32; Figure 1.4f). The number of identified rabbits per number of active burrows varied from 0.35-0.50 rabbits per burrow system ($\bar{X} = 0.43 \pm 0.08$ rabbits/burrow). Winter 2018-2019, following the initial release of rabbits into the area, resulted in 0.45 rabbits/burrow. By summer 2019, the average number of rabbits to active burrows was 0.50 rabbits/burrow and by winter 2019-2020, we observed the lowest value, 0.35

rabbits/burrow. The average across winter surveys only is 0.40 ± 0.07 rabbits/burrow (range 0.35-0.45) (Table 1.5). Rabbit density in the CHB recovery area remained consistent across years at 0.01 rabbit/ha. The M:F sex ratio has varied slightly but there were no significant differences from year to year due to the small sample size (Table 1.5).

Genetic Diversity and CB Ancestry

Genetic diversity across the CHB population has remained somewhat consistent, across the years, for H_o ($\overline{H_o} = 0.75 \pm 0.02$, range 0.73-0.77) (Table 1.5) with no significant differences detected from year to year or from initial establishment (2018) to winter 2019-2020 (p = 0.43-0.93). Mean AR at CHB (4.31) was similar to the mean AR of BH (4.31). AR values ranged from 3.71-4.65 from 2018-2020, with no significant differences detected (p = 0.87), until a decline in winter 2019-2020 (3.69, p = 0.02). AR values in CHB for were similar to AR in SBF, except for winter 2017-18 (p = 0.04). Winter 2019-2020 values for CHB were significantly lower than all years at SBF (p > 0.05). Due to the small sample size, N_e estimates could not be accurately estimated. From winter 2018-2019 to summer 2019, there was a significant change in CB ancestry (p = 0.01), with CB ancestry increasing from 14.85% (winter 2018-2019) to 20.89% (summer 2019) with no significant differences detected in subsequent surveys (Table 1.5). All individuals that have been detected and released into CHB have contained $\geq$ 5% CB ancestry, averaging $18.46 \pm 3.45\%$. The predominant ancestry in identified rabbits is Nevada/Oregon/Idaho ($\bar{X} = 67.77 \pm 6.41\%$, range 55.42%-77.36%) and the Wyoming/N. Utah ancestry is still represented across the years ($\bar{X} = 12.05 \pm 7.96\%$, ranging from 4.84%-27.10%). The S. Utah ancestry has nearly been removed from the CHB population. S. Utah estimates are below the 5% threshold considered to be significant ($\bar{X} = 1.73 \pm 0.80\%$, range 0.85-3.81%).

Release Pens

Release pens were augmented with approximately the same number of male and female rabbits during 2018-2020 (Table 1.6). Each year, release pens contained enclosure born individuals and juveniles translocated from the wild SBF population. Within CHB release pens, overall H_o level ranged from 0.72-0.78 across years (Table 1.6), with no significant differences detected from year to year (p = 0.30), or between release pens each year (p = 0.76-0.99). Overall CB ancestry among pens in CHB was significantly higher in 2018 (17.16%) than 2019 (21.78%) (p < 0.0001), and all individuals contained > 5% CB ancestry. CB ancestry persisted in each release pen, averaging $19.29 \pm 3.57\%$across the years (Table 1.6).

Enclosure Populations

Breeding enclosures SE and LE (located at SBF) were initiated in 2012, and population sizes increased steadily until 2015 when numbers began to decline (Table 1.7). Enclosures DE (Dormaier area) and BE (BH area) were initiated in 2013 and 2014, respectively, and population size was at its maximum during year 2 then declined sharply. Most of the rabbits in each enclosure were juveniles born that year and were either released into the wild or used as breeders in other enclosures. In 2015, managers chose to greatly reduce the populations in LE, SE, BE and DE to minimize the negative effects of the rabbits on the breeding enclosure habitat (Table 1.7). SE was being phased out in 2016 but as a result of the loss of BE due to the Sutherland Canyon fire (2017), surviving rabbits had to be translocated into SE, LE and DE. Additionally, with the loss of BE, a new mobile 3-acre breeding enclosure (MBE) was implemented in October 2017 (Table 1.7). In 2019, LE and SE were phased out, and all remaining adult rabbits were transferred to DE, and all juveniles were transferred to the mobile breeding enclosure or released into BH or CHB.

Genetic diversity was maintained in each enclosure with minimal differences occurring between initial establishment (2012) of enclosures and the end of this study for LE (2020; Table 1.7). H_o ($\overline{H_o} = 0.80 \pm 0.03$, range 0.76 - 0.83) and H_e ($\overline{H_e} = 0.79 \pm 0.02$, range 0.75 - 0.81) remained consistent in LE across the years with no significant differences from year to year or from initial establishment (2012) to the final cohort (2018; p = 0.09 - 0.94; Table 1.7) Over the years, AR averaged 5.00 ± 1.00 for LE with no differences occurring from initial establishment (2012) until the final cohort (2018; Table 1.7). CB ancestry was initially low in LE (2.77%) since only four individuals in the enclosure (3.4%) contained CB ancestry (Table 1.7) but increased in 2013 (17.70%, $p < 0.0001$) when 55.5% of individuals contained CB ancestry. In 2015, CB ancestry significantly increased (18.33%, p = 0.03) and 99.1% of individuals contained CB. By 2018, due to the limited number of individuals within LE, CB ancestry declined (17.32%, p = 0.01). All individuals from 2016-2018 contained > 5% CB ancestry. Overall, CB ancestry averaged $15.98 \pm 14.37\%$ in LE, Nevada/Oregon/Idaho ancestry averaged $64.34 \pm 21.51\%$%, Wyoming/N. Utah ancestry averaged $16.68 \pm 19.72\%$, and S. Utah ancestry averaged $2.68 \pm 11.00\%$.

Overall genetic diversity was maintained in the SE enclosure from 2012-2016, but as the breeding population declines, a loss of diversity was observed. H_o remained relatively consistent across the years in SE with no significant differences from year to year or from initial establishment (2012) to the final breeding population (2018) ($\overline{H_o} = 0.76 \pm 0.04$, range 0.70-0.80, p = 0.41 - 0.92; Table 1.7). AR averaged 4.38 ± 1.30 across years in SE (2012-2018), maintaining genetic diversity during peak years (2012-2016; Table 1.7). As SE began to be phased out in 2016, the number of

rabbits within SE was minimal, thus resulting in decreased AR (4.13, p = 0.06) in 2017, and then further decreasing to in 2018 (3.06, p = 0.01). The decreases in AR during 2017 and 2018 were significantly lower compared to the initial establishment of the enclosure (2012). CB ancestry in SE, initially averaged 25.58% where 62% of individuals contained CB ancestry (Table 1.7) but decreased in 2013 (16.77%, p < 0.001), although an increase in the proportion of individuals containing CB ancestry (76.5%) was observed. CB ancestry declined in 2014 (12.47%, p = 0.001) with a decline in the proportion of individuals containing CB ancestry (64.7%). During subsequent years, CB ancestry stabilized and all individuals within the breeding population contained CB ancestry (Table 1.7). Overall, CB ancestry averaged $16.82 \pm 16.92\%$% in SE, Nevada/Oregon/Idaho ancestry averaged $64.87 \pm 20.94\%$, Wyoming/N. Utah ancestry averaged $11.58 \pm 15.43\%$, and S. Utah ancestry averaged $6.59 \pm 11.08\%$.

Although there were significant changes detected from year to year in genetic diversity within DE, there were no differences from the initial establishment of the enclosure (2013) and the 2019 cohort. DE averaged 0.73 ± 0.03 for H_o, from 2012-2019 and 0.71 ± 0.04 for H_e (Table 1.7). During DE's inaugural year (2013) H_o was at its lowest (0.68) but increased in 2014 (0.74, p = 0.04) and H_o remained consistent in subsequent years (p = 0.60 - 0.93) or from initial establishment (2013) to the final breeding cohort (2019, p = 0.25). DE's AR averaged 4.17 ± 0.90 across the years. During the first year (2013), AR was 3.93 and then increased in 2013 (4.21, p = 0.03). During 2014-2016, a significant decrease in AR occurred, resulting in the lowest value across years (3.74, p = 0.03). In 2017, AR significantly increased (4.53, p-value=0.03) with no differences in subsequent years. AR did not differ significantly between the initial establishment of the enclosure (2013) and the 2019 population (p = 0.31). CB ancestry initially (2013) averaged 1.84% where 5.5% of individuals contained CB ancestry (Table 1.7). DE's initial augmentation was comprised of mostly out of state rabbits, with minimal rabbits containing CB ancestry but because of rabbits with higher CB ancestries being retained for breeding, CB ancestry increased in 2014 (9.69%, p < 0.0001) with an increase in the proportion of individuals containing CB ancestry (34.5%). In 2015, there was a single individual in DE containing 29.65% CB ancestry and then an increase from 2014 to 2016 (31.04%, p < 0.0001) but another decline in 2017 (23.74%, p = 0.03). The continued fluctuation in the breeding population resulted in a single individual (2017) containing 23.74% CB ancestry. There was a final increase in 2019 (22.07%, p = 0.004) as the rabbit population increased once again (Table 1.7). All individuals from 2015-2019 contained CB ancestry. During 2013-2019, CB ancestry averaged $8.39 \pm 12.93\%$, Nevada/Oregon/Idaho ancestry averaged $28.97 \pm 24.07\%$, Wyoming/N. Utah ancestry averaged $55.95 \pm 33.21\%$, and S. Utah ancestry averaged $6.39 \pm 13.84\%$.

Among enclosures, BE had the least variation in genetic diversity across years (2014-2017). H_o averaged 0.78 ± 0.02, H_e averaged 0.77 ± 0.01, and AR averaged 4.77 ± 1.00 with no significant differences from year to year (p = 0.24-0.94) or from initial establishment (2014) to the final cohort (2017; p = 0.98-1.00; Table 1.7). CB ancestry initially averaged 29.18% where 86.3% of individuals contained CB ancestry (Table 1.7) but declined in 2015 (22.93%, $p < 0.0001$) yet the proportion of individuals containing CB ancestry increased (99.1%). During subsequent years, CB ancestry was maintained, and all individuals contained detectable CB ancestry. During 2014-2017, CB ancestry averaged $24.10 \pm 13.31\%$, Nevada/Oregon/Idaho ancestry averaged $57.90 \pm 14.83\%$, Wyoming/N. Utah ancestry averaged $15.55 \pm 11.15\%$, and S. Utah ancestry averaged $2.12 \pm 3.33\%$.

With the loss of BE in June 2017, MBE was implemented in October 2017 and augmented with 14 rabbits from LE that escaped by winter 2017-2018; additional augmentations occurred in summer 2018 (Table 1.7). H_o and H_e varied significantly from 2017 to 2018 due to the different origin of the augmented rabbits (enclosure in 2017 vs wild in 2018). During 2017-2019, H_o averaged 0.80 ± 0.10 and H_e averaged 0.72 ± 0.07, with significant differences detected from 2017 (H_o = 0.92, H_e = 0.80) to 2018 (H_o = 0.75, H_e = 0.69, p = 0.004, 0.001). There were no significant changes in 2019 (p = 0.34 – 0.79) in H_o. AR averaged 4.59 ± 1.34 during 2017-2019. Upon initial establishment (2017), AR declined in 2018 (4.79, p = 0.19), and declined further in 2019 (3.57, p = 0.01; Table 1.7). CB ancestry averaged $21.87 \pm 4.24\%$, during 2017-2019 and was maintained across years where all individuals contained CB ancestry ($p < 0.05$; Table 1.7). During 2017-2019, Nevada/Oregon/Idaho ancestry averaged $62.04 \pm 8.85\%$, Wyoming/N. Utah ancestry averaged $12.70 \pm 6.52\%$, and S. Utah ancestry averaged $1.95 \pm 0.63\%$.

Apparent Survival

For rabbits released from 2012-2016 at SBF, the apparent survival of released rabbits to the following winter was 39%, 13%, 10%, 0.1%, and 9% respectively ($\bar{X} = 14 \pm 15\%$; Table 1.2). One hundred forty-one juveniles were detected from the 1354 juveniles that were released into the SBF area, resulting in an average juvenile apparent survival rate across all winter surveys of 10%. As for adults, 125 were released into the SBF area and 25 were detected, resulting in an averaged adult apparent survival rate of 20% across all years. Only five released juveniles were ever detected a second winter, resulting in an average adult apparent survival rate to their second winter of 4%; no released adults were ever detected a 2nd winter. As for wild-born rabbits, 420 wild-born individuals have been identified from winter monitoring surveys across the years (Table 1.2). 20 of the 420 wild-born individuals were detected a 2nd winter after their first detection, resulting in an average adult

apparent survival rate to their second winter of 4.8%, although the difference is not significant compared to released juveniles (Fisher's exact test, p-value=0.64). One juvenile that was detected during the summer 2018 survey was also detected during the winter 2019-2020 survey, 1.5 years later.

The year in which rabbits were released played a significant role in apparent survival for released juvenile rabbits in SBF (Table 1.11). During 2012-2014, there was a positive influence on apparent survival. 2015 had the largest negative effect on apparent survival but 2016 also reduced apparent survival, although estimates for 2016 overlapped zero. Released juvenile survival was positively influenced by release day, release weight, and genetic diversity (Figure 1.7, Table 8, Table 1.11). Weight and release day were moderately correlated (Pearson's correlation coefficient = 0.62, $p < 0.0001$). Rabbits that were released after the breeding season ended in July weighed more, driving this correlation. Sex and CB ancestry appeared in the top model sets but their addition to the top model did not improve the log-likelihood and 95% confidence intervals around the model-average estimates (Table 1.11). These parameter estimates overlapped zero suggesting that they are not actually significant in the model (Anderson 2008). Apparent survival of released adults in the winter following their release was influenced by release day only (Figure 1.7, Table 1.9, 1.11). Of the 44 individuals that were released earlier in the year in the larger data set (2014), only 7% were detected in winter; whereas the 45 adults released later in the year had a 47% detection rate. Genetic diversity showed weak evidence for a positive effect on adult survival (Table 1.11) but still overlapped zero for its parameter estimates.

Apparent survival in release pens from 2018-2019 varied between individual release pens and sites. At CHB, apparent survival ranged from 13% to 100% in 2018 ($\bar{X} = 54 \pm 44\%$), and 25% to 71% ($\bar{X} = 49 \pm 23\%$) in 2019 (Table 1.6). At BH, apparent survival ranged from 20% to 40% ($\bar{X} = 30 \pm 14\%$) in 2018 and 29% to 43% ($\bar{X} = 36 \pm 10\%$) in 2019 (Table 1.6). Apparent survival of juvenile juveniles released into pens was only slightly influenced by release day and the 95% confidence interval overlaps zero slightly, attributing to the minor significance of the parameter (Table 11). All the parameters that were included in the released juvenile models at SBF were examined but no other parameters made it into the top model set, where the null model followed just behind the top model (Table 1.10). Overall release pen (soft release) apparent survival ($\bar{X} = 44 \pm 26.13\%$) was significantly higher compared to the apparent survival of released juveniles in SBF (hard release) ($\bar{X} = 14 \pm 15\%$; $p < 0.0001$; Tables 1.2,1.6).

Discussion

Our study intensively and effectively applied genetic tools to monitor demographic and genetic parameters of the Columbia Basin pygmy rabbit recovery program for eight years following

reintroductions. Monitoring methods were designed in collaboration with managers and frequent updates of results were provided to allow continuous adaptive management of this endangered population. Genetic samples were obtained from all enclosure-born individuals, which allowed us to assess the genetic diversity, parentage, and ancestry composition of each enclosure and implement adaptive management protocols necessary to retain individuals of higher CB ancestry. Noninvasive genetic sampling has allowed us to monitor the wild populations for spatial expansion, apparent survival of released individuals, dispersal distances, overall genetic health and ancestry, minimum population size, and document reproduction in the wild, a critical parameter for success. We documented the persistence of CB ancestry in wild populations over the eight years since the first reintroduction, where by 2015, all individuals detected in winter survey efforts contained detectable CB ancestry > 5%. Through noninvasive genetic sampling and winter survey efforts, we documented dispersal distances of released and wild-born rabbits in SBF, BH, and CHB, allowing us to assess the difference in dispersal between hard releases at SBF, and soft releases, using release pens, at BH and CHB. Released juvenile and adult rabbits dispersed average distances of just under 1km in SBF, whereas dispersal distances in BH and CHB, averaged >300m. We monitored the spatial distribution of rabbits across SBF, identifying a striking shift in the use of habitat in 2015 to CRP fields, providing insight into possible future release site habitat preference. By monitoring individual rabbits, we documented reproduction in the wild and determined that survival to a second detection year did not significantly differ between wild-born rabbits and released rabbits.

Through our genetic monitoring, we modeled apparent survival in SBF and determine that release day, release weight, and genetic diversity positively influence apparent survival in released juvenile rabbits, and only release day positively influenced apparent survival in released adults. The significant relationship between apparent survival and individual homozygosity supports the conclusion that genetic rescue is effective in this population, showing that fitness may be increased by increasing heterozygosity. This information can be used to manage releases and possibly increase survivorship in all released rabbits. Additionally, we were able to determine an average number of rabbits to the number of active burrows found in each survey region, allowing management to determine an approximate number of rabbits in each area from field surveys, if genetic monitoring efforts cannot be conducted. Using genetic sampling of both tissue and fecal pellets, we have effectively and efficiently monitored the endangered Columbia Basin populations, providing the necessary insight to properly manage and reintroduce this species.

Spatial Distribution, Post-release dispersal, Reproduction and Apparent Survival

Contrary to results of other studies (Pierce et al. 2011), our data shows more active burrows in disturbed habitats (defined as any non-shrub-steppe/CRP habitat within a seasonal home range radius around the active burrow) than intact native shrub-steppe habitat found within SBF. As hypothesized, active burrows within the SBF population were predominantly detected within the SBF native shrub-steppe habitat from 2012-2014, with minimal detections in CRP. Yet as the population began to increase, the shift to CRP became predominant in 2015 with subsequent years resulting in predominant CRP burrow establishment. As wild-born rabbits continued to expand their distribution and recolonize habitat, this shift to early successional stages of replanted sagebrush raises many questions as to the reasons behind the move. WDFW considers CRP habitat to be highly fragmented and patchy, since patch sizes are small and most areas are surrounded by agricultural fields (Gallie and Zinke 2019). Most literature suggests fragmentation negatively affects specialist species including pygmy rabbits (Pierce et al. 2011). Many sagebrush steppe species including sage grouse (*Centrocercus urophasianus*; Schroeder and Vander Haegen 2011), mule deer (*Odocoileus hemionus*) and jackrabbits (*Lepus californicus*, *Lepus townsendii;* Schroeder and Vander Haegen 2006) have chosen to occupy CRP habitat, containing sagebrush, over adjacent, undisturbed sagebrush habitat. Additionally, increased nest survival has been documented in CRP habitat in Brewer's sparrows (*Spizella breweri*) and sage thrashers (*Oreoscoptes montanu*s; Vander Haegen et al. 2000). CRP fields may help connect fragmented patches of shrub-steppe habitat, creating a relatively continuous vegetative community for the dispersal of sagebrush obligates (i.e., Lupis et al. 2006).

During our study in Washington, pygmy rabbits were identified at 1-6 burrow systems within a winter survey period; this finding is comparable to the number of burrow systems used by rabbits within their home range during non-breeding seasons in Idaho pygmy rabbits (Sanchez and Rachlow 2008). Pygmy rabbits are typically not observed together at burrow systems and are known to occupy more than one burrow system, swapping throughout the year (Wilde 1978; Sanchez and Rachlow 2008). Home ranges during winter months have been shown to be more restricted than other seasons (Sanchez 2007; Sanchez and Rachlow 2008). Through our pellet surveys, from 2012-2020, the number rabbits identified per the number of active burrows identified averaged 0.63 rabbits/burrow with a range of 0.33-1.00 across all populations. Further analysis of this information can provide a means for estimating the relative abundance through burrow counts, rather than relying strictly on genetic monitoring. Burrow counts for indexing abundance have been evaluated previously in pygmy rabbits and revealed that the density of burrows can serve as an index for monitoring changes in abundance of pygmy rabbits in eastern Idaho, although their models were based on radio-collared rabbits (Price and Rachlow 2011).

SBF/CRP density estimates ranged from 0.01-0.09 rabbits/ha, CHB was 0.01 rabbits/ha, and BH ranged from 0.04-0.09 rabbits/ha suggesting that our findings are similar to lower density estimates for specific regions in Idaho. Density estimates for seven established populations within east central Idaho ranged from 0.02-0.46 rabbits/hectare (Price and Rachlow 2011). CHB estimates are low compared to the Idaho, SBF/CRP, and BH estimates, but this population is only in its first 2 years of establishment. The CHB habitat has the greatest potential for pygmy rabbits due to the continuous sagebrush-steppe habitat in the area (Gallie and Zinke 2019). BH on the other hand, has higher density estimates due to the much smaller size of the recovery area (79ha). Potential habitat has been identified in surrounding private land parcels, in which a larger number of active pygmy rabbits' burrows have been identified to the east of the BH recovery area.

As the shift to CRP habitat began in 2015 and release sites were located in native shrub-steppe habitat in SBF, the dispersal distances of the released juveniles nearly doubled (1947m) compared to the mean distance observed in 2012-2015. DeMay et al. (2017) found that dispersal rates of released juveniles in SBF from 2012-2015 mimic natural natal dispersal distances in Idaho populations. Juveniles and adults hard released from 2012-2015 settled relatively close to their release sites, and median distances were < 1 km (mean dispersal distance of juveniles was 988 m, and adults were 783 m). The juveniles and adults were all taken from on-site breeding enclosures with habitat similar to that of the release sites; yet dispersal to the CRP habitat appeared to be a priority. The mechanisms driving this shift are unclear but could be explained by differences in habitat quality, rabbit population density, predator abundance or other environmental conditions.

In our study, we found a trend towards longer dispersal distances in male released juveniles and adults in the SBF population compared to females, but contrary to our hypothesis, there was a high degree of variability and these differences were not statistically significant. Yet, when evaluating average dispersal distance post settlement (i.e., from year 1 to year 2), we detected a significant pattern of increased dispersal distances in males (804m) compared to females (351m). Sex-biased dispersal was observed in pygmy rabbit natal dispersal where females dispersed greater distances after emerging from the natal burrow (Estes-Zumpf and Rachlow 2009). Greater dispersal distances by females are suggested to be due to competition for resources, whereas greater male dispersal may be due to inbreeding avoidance and greater potential for mates (Jones et al. 1988; Bray et al. 2007). The greater dispersal in male rabbits during breeding season may explain the differences observed in our M:F ratio in summer 2018, where female rabbits were detected in higher proportion than males in SBF/CRP; yet overall winter M:F ratios reflected the 1:1 relationship. In all cases of second year detection dispersal, rabbits moved toward areas of higher rabbit density, suggesting environmental conditions and habitat

quality may differ in SBF compared to the CRP habitat. Further investigation into differences in environmental conditions and habitat quality should be a management priority to gain a better understanding of the mechanisms behind these dispersals and shift in habitat by pygmy rabbits.

Allowing animals to acclimate in specially constructed release pens in the new environment before release, is generally assumed to increase translocation and reintroduction viability by reducing the biological cost of release experienced by individuals (Carbyn et al. 1994). With the limited number of rabbits within the breeding enclosures and the loss of the BE enclosure to fire, a soft release approach using temporary release pens was implemented in 2017 with the goal of increasing survival rates of released rabbits. Previous translocation studies on rabbits identified a 57% mortality rate within the first three days after hard release of European rabbits (*Oryctolagus cuniculus*) into a new habitat (Letty 1998). Soft release studies on the European rabbit showed higher rates of survival in female rabbits that were acclimatized in soft release pens and the complete opposite for males (Letty et al. 2000). Soft release methods were used in the translocation of the endangered western burrowing owl, in which soft release protocols limited dispersal (86% stayed near release sites) and increased survivorship by 20% compared to hard release methods (Mitchell et al. 2011). Pygmy rabbits have a very high and variable annual survivorship rate, documented at 0.3%-17% in Nevada/Oregon (Crawford et al. 2010) and 7 -45% in Idaho (Sanchez 2007). Juvenile mortality in Idaho was 69% and 89% for male and females, respectively, with the highest mortality occurring within the first two months of emergence from natal burrows (Estes-Zumpf and Rachlow 2009). The average survival rate for hard releases in the SBF population was 14%, and survival rates using the release pens increased survivorship of released juveniles to 43% and 33% for CHB and BH, respectively, supporting our hypothesis that release pens increased survival rates. It is important to note that SBF, CHB, and BH are different treatment sites but BH and CHB were chosen by managers as release sites because they contained habitat variables that were thought to be optimal and comparable to preferred pygmy rabbit sites at SBF (Gallie and Zinke 2018, 2019). Initial apparent survival rates in SBF (2012) for juvenile pygmy rabbits was 39%, comparable to the results observed in CHB and BH. Yet by the second year of releases into SBF, juvenile apparent survival dropped to 12%. Contrary to SBF, CHB and BH increased apparent survival during the second-year releases, suggesting that the release pens are maximizing survivorship across multiple years.

Rabbits released into soft release pens had maximal dispersal distances of 530m and 588m for CHB and BH, respectively. Experimental reintroductions for other wildlife species have shown that another benefit of the soft release method is that it can enhance site affinity and social group cohesion (Price 1989; Bright and Morris 1994; Wanless et al. 2002; Sasmal et al. 2015). In contrast, animals

that are hard released are expected to display greater dispersal rates and distances when released into an unfamiliar environment. This dispersal away from the chosen release environment can result in higher individual mortality (Bright and Morris 1994). Juvenile pygmy rabbits have been known to disperse large distances greater than 7km (Estes-Zumpf & Rachlow 2009; DeMay et al. 2015), and the release pen implementation appears to have minimized dispersal distance which is necessary in the newer recovery areas where private land safe harbor agreements must be developed (Figure 1.4g).

Within the SBF wild population, the average survival rate of identified individuals during winter monitoring surveys was 14% from 2012-2016. Each year that rabbits were released, we detected a decreasing trend of survivorship of released individuals. The very low 0.1% apparent survival rate of released individuals in 2015 may be attributed to a combination of the sub-lethal to lethal levels of coccidia identified in released individuals, a drought year, low individual identification success rates due to unfavorable weather conditions and lower survey efforts compared to 2014 (Gallie 2016; Gallie and Zinke 2018). The decreasing trend of survivorship among enclosure born, released rabbits continued with 2nd year detection, although the 4% 2nd year detection was comparable to wild-born rabbits (5%). The release year for juvenile rabbits in SBF was highly significant and was retained in each of the apparent survival models, suggesting that other environmental variables across that landscape each year may play a role in the apparent survival of released rabbits. The decrease in survival after the first year could be explained by an increased response of predators across the reintroduction landscape as has been documented in other studies (Korpimaki and Norrdahl 1991; Sinclair et al. 1998; Gilg et al. 2006). Differences in predator densities may have also led to the shift in spatial distribution across the landscape in 2015. Preliminary data of terrestrial predator visitations at pygmy rabbit burrows in 2017-2018, using game cameras, revealed significantly fewer terrestrial predator occurrences near CRP burrows compared to SBF burrows in summer and fall months, but by winter, predator occurrences at burrow sites were similar between SBF and CRP (Gallie and Zinke 2019).

Timing of the release date for both adults and juveniles significantly influenced apparent survival at the SBF population and in the release pens at CHB and BH. The later they were released, the greater their chances of being detected in winter survey efforts likely due to the decreased intervals of being exposed to predation, especially raptors (Goodrich and Smith 2008; Crawford et al. 2010) and other mortality sources. High mortality rates typically occur in juveniles during the two months following emergence from natal burrows (Estes-Zumpf and Rachlow 2009). Thus, allowing rabbits to develop longer in the breeding enclosures or temporary release pens may increase their overall survivorship to winter. Multiple factors might influence variation in survival of leporids spatially and

temporally, including variability in predator populations, climatic conditions, forage quality or quantity, soil characteristics, parasites, and disease (O'Donoghue et al. 1997; Gillis 1998; Bond et al. 2001; Rödel et al. 2004). Juvenile apparent survival was also positively influenced by release weight, and release age; the older the rabbit, the more it weighs. DeMay et al. (2017) raised the concern that juveniles kept in enclosures longer may have lower survival due to possible acclimatization to humans and decreased exposure to predators, but as their model and ours showed, there appears to be no effect. By keeping juveniles in the enclosures longer, juvenile body condition and weight could increase, increasing their overall chances of survival.

Increased genetic diversity positively and significantly influenced the apparent survival rate of juveniles in the SBF population. Although not significant, increased genetic diversity followed the top model in adults as well, suggesting overall genetic diversity may play a role in survivorship of all life stages. Increased individual heterozygosity has also been shown to be a an important indicator of survival in the translocation of Mojave desert tortoises (*Gopherus agassizii*) (Scott et al. 2020). The influences of increased genetic diversity may be attributed to preventing effects of inbreeding in the population, favoring those of higher genetic diversity from random mating (Willi et al. 2006), and thus preventing a deleterious effect on population fitness (O'Grady et al. 2004). Low levels of genetic diversity and high levels of inbreeding are thought to have contributed to the low reproductive success and juvenile survival in the captive breeding program (U.S. Fish and Wildlife Service 2012; Elias et al. 2013). The genetic rescue conducted in 2001-2002, helped increase genetic diversity, increasing pregnancy rates and juvenile growth and survivorship within the captive breeding program (Elias et al. 2013).

During 2012-2014, the number of wild-born rabbits identified was only 14, and as DeMay et al. (2017) suggested, the SBF population did not appear to be a sustainable wild population due to the low apparent survival and reproduction rates. In 2015, the wild-born rabbit total (16) surpassed the 2012-2014 total and continued to increase during 2015-2018 with 401 wild-born rabbits identified. This suggests that the SBF population may be in early stages of being a sustainable wild population since reproduction rates have significantly increased since the findings in DeMay et al. (2017), although apparent survival rates have only slightly increased from 13% to 14%. Additionally, due to the small population size at SBF, the population is vulnerable to other stochastic effects, as we saw in winter 2019-2020's decline. Most rabbits that are found each year during survey efforts appear to be new wild-born juveniles, rather than adults that have survived multiple years. Annual survival rates of radio-collared pygmy rabbits ranged from 7 – 45% in Idaho (Sanchez 2007), and from 0.3 – 17% in Oregon and Nevada (Crawford et al. 2010). Although we are using pellets to assess apparent survival

and thus likely underestimate survival, our estimates fall in these ranges demonstrating the value of this noninvasive genetic sampling approach.

As a result of the increased reproductive productivity observed in the SBF wild population and declining numbers in the breeding enclosures, supplemental releases were halted in 2017. Unfortunately, a significant decrease in numbers of rabbits (~94% decline in individuals detected) was detected in winter 2019-2020. The causes of this decline are unknown. One possibility is that heavy flooding in March 2019 from the large amount of snow received in February occurred within the SBF recovery area, possibly negatively affecting the natal burrows and overall spatial distribution within SBF. Given the sudden shift in the use of habitat seen from 2015 onward, it is also possible that managers may not be looking in the right areas during survey efforts. 26% of released adults and 38% of released juveniles dispersed ≥1km, suggesting that rabbits may have dispersed beyond the SBF/CRP survey areas. Further efforts, such as helicopter surveys and the use of conservation canine units could increase the efficiency and spatial extent of the search for active burrows. In fact, during the winter 2020-2021 surveys, a new population of pygmy rabbits was identified ~5.63km north-east of the SBF/CRP population, on private CRP habitat. Further analysis of genetic data will reveal if this population is an extension of the SBF/CRP population or remnants of the original pure CB population thought to be extirpated in 2001.

At the end of our study, BH and CHB populations were still in early stages of establishment and could not yet be considered sustainable wild populations. Pygmy rabbits had not resided in CHB since the 1980s and most of the burrows that were identified during surveys were either newly created or modified badger digs. By 2019, no wild-born individuals were detected in CHB and only two wild-born rabbits were detected in BH. In the SBF population, wild-born rabbit production did not significantly increase until its fifth year, thus we can expect a similar pattern in these reintroduction areas. Initial attempts in 2015 to establish a population in BH were unsuccessful due to translocated juveniles being infected with coccidia (Gallie and Zinke 2018). Both areas are still early on in their establishment (≤5 years) and follow similar trends from the SBF population, in which most rabbits detected were ones released that same year (Table 1.2). Summer monitoring of both populations was completed in 2020 and will provide insight into how rabbits are spatially distributing across the habitat and if wild reproduction is beginning to increase. Unfortunately for the CHB population, in September 2020, the Pearl Hill fire swept through the CHB area, destroying nearly 97,124 ha (InciWeb 2020, https://inciweb.nwcg.gov/incident/7169/). All wild rabbits, release pen rabbits, mobile enclosures, and the DE enclosure were destroyed. CHB had the greatest overall potential for

expansion due to the large amount of connected sagebrush steppe habitat in the state of Washington. This loss was a great hit to the Columbia Basin pygmy rabbit recovery program.

Ancestry and Genetic Diversity

The CB pygmy rabbit population has undergone both genetic (2001) and demographic rescues (2011), contributing to the increase of genetic diversity compared to that observed in the original CB populations (Warheit 2001). Our analysis allowed us to tease apart the CB and Idaho ancestry that was examined in DeMay et al. (2015), providing a genetic estimate of CB ancestry for all pygmy rabbits in the project. We determined that four distinct genetic ancestries were represented in our mixed ancestry rabbits, (1) CB, (2) Nevada, Oregon, and Idaho, (3) northern Utah and Wyoming, and (4) southern Utah. Although CB ancestry played a role in the second top model in juvenile apparent survival in SBF, it did not significantly influence apparent survival of adults or juveniles in any of the models. DeMay et al. (2015) provided evidence of fitness benefit associated with Columbia Basin ancestry with the enclosure populations. Males with Columbia Basin ancestry estimates had increased reproductive output whereas males with high levels of northern Utah/Wyoming ancestry and females with high levels of Nevada/Oregon ancestries had decreased levels of reproductive output. Within the SBF population, all wild-born rabbits detected, other than two individuals in winter 2014-15, contained CB ancestry. Since 2015, all individuals detected during winter or summer monitoring surveys contained CB ancestry suggesting that selection may be favoring ancestry in the wild population. Managers must balance the needs for demographic rescue and numbers of reintroduced individuals with the preservation of locally adapted genes. Introducing genetically divergent or geographically distant individuals into a population can cause outbreeding depression, a decrease in fitness caused by the breaking up of co-adapted traits or the loss of locally adapted alleles (Lynch 1991; Tallmon et al. 2004; Hedrick et al. 2011).

Yet, overall estimates of heterozygosity and AR did not significantly differ across the 8 years within SBF/CRP providing evidence that genetic diversity has been maintained within the wild population. AR values within the SBF/CRP population reflected the AR values from the breeding enclosures, since the population was founded and augmented with individuals from each enclosure. Heterozygosity levels for both the wild and enclosure populations have nearly doubled (H_o=0.62-0.84) compared to the estimates from the remnant SBF population in 2001 (H_o=0.40; Warheit 2001). Reintroduction efforts are often challenged by a small number of founders and the rapid loss of genetic diversity (Leberg 1993; Earnhardt 1999; Miller et al. 2009, 2012). Additionally, reintroductions of pygmy rabbits are often accompanied by high mortality rates during the rabbit's first year (Estes-

Zumpf and Rachlow 2009; Crawford et al. 2010; DeMay et al. 2015, 2017), which may reduce the effective population size and genetic diversity within the wild population.

Genetic diversity was maintained in the enclosure populations, with little differences across years from initial establishment to the final enclosure populations. A loss of diversity in 2018 was detected in SE, but this was mainly due to limited number of individuals within the enclosure and the relatedness of a single male to many of the rabbits within the enclosure. Other male rabbits, who were survivors of the BE enclosure fire, had been translocated into SE in 2017, but many died of smoke inhalation complications weeks to months after translocation (Gallie and Zinke 2019). The variation and significant changes in DE's AR values from 2013-2019 can be attributed to either augmentation of more diverse individuals from other enclosures in 2013 and 2017, or the decline of breeders as the case in 2016. In 2017, survivors of the BE enclosure fire were also translocated to DE enclosure, in which over 20 rabbits could be accounted for in February 2018, but for unknown reasons, only a single female could be documented by late April 2018. Although male augmentation from the LE enclosure was immediately performed, no juveniles were produced in the DE enclosure during 2018.

The BH and CHB populations had many fewer founders compared to the SBF population, resulting in the lower AR (4.31 alleles per locus), yet observed heterozygosity (H_o=0.74) was comparable to SBF/CRP. AR and heterozygosity were comparable to estimates found in Idaho (H_e=0.73 across all sites, allelic richness = 4.3-5.6; Estes-Zumpf et al. 2010), but much higher compared to the Wyoming populations (H_e=0.58, AR = 2.8-3.1; Thimmayya and Buskirk 2012). However, results are not directly comparable because both studies used a subset of our loci (10). The genetic and demographic rescues performed in 2001 and 2011, respectively, successfully increased and maintained the genetic diversity within the captive and wild CB populations.

All N_e point estimates for the SBF/CRP populations were under 50 individuals. For many species, an effective population size greater than N_e >100 is considered sufficient for short term persistence of a population, preventing inbreeding depression (Frankham et al. 2014), yet in highly dynamic populations, N_e >300 is recommended (Newmark 1995; Fenderson et al. 2014). Yearly N_e estimates within SBF are similar to those found in the small and endangered (state listed) populations of New England cottontail (average N_e = 3.2-36.7; Fenderson et al. 2014; Bauer 2018). Concern about the persistence of all CB pygmy rabbit populations should be a major priority and augmentation into each of the populations may be necessary to maintain genetic diversity for the unforeseeable future, until N_e estimates increase.

Conservation Management

Genetic monitoring has been an increased focus in conservation biology and wildlife management (Schwartz et al. 2007; Stetz et al. 2011) and is becoming widely used in monitoring and adaptive management of reintroduced populations (Adams et al. 2007; De Barba et al. 2010; Bohling et al. 2013; Gese et al. 2015; Woodruff et al. 2015, 2016; Clendenin et al. 2020). Our study has helped effectively guide conservation management strategies for the Columbia Basin pygmy rabbit recovery program. Characteristics of the pygmy rabbit reintroduction that helped retain high genetic diversity included a large founding population from multiple sources, supplementations of more animals into the wild each year, short generation times, promiscuous mating systems (DeMay et al. 2016, 2017), and high reproductive output. Additionally, a portion of juveniles known to have high Columbia Basin ancestry were retained in the breeding enclosures each year for future breeding in relatively safe conditions compared to the wild, thereby retaining more Columbia Basin ancestry for future releases. Conversely, low survival rates and dispersal away from the release site remove potential breeders from the population and contribute to the loss of genetic diversity.

Evaluating the genetic diversity present in both the founding population and subsequent generations of the reintroduced populations allowed us to monitor the population's genetic response to reintroduction and assess the success of the reintroduction in genetic and demographic terms. One of the main goals of the Columbia Basin pygmy rabbit recovery program was to maintain the Columbia Basin ancestry, and our monitoring data has shown that nearly all wild-born rabbits (99.3%) have maintained > 5% native CB ancestry. We acknowledge that ancestry estimates based on 18 microsatellite loci can be imprecise and have wide confidence intervals, thus we are currently using RADseq approaches (Ali et al. 2016) to identify thousands of single nucleotide polymorphism loci (SNPs) from the founders of this population that can be used for future ancestry estimates. Also, further investigation of adaptive loci is necessary to understand which regions of the genome are under selection within the Columbia Basin population.

The SBF population showed initial signs of being a sustainable wild population based on high reproductive rates, moderate survival rates and large numbers of wild-born rabbits identified from 2015-2019 but based on the population crash in 2019 and N_e estimates, augmentation of the populations will likely be needed in the future. Noninvasive genetic sampling has proven to be an effective and efficient tool in monitoring this reintroduced population an in helping managers address the goal of the Columbia Basin recovery project of establishing multiple sustainable wild populations

within the sagebrush steppe-habitat of Washington. The results of this study have helped effectively guide monitoring strategies in the past and can be used to inform future recovery efforts for the CB pygmy rabbit. This study can also be used as a guide for other genetic management studies.

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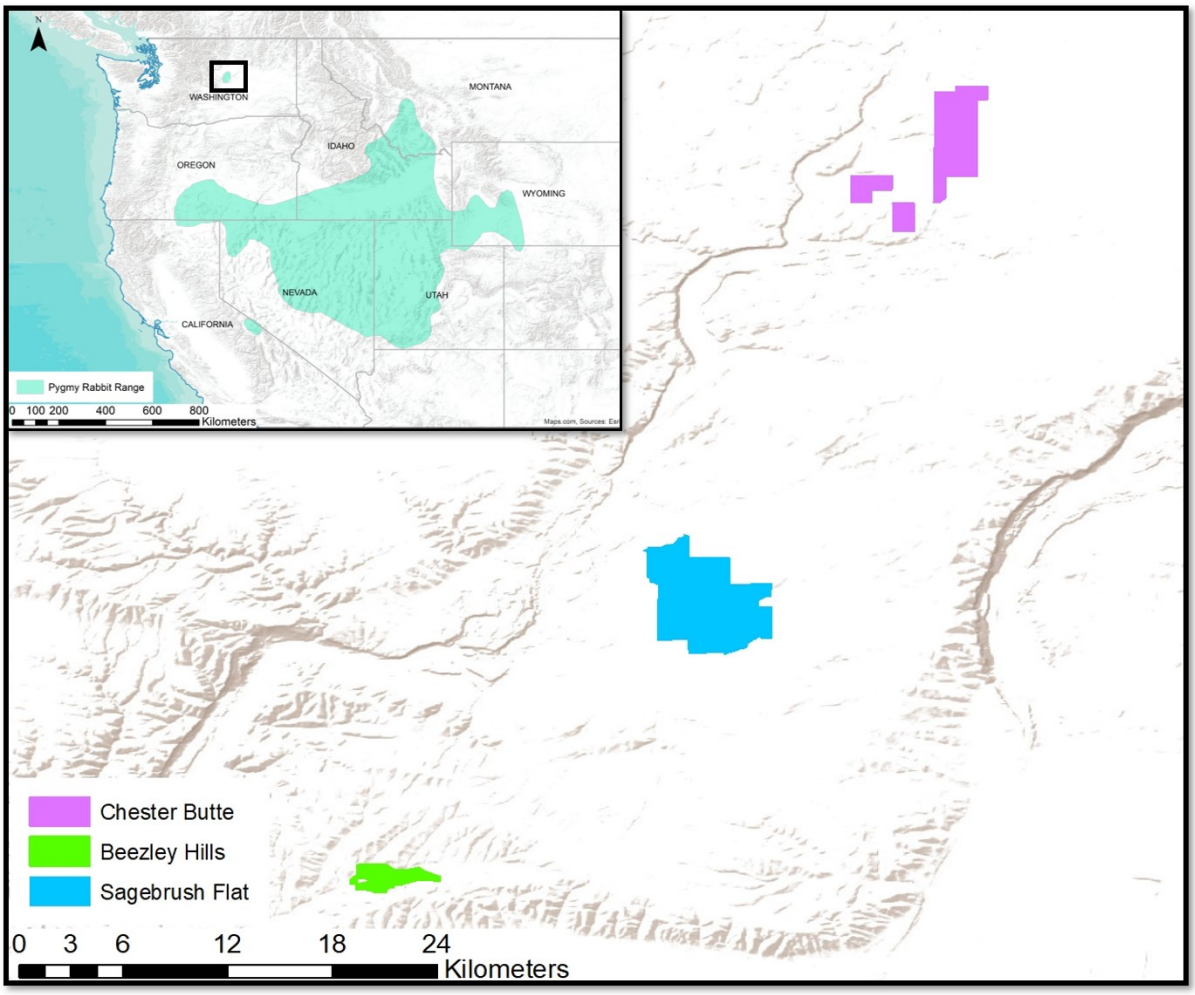

Figure 1.1. Geographic location of the reintroduced Columbia Basin pygmy rabbit (*Brachylagus idahoensis*) populations in Washington, USA, and locations of Sagebrush Flat, Beezley Hills, and Chester Butte recovery areas.

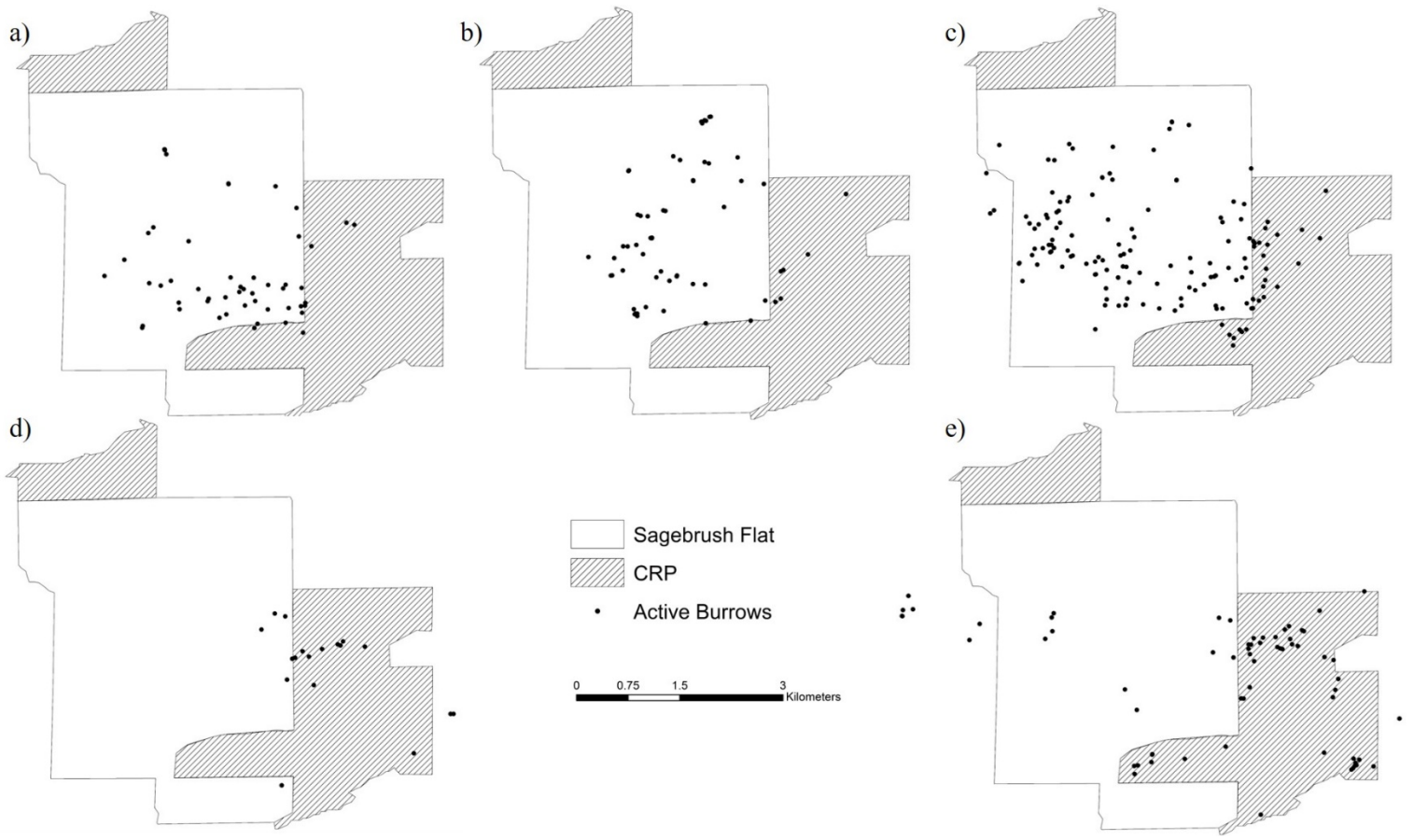

Figure 1.2. Location of Sagebrush Flat (SBF) wildlife area in Washington, USA, Conservation Reserve Program habitat (CRP), and active pygmy rabbit (Brachylagus idahoensis) burrows (•) identified during winter monitoring surveys during (a) winter 2012-2013, (b) winter 2013-2014, (c) winter 2014-2015, (d) winter 2015-2016, and (e) winter 2016-2017. Area to the left of SBF represents private land.

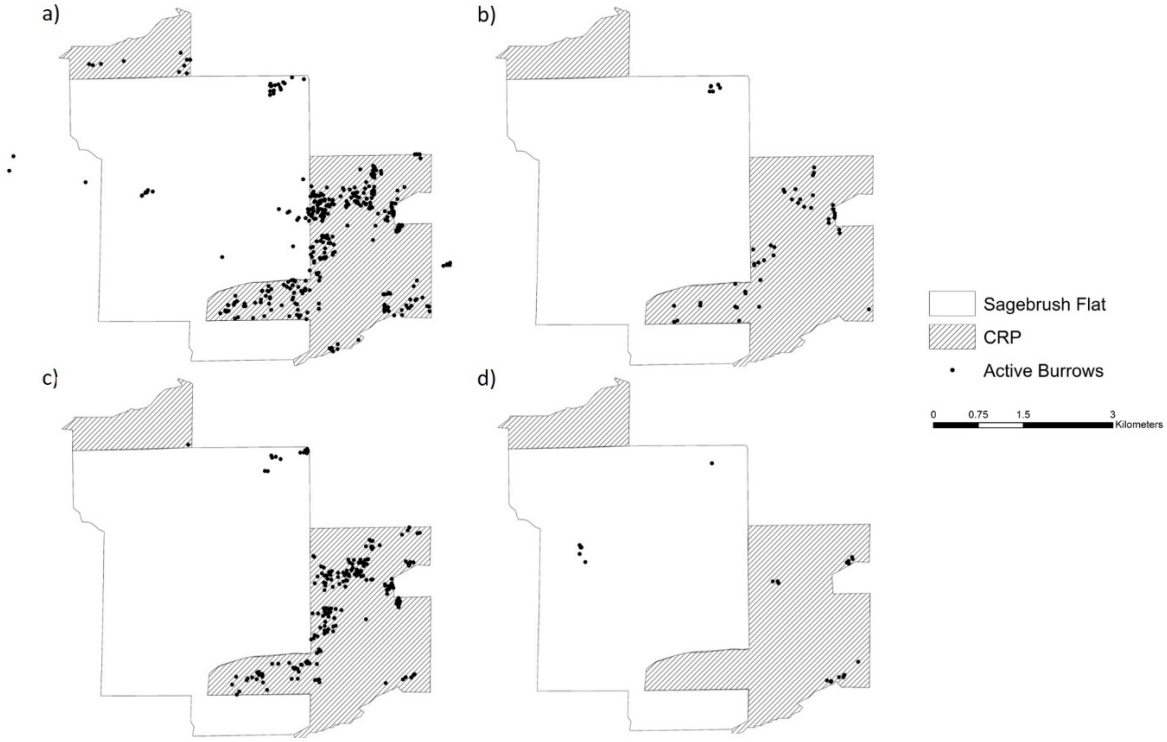

Figure 1.3. Location of Sagebrush Flat (SBF) wildlife area in Washington, USA, Conservation Reserve Program habitat (CRP), and active pygmy rabbit (*Brachylagus idahoensis*) burrows (•) across the habitat identified during monitoring surveys during (a) winter 2017-2018, (b) summer 2018, (c) winter 2018-2019, and (d) winter 2019-2020. Area to the left of SBF represents private land.

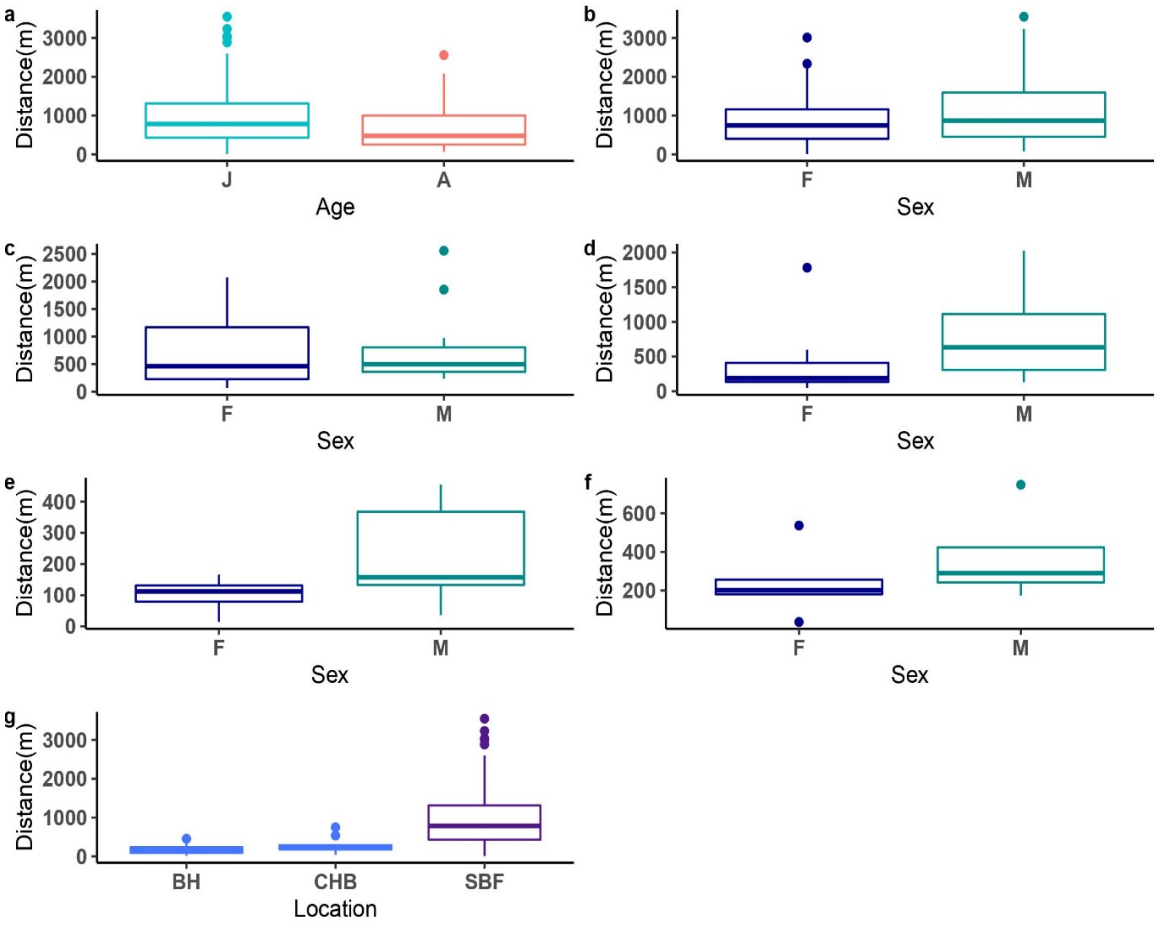

Figure 1.4. Box plots showing dispersal distances of pygmy rabbits (*Brachylagus idahoensis*): (a) from release site to winter burrow detection in released juveniles (J) vs adults (A) in SBF population from 2012-2016, (b) from release site to winter burrow detection in released juvenile females (F) vs juvenile males (M) in SBF population from 2012-2016, (c) from release site to winter burrow detection in released adult females (F) vs adult males (M) in SBF population from 2012-2016, (d) of second year detection adult females (F) vs adult males (M) in SBF population from 2012-2020, (e) from temporary release pen for juvenile females (F) and juvenile males (M) in Beezley Hills (BH) recovery area from 2017-2020, (f) from temporary release pen for juvenile females (F) and juvenile males (M) in Chester Butte (CHB) recovery area from 2018-2020, and (g) between released juveniles in soft releases (CHB and BH) vs hard releases (SBF). There were no significant differences in dispersal distances between age or sex in released individuals. Male rabbits dispersed significantly farther than female rabbits ($p = 0.04$) in second year detections. Soft release dispersal distances were significantly less than hard releases ($p < 0.0001$).

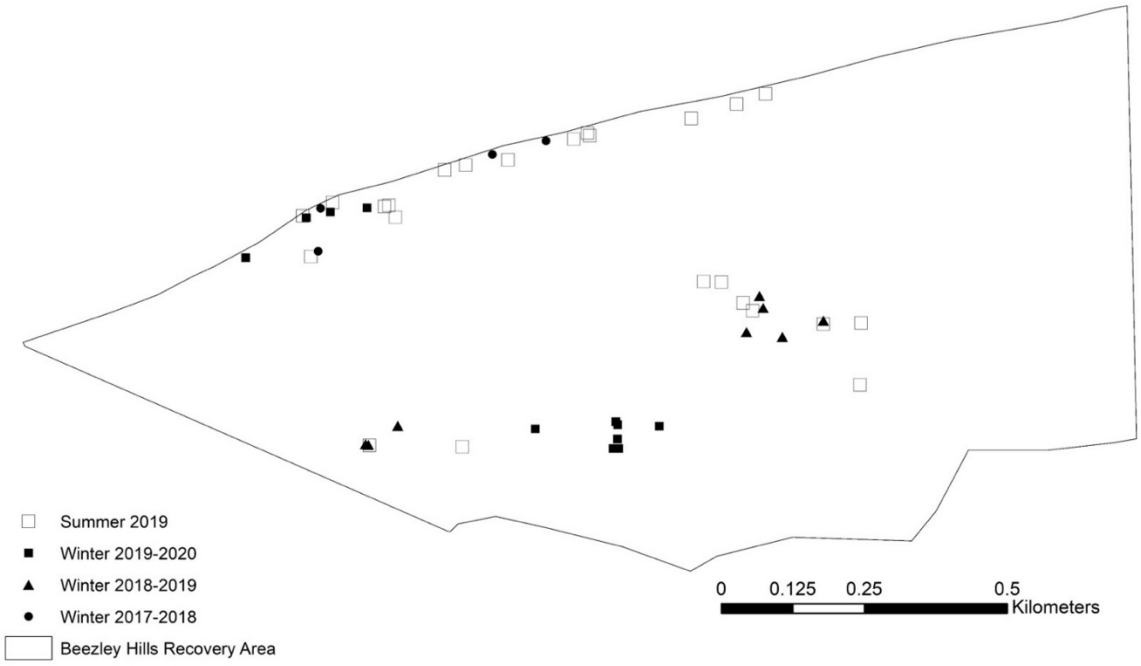

Figure 1.5. Location of active pygmy rabbit (*Brachylagus idahoensis*) burrows across the habitat identified during monitoring surveys in the Beezley Hills recovery area in central Washington state, during winter 2017-2018 (•), winter 2018-2019 (▲), summer 2019 (■), and winter 2019-2020 (□).

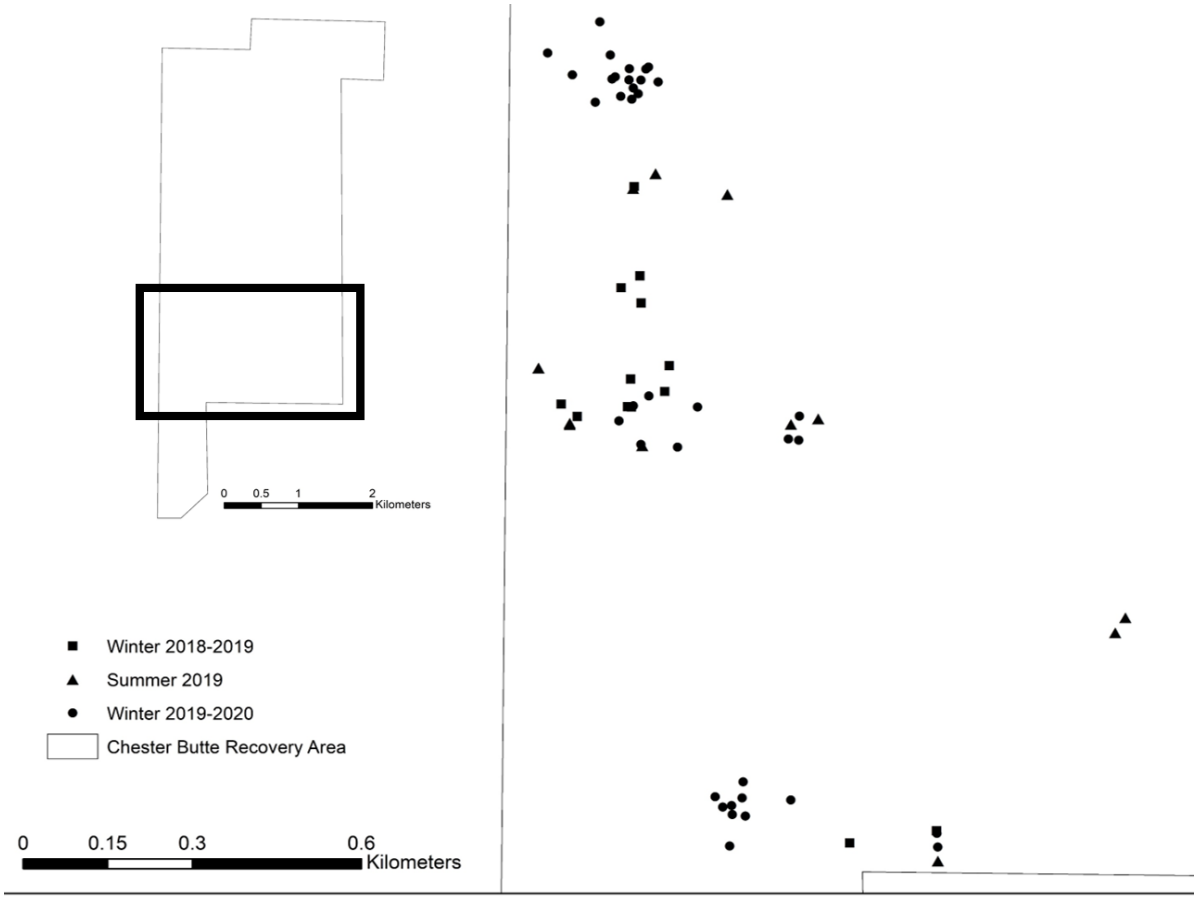

Figure 1.6. Location of active pygmy rabbit (*Brachylagus idahoensis*) burrows across the habitat identified during monitoring surveys in the Chester Butte recovery area, in central Washington state, during winter 2018-2019 (■), summer 2019 (▲), and winter 2019-2020 (•).

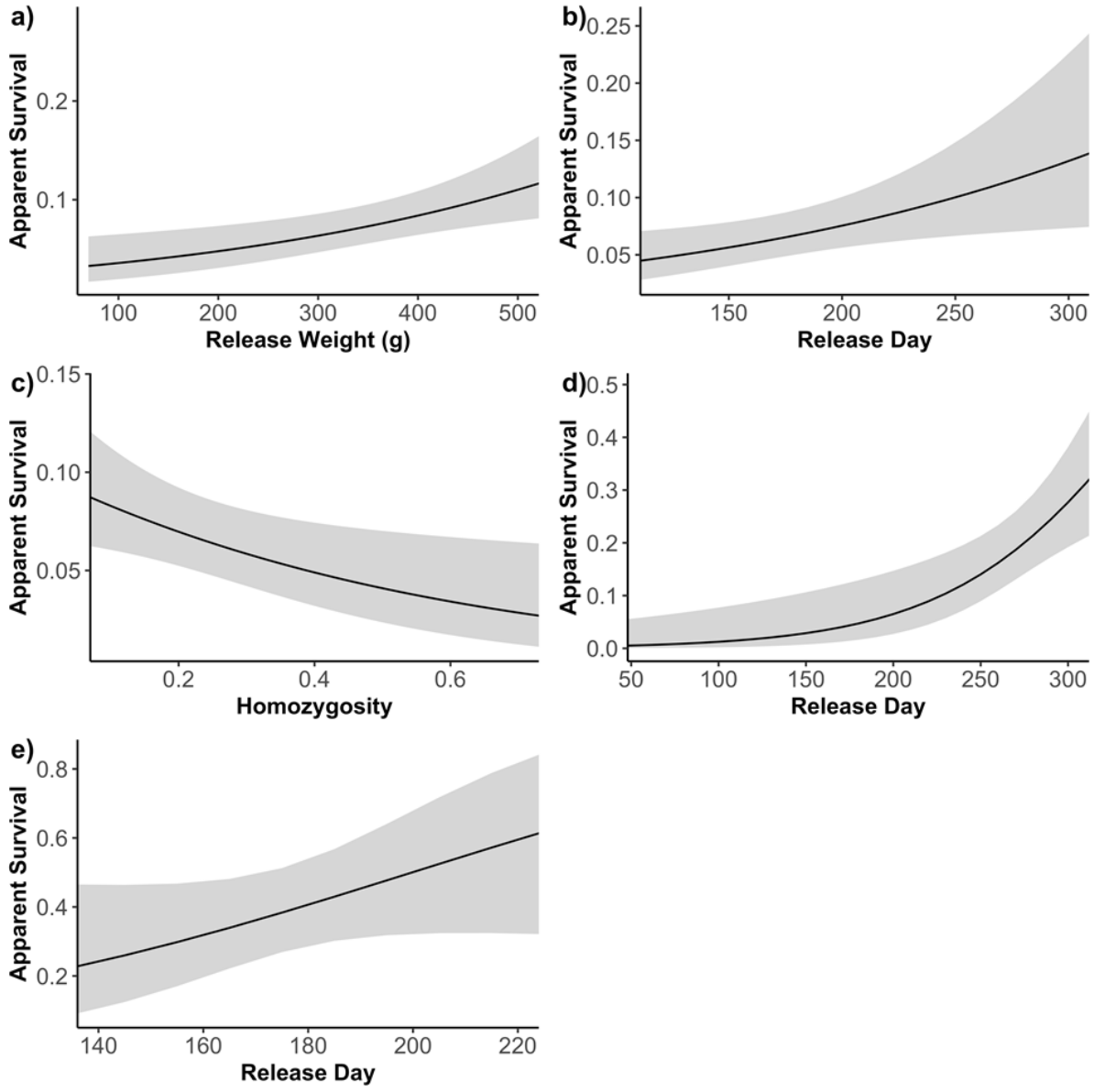

Figure 1.7. Predicted probabilities of apparent survival rates for pygmy rabbits (*Brachylagus idahoensis*) introduced into the central WA for significant variables in each model. (a) Juvenile survival rate by release weight for released juveniles in the Sagebrush Flat recover area, (b) Juvenile survival rate by release day for released juveniles in the Sagebrush Flat recovery area, (c) Juvenile survival rate by homozygosity for released juveniles in the Sagebrush Flat recovery area, (d) adult survival rate by release weight for released adults in the Sagebrush Flat recover area, (e) Juvenile survival rate by release day for juveniles released into pens in the Beezley Hills and Chester Butte recovery areas. Predicted probabilities plots were generated from top models for juvenile and adult survival.

Table 1.1. Study objectives and parameters examined for endangered Columbia Basin pygmy rabbits (Brachylagus idahoensis) of Washington and the genetic monitoring approaches used to address each parameter. Wild population is defined as all free-ranging rabbit on the Sagebrush Flat wildlife area in central Washington.

Objective	Parameter	Genetic Monitoring Approach
Wild Apparent Survival	Released Individuals	Compare tissue sample genotypes to winter pellet genotypes
	Adults after 1st winter	Compare tissue sample genotypes to winter pellet genotypes
	Factors Influencing Survival Rates (Genetic)	Logistic regression models of winter monitoring data
Wild Population Information	Habitat Occupancy and Spatial distribution	Compare GPS locations of burrows and identified species and individuals from winter monitoring data each year
	Minimum population size	Winter monitoring fecal DNA genotyping
	Sex ratios in wild population	Ratio of male/female individuals identified in winter monitoring surveys
	Rabbits per active burrow	Ratio of # of rabbits identified to total number of active burrows located during winter monitoring surveys
	Genetic Diversity	Estimates of observed and expected heterozygosity, and allelic richness from winter and summer fecal DNA genotypes
	CB ancestry	Genetic estimates based on winter and summer monitoring fecal DNA genotypes
	Effective Population Size	Parametric point estimates using linkage disequilibrium method and minor allele frequency 0.05, from winter and summer monitoring fecal DNA genotypes
Enclosure Population Information	CB ancestry	Genetic estimates based on tissue genotypes of trapped enclosure rabbits
	Genetic Diversity	Estimates of observed and expected heterozygosity, and allelic richness using programs from tissue genotypes of trapped enclosure rabbits
Comparison of Release Types	Apparent Survival in Soft Releases (Release Pens) vs Hard Releases	Comparison of individuals detected during winter monitoring data vs the total number of individuals released
	Factors Affecting Survivorship in Release Pens	Logistic regression models of winter monitoring data

Table 1.2. Details for winter and summer monitoring of pygmy rabbits (*Brachylagus idahoensis*) from 2012-2020 for Sagebrush Flat/Conservation Reserve Program (CRP) areas in central Washington, USA. Area surveyed represents ground survey efforts. Release period indicates when juveniles and adults were released into the SBF area. Total fecal samples represent all pellet samples collected and pygmy fecal samples represents pellets that were determined to be pygmy rabbit through species ID or microsatellite panels. Species ID success rates were not formally

Breeding Season	# of enclosures	Release Period	# Released	Survey Period	Area Surveyed	Total Fecal Samples Collected	Pygmy Fecal Samples Identified	SPID Success Rate	Individual ID Success Rate	Individuals Detected (Year Released or First Detected)	Number of breeders of wild-born juveniles
2012	2	May-Jul	104 juveniles 0 adults	Dec 2012-Jan 2013	9.71 km^2	117	111	NA	78%	45 41 released (2012) 4 wild-born (2012)	1 female 1 male
2013	3	May-Aug	265 juveniles 7 adults	Jan-Feb 2014	10.52 km^2	296	273	NA	46%	44 3 released (2012) 34 released (2013) 7 wild-born (2013)	7 females 7 males
2014	4	Mar-Nov	717 juveniles 113 adults	Jan-Mar 2015	13.76 km^2	265	212	NA	76%	91 1 released (2013) 87 released (2014) 3 wild-born (2014)	2 females 3 males
2015	4	Feb-Oct	149 juveniles 4 adults	Jan-Feb 2016	10.84 km^2	105	105	NA	20%	18 1 released (2014) 1 released (2015) 16 wild-born (2015)	11 females (1 unknown) 8 males (5 unknown)
2016	4	May-Oct	119 juveniles 1 adult	Dec 2016-Mar 2017	24.28 km^2	193	124	46%	52%	60 1 wild-born (2015) 5 released (2016) 54 wild-born (2016)	18 females (25 unknown) 17 males (32 unknown)
2017	4	May-October	0 juveniles 0 adults	Dec 2017-Mar 2018	14.67 km^2	357	296	72%	56%	158 2 wild-born (2016) 156 wild-born (2017)	47 females (98 unknowns) 46 fathers (92 unknowns)
2018	2	May-August	0 juveniles 0 adults	June-Aug 2018	7.40 km^2	98	98	NA	56%	54 2 wild-born (2017) 49 wild-born adults (2017) 3 wild-born juveniles (2018)	19 females (33 unknowns) 17 males (31 unknowns)
				Dec 2018-Apr 2019	11.51 km^2	447	296	77%	73%	138 1 wild-born (2016) 14 wild-born (2017) 123 wild-born (2018)	19 females (88 unknowns) 20 males (81 unknowns)
2019	2	May-August	0 juveniles 0 adults	Jan-Mar 2020	5.89 km^2	59	27	97%	83%	8 3 wild-born (2018) 5 wild-born (2019)	2 females (3 unknowns) 3 males (2 unknowns)

Table 1.3. Winter and summer monitoring of pygmy rabbits (Brachylagus idahoensis) information from 2012-2020 for Beezley Hills (BH) and Chester Butte (CHB) recovery areas. Area surveyed represents formal survey efforts and helicopter surveys. Release period indicated when juveniles and adults were released into the SBF area. Total fecal samples represent all pellet samples collected and pygmy fecal samples represents pellets that were determined to be pygmy rabbit through species ID or microsatellite panels. Species ID success rates were not formally introduced until winter 2018-19 survey year. Individual ID success rates are based on the number of individuals identified from confirmed pygmy rabbit samples. Unknown parentage represents individuals whose parents could not be assigned at the 95% confidence

Breeding Season	Release Area	Release Period	# Released	Survey Period	Area Surveyed	Total Fecal Samples Collected	Pygmy Fecal Samples Collected	SPID Success Rate	Individuals ID Success Rate	Individuals Detected (Year Released)	Contributing breeders to wild-born juveniles
2015	BH	Feb-May	369 juveniles 51 adults	Jan-Feb 2016	3.09km²	0	0	-	-	-	-
2017	BH	May-October	14 juveniles 0 adults	Dec 2017-Mar 2018	0.21km²	9	8	-	75%	5 5 released (2017)	0 females 0 males
2018	BH	May-August	10 juveniles 7 enclosure 3 wild	Dec 2018-Apr 2019	0.69km²	10	8	80%	88%	3 2 released enclosure (2018) 1 released wild (2018)	0 females 0 males
	CHB	May-August	17 juveniles 8 enclosure 9 wild	Dec 2018-Apr 2019	1.07km²	20	19	95%	84%	6 1 released enclosure (2018) 5 released wild (2018)	0 females 0 males
2019	BH	May-August	17 juveniles 10 enclosure 4 wild	June-Sept 2019	0.69km²	34	27	85%	67%	7 2 released enclosure (2019) 3 escaped enclosure (2019) 2 wild-born (2019)	1 female (1 unknown) 1 male (1 unknown)
	CHB	May-August	21 juveniles 19 enclosure 1 wild	June-Sept 2019	1.53km²	20	14	80%	93%	5 5 released enclosure (2019)	0 females 0 males
	BH	May-August	17 juveniles 10 enclosure 4 wild	Oct 2019-Feb 2020	0.69km²	15	13	93%	92%	5 4 released enclosure (2019) 1 released wild (2019)	0 females 0 males
	CHB	May-August	21 juveniles 19 enclosure 1 wild	Oct 2019-Feb 2020	2.43km²	39	37	97%	81%	10 9 released enclosure (2019) 1 released wild (2019)	0 females 0 males

Table 1.4. Winter and summer monitoring of pygmy rabbits (*Brachylagus idahoensis*) for Sagebrush Flat/CRP recovery area, in central Washington state, from 2012-2019. Minimum count is established through the number of identified pygmy rabbits (Brachylagus idahoensis) through genotyping using a microsatellite panel. The number of rabbits per number of active burrows identified is based on the minimum count of rabbits/total number of active burrows located. Density estimates (rabbits/ha) are based on the minimum count/total potential habitat in SBF/CRP (1780ha). Individuals containing CB ancestry are defined as rabbits with 5%- 80% CB ancestry using STRUCTURE. Genetic diversity estimates are summarized as observed heterozygosity, expected heterozygosity, and allelic richness. Effective population size is represented at the point estimate and the 95% confidence interval in parentheses using the linkage disequilibrium method and minor allele frequency of 0.05.

		YEAR								
		2012	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**		**2019**
		SURVEY PERIOD								
Category	**Parameter**	Winter	Winter	Winter	Winter	Winter	Winter	Summer	Winter	Winter
Demographic	Minimum count	45	44	91	18	60	158	54	138	8
	M:F Sex Ratio (actual #s)	1:1.5 (18:27)	1:1 (22:22)	1:1.1 (44:47)	1:1.6 (7:11)	1.1:1 (32:28)	1.8:1 (101:57)	1:1.8 (19:35)	1.9:1 (91:47)	1:1 (4:4)
	Rabbits/Active Burrow	0.87	0.75	0.63	1	0.92	0.96	0.56	0.65	0.33
	Density (rabbits/ha)	0.03	0.02	0.05	0.01	0.03	0.09	0.03	0.08	0.004
Genetic	Average CB ancestry	19.69%	19.72%	19.10%	21.06%	21.89%	15.31%	18.48%%	17.97%	16.1%
	Proportion of identified individuals containing CB ancestry	48.89%	88.64%	71.43%	100%	100%	100%	100%%	100%	100%
	Proportion of wild-born individuals containing CB ancestry	100%%	100%	33.33%	100%	100%	100%	100%%	100%	100%
	Effective population size (95% Confidence Interval)	15.4 (13.7-17.3)	29.6 (25.3-34.9)	30.4 (27.7-33.5)	19.3 (14.7-27.0)	40.7 (35.0-47.9)	44.3 (40.6-48.5)	36.9 (23.7-30.8)	27.6 (25.5-29.9)	12.3 (7.0-26.8)
	Observed Heterozygosity	0.76	0.81	0.81	0.75	0.7	0.84	0.76	0.62	0.64
	Expected Heterozygosity	0.8	0.79	0.8	0.8	0.8	0.82	0.79	0.79	0.72
	Allelic Richness	5.22	5.15	5.13	5.29	5.16	5.35	5.00	4.95	4.67

Table 1.5. Winter and summer monitoring of pygmy rabbits (*Brachylagus idahoensis*) for Beezley Hills (BH) and Chester Butte (CHB) recovery areas in central Washington state from 2017-2019. Minimum count is established through the number of identified pygmy rabbits (Brachylagus idahoensis) through genotyping of the microsatellite panel. The number of rabbits per number of active burrows identified is based on the minimum count of rabbits/total number of active burrows located. Density estimates (rabbits/ha) are based on the minimum count/total potential habitat in BH (83ha) or CHB (893ha). Individuals containing CB ancestry are defined as rabbits with 10.8%- 42.74% CB ancestry using STRUCTURE. Genetic diversity estimates are given through observed heterozygosity, and allelic richness. Expected heterozygosity is only given when sample sizes are ≥5. Effective population estimates were based on the linkage disequilibrium method and minor allele frequency of 0.05 for minimum counts ≥7.

		SURVEY PERIOD							
		Winter 2017-18		***Winter 2018-19***		***Summer 2019***		***Winter 2019-20***	
	LOCATION	***CHB***	***BH***	***CHB***	***BH***	***CHB***	***BH***	***CHB***	***BH***
Demographic Parameters									
Minimum count		-	5	5	3	5	7	10	5
M:F Sex Ratio (actual #s)		-	1:1.5 (2:3)	1.5:1 (3:2)	1:2 (1:2)	1:1.5 (2:3)	6:1 (6:1)	1:1.5 (4:6)	1:1.5 (2:3)
Rabbits/Active Burrow		-	0.42	0.45	0.43	0.5	0.7	0.35	0.5
Density (rabbits/ha)		-	0.06	0.01	0.04	0.01	0.09	0.01	0.04
Genetic Parameters									
Average CB ancestry		-	23.98%	14.85%	23.97%	20.89%	22.87%	19.04%	27.46%
Proportion of identified individuals containing CB ancestry		-	100%	100%	100%	100%	100%	100%	100%
Effective population size (95% Confidence Interval)		-	-	-	-	-	9.3 (3.3-32.0)	12.5 (7.1-26.6)	-
Observed Heterozygosity		-	0.78	0.77	0.75	0.73	0.8	0.74	0.59
Expected Heterozygosity		-	0.71	0.71	-	0.66	0.66	0.64	0.64
Allelic Richness		-	5.41	4.65	-	4.59	3.82	3.69	3.71

Table 1.6. Sample size (n), genetic estimates of observed heterozygosity (Ho), and Columbia Basin (CB) ancestry composition of each of the pygmy rabbit (*Brachylagus idahoensis*) release pens from 2018-2019 through stocking in summer and pellet surveys during winter monitoring in the Chester Butte (CHB) and Beezley Hills (BH) recovery areas. Overall estimates include all release pens within a given recovery area. Winter survivorship was determined through identified individuals in winter surveys.

	2018							2019						
	Summer			Winter				Summer			Winter			
Release Pen	**n**	**Ho**	**CB**	**n**	**Ho**	**CB**	**Survivorship**	**n**	**Ho**	**CB**	**n**	**Ho**	**CB**	**Survivorship**
CHB-1	8	0.77	18.18%	1	-	15.17%	12.50%	7	0.71	21.23%	5	0.73	18.04%	71.43%
CHB-2	8	0.76	16.64%	4	0.74	16.96%	50.00%	8	0.74	22.51%	2	0.77	17.92%	25.00%
CHB-3	1	-	13.19%	1	-	13.19%	100.00%	6	0.72	21.47%	3	0.73	21.47%	50.00%
BH-1	5	0.78	20.72%	2	0.77	21.62%	40.00%	7	0.7	21.16%	2	0.71	23.75%	28.57%
BH-2	5	0.77	16.35%	1	-	18.23%	20.00%	7	0.61	22.93%	3	0.51	29.93%	42.86%
Overall CHB	16	0.78	17.16%	6	0.80	16.04%	37.50%	21	0.72	21.78%	10	0.74	19.04%	47.62%
Overall BH	10	0.77	18.54%	3	0.745	20.49%	30.00%	14	0.65	21.60%	5	0.59	27.46%	35.71%

Table 1.7. Genetic characteristics and ancestry composition of pygmy rabbits (Brachylagus idahoensis) in each of the captive breeding enclosures from years (YR) 2012-2019 in central Washington, USA. Enclosure populations (n) included all individuals born each year; adults detected as being parents in a given year through parentage analysis. Genetic diversity estimates are given through observed heterozygosity (Ho), expected heterozygosity (He), and allelic richness (AR). Columbia Basin (CB%) ancestry estimates are genetic estimates based on program STRUCTURE.

	Enclosure 1 LE					Enclosure 2 SE					Enclosure 3 DE					Enclosure 4 BE					Mobile Enclosure 1				
YR	**n**	**H_e**	**H_o**	**CB%**	**AR**	**n**	**He**	**Ho**	**CB%**	**AR**	**n**	**He**	**Ho**	**CB%**	**AR**	**n**	**He**	**Ho**	**CB%**	**AR**	**n**	**He**	**Ho**	**CB%**	**AR**
2012	117	0.79	0.76	2.77	5.13	121	0.79	0.78	25.58	4.95	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2013	146	0.79	0.81	17.70	4.96	221	0.78	0.80	16.77	4.93	181	0.70	0.68	1.84	3.93	-	-	-	-	-	-	-	-	-	-
2014	379	0.81	0.83	16.20	5.08	232	0.77	0.79	12.47	4.85	275	0.75	0.74	9.6	4.43	139	0.77	0.79	29.18	4.88	-	-	-	-	-
2015	232	0.79	0.80	18.33	4.97	2	-	0.74	9.12	-	1	-	-	29.65	-	457	0.78	0.79	22.93	4.72	-	-	-	-	-
2016	93	0.78	0.76	20.10	4.84	1	-	-	31.81	-	11	0.66	0.76	31.04	3.74	121	0.76	0.74	23.48	4.72	-	-	-	-	-
2017	54	0.81	0.82	20.64	5.29	11	0.68	0.75	15.22	4.13	26	0.74	0.74	23.74	4.53	41	0.76	0.78	21.80	4.75	14	0.80	0.92	23.22	5.39
2018	23	0.75	0.79	17.32	4.74	6	0.54	0.70	13.37	3.06	1	-	-	13.67	-	-	-	-	-	-	6	0.69	0.75	17.43	4.79
2019	-	-	-	-	-	-	-	-	-	-	14	0.69	0.73	22.07	4.21	-	-	-	-	-	38	0.66	0.73	19.46	3.57

Table 1.8. AICc values, ΔAICc, model weights, cumulative model weights, and log-likelihood values at the 95% confidence interval of the top models describing apparent survival rate of juvenile pygmy rabbits (*Brachylagus idahoensis*) after reintroduction into Sagebrush Flat/CRP sits in central Washington, USA, from 2012-2016. See Figure 1.7 for relationships between survival and significant variables in the top model. Year refers to the release year and was included in all models because it was highly significant. Day represents the release day based on Julian calendar, and weight represents the weight (g) of a juvenile at time of release. Columbia Basin ancestry (CB Ancestry) was a genetic estimate based on program STRUCTURE. Homozygosity was determined from genotypes of individuals in R-package GENHET. Only models that that performed better than the intercept only (due to random factors) were included.

Model	n	Variables	AIC	ΔAIC	wi	∑wi	Log-Likelihood
Juvenile Survival - Sagebrush Flat/CRP	1660	Year + Day + Weight + Homozygosity	781.33	0	0.43	0.43	-382.62
		Year + Day + Weight + Sex + Homozygosity	783.35	2.02	0.16	0.59	-382.62
		Year + Weight + Homozygosity	784.90	3.57	0.07	0.67	-385.42
		Year + Day + Weight + Sex + CB Ancestry + Homozygosity	784.99	3.66	0.07	0.74	-382.43
		Year + Day + Weight	785.02	3.69	0.07	0.80	-385.48
		Year + Day + Weight + CB Ancestry	786.68	5.35	0.03	0.83	-385.30
		Year + Weight + CB Ancestry + Homozygosity	786.78	5.45	0.03	0.86	-385.35
		Year + Weight + Sex + Homozygosity	786.85	5.52	0.03	0.89	-385.38
		Year + Day + Weight + Sex	787.04	5.71	0.03	0.92	-385.48
		Year + Day + Homozygosity	788.23	6.90	0.01	0.93	-387.08
		Year + Weight	788.50	7.17	0.01	0.94	-388.23
		Year + Day + CB Ancestry + Homozygosity	788.58	7.25	0.01	0.95	-386.25
		Year + Day + Weight + Sex + CB Ancestry	788.70	7.37	0.01	0.96	-385.29
		Year + Weight + Sex + CB Ancestry + Homozygosity	788.74	7.41	0.01	0.97	-385.31
		Year + Day + Sex + Homozygosity	790.15	8.82	0.01	0.98	-387.03
		Year + Weight + CB Ancestry	790.39	9.06	0.00	0.98	-388.16
		Year + Weight + Sex	790.45	9.12	0.00	0.99	-388.19
		Year + Day + Sex + CB Ancestry + Homozygosity	790.53	9.20	0.00	0.99	-386.21
		Year + Day	792.24	10.91	0.00	1.00	-390.09
		Year + Weight + Sex + CB Ancestry	792.34	11.01	0.00	1.00	-388.13
		Year + Day + CB Ancestry	792.62	11.29	0.00	1.00	-389.28
		Year + Day + Sex	794.16	12.83	0.00	1.00	-390.04
		Year + Day + Sex + CB Ancestry	794.57	13.24	0.00	1.00	-389.24
		Year + Homozygosity	805.38	24.05	0.00	1.00	-396.66
		Year + CB Ancestry + Homozygosity	805.61	24.28	0.00	1.00	-395.77
		Year + Sex + Homozygosity	807.37	26.05	0.00	1.00	-396.65
		Year + Sex + CB Ancestry + Homozygosity	807.62	26.29	0.00	1.00	-395.76
		Year	809.50	28.17	0.00	1.00	-399.73
		Year + CB Ancestry	809.75	28.42	0.00	1.00	-398.85
		Year + Sex + CB Ancestry	811.51	30.18	0.00	1.00	-399.73
		Year + Sex + CB Ancestry	811.76	30.43	0.00	1.00	-398.85
		Intercept Only	957.49	176.16	0.00	1.00	-477.74

Table 1.9. AICc values, ΔAICc, model weights, cumulative model weights, and log-likelihood values at the 95% confidence interval of the top models describing apparent adult survival rate of adult pygmy rabbits (*Brachylagus idahoensis*) after reintroduction into Sagebrush Flat/CRP sits in central Washington (2012-2016). See Figure 1.7 for the top model. Year was not included in the model because the number of released adults each year varied greatly. Day represents the release day based on Julian calendar, and weight represents the weight (g) of an adult at time of release. Columbia Basin ancestry (CB Ancestry) were genetic estimates based on program STRUCTURE. Homozygosity was determined from genotypes of individuals in R-package GENHET. Only models that that performed better than the intercept only (due to random factors) were included.

Model	n	Variables	AIC	Δ AIC	wi	∑wi	Log-Likelihood
Adult Survival -	177	Day	144.36	0.00	0.20	0.20	-70.15
Sagebrush Flat/CRP		Day + Homozygosity	144.73	0.37	0.17	0.37	-69.30
		Day + CB Ancestry	145.83	1.47	0.10	0.46	-69.84
		Day + CB Ancestry + Homozygosity	145.84	1.48	0.10	0.56	-68.81
		Day + Sex	146.37	2.01	0.07	0.63	-70.12
		Day + Weight	146.41	2.05	0.07	0.70	-70.13
		Day + Sex + Homozygosity	146.66	2.29	0.06	0.77	-69.21
		Day + Weight + Homozygosity	146.75	2.39	0.06	0.83	-69.26
		Day + Sex + CB Ancestry + Homozygosity	147.91	3.55	0.03	0.86	-68.78
		Day + Sex + CB Ancestry	147.91	3.55	0.03	0.89	-69.84
		Day + Weight + CB Ancestry	147.92	3.56	0.03	0.93	-69.84
		Day + Weight + Sex	148.46	4.10	0.03	0.95	-70.12
		Day + Weight + Sex + Homozygosity	148.76	4.40	0.02	0.98	-69.21
		Day + Weight+ Sex + CB Ancestry	150.03	5.67	0.01	0.99	-69.84
		Day + Weight + Sex + CB Ancestry + Homozygosity	150.05	5.69	0.01	1.00	-68.78
		Homozygosity	158.98	14.62	0.00	1.00	-77.45
		Intercept Only	159.90	15.54	0.00	1.00	-78.94

Table 1.10. AICc values, ΔAICc, model weights, cumulative model weights, and log-likelihood values at the 95% confidence interval of the top model describing apparent juvenile survival rate of pygmy rabbits (*Brachylagus idahoensis*) after reintroduction into temporary release pens at Chester Butte and Beezley Hills recovery areas in central Washington (2018-2019). All combination of release year, release day, release weight, Columbia Basin ancestry, sex, and homozygosity were examined but only release day slightly outperformed the intercept only model (random factors).

Model	n	Variables	AIC	Δ AIC	wi	∑wi	Log-Likelihood
Juvenile Apparent Survival Release Pens	62	Release Day	84.19	0.00	0.16	0.16	-40.00
		Intercept Only	84.83	0.63	0.11	0.27	-41.38

Table 1.11. Model averaged parameter estimates for each of the parameters describing apparent survival in juvenile and adult released pygmy rabbits into the Sagebrush Flat/CRP recovery area (2012-2016) and the apparent survival rates in juvenile rabbits released into pens at the Chester Butte and Beezley Hills recovery areas (2018-2019). Parameter estimates were averaged across all of the candidate models which was generated by adding weight to the top model according to AICc values. Parameters that overlap zero do not fall into the 95% confidence interval. HL represents homozygosity per locus, an estimate of the genetic diversity and Columbia Basin ancestry (CB) represents the proportion of ancestry.

Variable	Juvenile Estimate (SBF)	95% CI		Adult Estimate (SBF)	95% CI		Juvenile Estimate (Release Pens)	95% CI	
		Lower	Upper		Lower	Upper		Lower	Upper
Release Day	0.010	0.005	0.014	0.017	0.007	0.027	0.020	-0.005	0.046
Release Weight	0.003	0.001	0.005	0.000	-0.005	0.005	0.000	-0.007	0.007
Sex (female)	0.056	-0.318	0.430	0.109	-0.768	0.986	0.282	-0.786	1.350
HL	-1.905	-3.465	-0.346	-2.442	-6.065	1.180	1.537	-3.588	6.661
CB	0.007	-0.004	0.018	-0.012	-0.039	0.016	-0.038	-0.184	0.109
Year 2012	2.227	1.717	2.737	NA	-	-	NA	-	-
Year 2013	0.544	0.066	1.021	NA	-	-	NA	-	-
Year 2015	-3.982	-5.964	-2.000	NA	-	-	NA	-	-
Year 2016	-0.586	-1.638	0.465	NA	-	-	NA	-	-

Chapter 2: Range wide genomic analysis of pygmy rabbits reveals genetic distinctiveness of the endangered Columbia Basin pygmy rabbit (*Brachylagus idahoensis*).

Abstract

Loss and fragmentation of habitat has led to the near extirpation of the isolated pygmy rabbit (*Brachylagus idahoensis*) population in the Columbia Basin of Washington, USA. In 2003, the Columbia Basin pygmy rabbit (hereafter known as the Washington population) was listed as an endangered distinct population segment under the US Endangered Species Act. In 2001, 16 rabbits were taken from the last remaining population in Washington to start a captive breeding program, and four rabbits from Idaho, USA, were added to counteract the effects of inbreeding. Rabbits were moved to semi-wild breeding enclosures in 2011, and additional rabbits were translocated from other populations within the western United States. Since then, ~2000 admixed rabbits have been released into the wild. We used a restriction site-associated DNA sequencing (RADseq) approach on 232 rabbit samples to identify single nucleotide polymorphisms (SNPs) and determine population genetic structure across the pygmy rabbit range, assess the distinctiveness of the Washington population, and test for genomic signatures of adaptive divergence among populations. Using 12,084 SNPs, model-based and non-model-based analyses identified four distinct genetic groups: (1) Washington, (2) Great Basin (California, Nevada, Idaho, Montana), (3) northern Utah/Wyoming and (4) southern Utah, and the Washington group was the first to separate from the other groups at K=2. Moderate to significant levels of genetic structure exist between each of the populations (F_{ST}=0.04-0.27, θ_{ST}=0.09-0.36), with the greatest occurring between Washington and the other regions. We identified signatures of adaptive differentiation among populations, most of which were associated with cellular processes but 8.2% of the SNPs were associated with metabolic processes. Identifying genetic markers for ancestry from the multiple pygmy rabbit populations will help monitor variation in the admixed Washington population and assess the consequences of genetic rescue efforts.

Introduction

Landscape structure can affect population connectivity and population size, and in turn affect the distribution of genetic diversity among populations (Manel et al. 2003; Storfer et al. 2007).

Landscape composition and structure can also affect the local abundance and demography of populations (Brown 1984), which can influence gene flow and demography of populations, and their ability to persist and adapt to environmental change (Slatkin 1987). Notable differences in genetic and demographic factors can occur between populations that inhabit the more stable, well-connected, optimal habitat of the interior regions of a species' distribution. Populations near the range margin that are often patchily distributed, are subjected to greater isolation, more limited resources, and greater habitat and environmental variability (Brown 1984; Brussard 1984; Eckert et al. 2008). Understanding the patterns and processes associated with geographical variation in population genetic structure across species' ranges also provides important information for conservation and management. Peripheral populations are often rare representatives of relatively widespread species, often leading to extirpation or conservation concern for the peripheral population (Bunnell et al. 2011). Washington pygmy rabbits (*Brachylagus idahoensis*) are an example of a peripheral population that is the focus of intense conservation efforts.

Pygmy rabbits are the smallest rabbit in the world and a sagebrush (*Artemesia tridentata*) obligate species. The historical distribution of pygmy rabbits was highly patchy across the sagebrush steppe habitat of the western United States, with populations in Washington, Idaho, Oregon, Utah, Nevada, Wyoming, Montana, and California (Hall and Kelson 1959; Green and Flinders 1980; Campbell et al. 1982). Sagebrush steppe ecosystems in the United States currently occur on less than half of their historical distribution due mostly to changes in land use, degradation of habitat from invasive species, and urban development (Pyke et al. 2018). Although the geographic distribution of the pygmy rabbit includes most of the Great Basin (Figure 2.1), its specialized habitat requirements (e.g., requirement for deep soil for burrowing) restrict it to a only a small fraction of sites within the geographic range (Smith et al. 2019). Loss and fragmentation of sagebrush is considered the main contributor to pygmy rabbit population declines throughout their range (Weiss and Verts 1984; Siegel Thines et al. 2004; Larrucea and Brussard 2008). As a result, the entire species has been proposed for federal endangered status, but the US Fish and Wildlife Service concluded a range-wide listing was not warranted (U.S. Fish and Wildlife Service 2010).

The Columbia Basin pygmy rabbit (hereafter known as the Washington population) was listed under the Endangered Species Act as a distinct population segment (U.S. Fish and Wildlife Service 2003; Chambers and Wisdom 2009; Meinke et al. 2009). Genetic studies of pygmy rabbits have been conducted using nuclear DNA microsatellite loci for the endangered Washington population (Warheit 2001; DeMay et al. 2016, 2017), and populations in Idaho (Estes-Zumpf et al. 2010), Wyoming (Thimmayya and Buskirk 2012), Oregon (Warheit 2001), and Nevada/California (Larrucea et al.

2018). DeMay et al. (2016) identified four distinct genetic groups when assessing rabbits from different regional populations; (1) Washington /Idaho, (2) Nevada/Oregon, (3) northern Utah/Wyoming, and (4) southern Utah; although they had no pure Washington samples to analyze. Warheit (2001) suggested that the Washington population may have been isolated from the Idaho and Montana populations for nearly 40,000 years, based on mitochondrial DNA *cytochrome b* sequence data.

Because of loss and fragmentation of sagebrush steppe habitat, the Washington pygmy rabbits were restricted to a single population by 2001 (U.S. Fish and Wildlife Service 2003). The last 16 Washington pygmy rabbits were removed from the wild in 2001 to save this subpopulation and placed into a captive breeding program. To counteract the effects of low reproductive rates and assumed inbreeding depression, 4 Idaho pygmy rabbits were augmented into the captive population in 2003 (Elias et al. 2013). By 2006, the last remaining 100% Washington pygmy rabbit had died and all rabbits in the program were now admixed (Washington /Idaho). With the goal of re-establishing rabbits back to the state of Washington, the captive breeding program transitioned to on-site breeding enclosures in central Washington in 2011, and 111 additional pygmy rabbits from other regions (Oregon, Nevada, Wyoming, and Utah) were trapped and translocated to the breeding program. Pygmy rabbits containing higher levels of Washington ancestry were selected as breeders, but most of the juveniles produced were admixed (Washington, Idaho, Nevada, Oregon, Wyoming, and/or Utah; USFW 2012; DeMay et al. 2016, 2017). Since 2012, nearly 2000 admixed pygmy rabbits have been released back to the state of Washington (Gallie and Zinke 2019).

With the continued loss and fragmentation of the sagebrush habitat (Knick and Rotenberry 1997), assessing connectivity among pygmy rabbit populations is important for future conservation planning (Estes-Zumpf et al. 2010; Smith et al. 2019). Additionally, to improve genetic monitoring and management efforts within our admixed, reintroduced populations within Washington, requires understanding the genetic diversity within the regional pygmy rabbits' populations across the western United States that acted as founders. Here, we use a reduced representation sequencing approach to identify single nucleotide polymorphism loci (SNPs) and (1) determine population genetic structure across the pygmy rabbit range, (2) assess the distinctiveness of the Washington population, and (3) test for genomic signatures of adaptive divergence among populations. We predicted that, consistent with previous microsatellite results, a genome-wide set of SNPs would show the Washington population to be a highly divergent. In addition, we expected the Washington population to be the most genetically differentiated and have the highest pairwise F_{ST} values to other populations, whereas the Idaho rabbits would be more similar to regions within the Great Basin, separating the Washington/Idaho ancestry

group identified in DeMay et al. (2016). We expected low to moderate levels of genetic structure to be detected between the other identified populations. Ancestrally, the Great Basin region allowed for connectivity but over the past century, the shrub-steppe habitat has been reduced and degraded by wildfire, urban development, livestock grazing practices, and under-informed management practice (Wisdom et al. 2005). As the loss of habitat and continued fragmentation occurred across the sagebrush steppe biome, gene flow between populations has been greatly reduced increasing genetic structure (Larrucea et al. 2018). This restriction of gene flow and resulting genetic drift should produce unique alleles specific to each region and differences in local selection pressures might have generated detectable locally adaptive differences across the range.

Materials and Methods

Tissue Samples, DNA Extraction, and RADSeq Library Preparation

Researchers and mangers collected 232 tissue samples by either (1) 2mm ear biopsies from pygmy rabbits across the region (Figure 2.1) from 2001-2013 (n=209), or (2) necropsy organ tissue samples (n=23) collected from Oregon Zoo for individuals that were pure Washington ancestry (either individuals taken from the wild or their offspring that were 100% Washington). We included all 111 pygmy rabbits that were translocated into the on-site breeding enclosures among the samples, as were tissue samples from the last 16 Washington pygmy rabbits taken from the wild in 2001. Samples were stored in ethanol and kept at -20°C until a DNA extraction was performed. Genomic DNA was extracted using Qiagen Blood and Tissue extraction kit (QIAGEN, Valencia, CA) following the manufactures' recommended protocol or through phenol-chloroform extractions. We evaluated quality control of each sample using Qubit Fluorometer and a 1.0% agarose gel electrophoresis. RAD sequencing library preparation was performed according to the methods describe in Ali et al. (2016) without the sequence capture steps. Libraries were sequenced using Illumnia HiSeq 4000 and Illumnia NovaSeq 6000 with 150 pair-end reads at the Vincent J. Coates Genomics Sequencing Laboratory at University of California, Berkeley.

RAD Sequence Filtering and Genotyping

Based on the Ali et al. (2016) method, the barcode and partial restriction site can occur on either the forward or reverse Illumina sequence reads. We used a custom perl script to re-orient the raw sequence reads so that all reads starting at the restriction cut site were in one file, while the other reads were contained in a second file. Using STACKS version 2.54 (Catchen et al. 2011, 2013; Rochette et al. 2019) PROCESS_RADTAGS function, reads were demultiplexed by barcode and removed reads with uncalled bases or poor sequence quality based on Phred scores less than 10 (90%

probability of being correct). PCR duplicates were removed using the *CLONE_FILTER* function in STACKS version 2.54.

To minimize missing data and optimize alignments, samples containing fewer than 200,000 reads were removed and all remaining sequences reads were aligned to the European rabbit genome (*Oryctolagus cuniculus*, OryCun 2.0) using BOWTIE2 v.2.2.9 (Langmead and Salzberg 2012) with the following parameters: -very-sensitive, -end-to-end, -X 900. The resulting alignments were converted from sequence alignment/map format (SAM) to binary alignment map (BAM) format using SAMTOOLS (Li et al. 2009). The function *REF_MAP* in STACKS version 2.54 was used to identify SNPs for each individual sample from the reference aligned sequence reads using a maximum likelihood approach. We used *REF_MAP* function in STACKS version 2.54 to identify SNPs for each individual; requiring a minimum of three identical reads to create a stack and used an upper bound for the sequence error rate at 0.01. To optimize parameters for calling genotypes, we used six samples in which replicates were run independently during library prep and sequencing. We assessed the mismatch rate among the replicate pairs, using a modified version of a R script described in Mastretta-Yanes et al. (2015). We estimated genotype mismatch rates between the replicate pairs across 25 parameter sets, varying the minimum depth of coverage from five to nine and varying the minimum percent of individuals genotyped at a locus from 50% to 90%. The mismatch rate for each replicate pair was calculated as the number of loci for which the genotypes were different between replicates, divided by the total number of loci typed for both replicates. Data for replicate samples were then merged for inclusion in downstream analysis.

To identify SNPs among our sample we used the optimized parameters values that generated the lowest genotype mismatch rates (minimum depth of coverage = 5, and minimum percent of individuals genotyped at a locus = 70%) in *POPULATIONS* function of STACKS. RAD loci had to be present in 70% of the individuals to be kept (-r 0.7), biallelic SNP's minor allele frequency was set to 0.05 (--min-maf 0.05), and one SNP per locus to limit the number of linked loci retained (--write_single_snp). This dataset was used to estimate genetic structure among the regions. Since our Washington samples had high levels of inbreeding, we filtered the data set to include (1) all individuals – no filter, (2) a relatedness cutoff value of 0.33, and (3) a relatedness cutoff value of 0.4. To determine relatedness, we used the –relatedness option in VCFTOOLS v.0.1.16 (Danecek et al. 2011) which produced unbiased ajk values. Cutoffs for relatedness values were assessed at the 0.33 and 0.4 cutoff, including any known half-sibling and full-sibling pairs. Values just under 0.33 were observed in some individuals that were in different populations, possibly resulting from ancestral admixture; thus we set a cutoff at 0.33. After comparing the three different datasets, we found no

differences in the results and chose to use the full set of individuals with no filters. The final dataset was then further filtered to remove any individuals with more than 50% missing data, in VCFTOOLS v.0.1.16, to provide us with a list of SNPs and samples to be used for further downstream analysis.

Genetic Structure

To assess the number of distinct genetic groups across the pygmy rabbit range, the --write_single snp and –ordered_export option in the POPULATIONS function were used to minimize linkage disequilibrium among the identified loci. We used the same criteria described above in VCFTOOLS v.0.1.16, to create a list of SNP loci of interest, and a population map containing the samples that had met our filtering criteria. We ran 10 repetitions of STRUCTURE for *K*= 1-10, with a burn-in of 100,000 iterations (BURNIN = 100 000) and MCMC length of 500,000 iterations (NUMREPS = 500 000). These runs used the admixture model, correlated allele frequencies among populations, and did not assume prior population information. All other parameters were left at their default values. We used mean log likelihood values (Pritchard et al. 2000) and the ΔK statistic (Evanno et al. 2005) in STRUCTURE HARVESTER (Earl and vonHoldt 2012). STRUCTURE results were visualized in the Rstudio package *Pophelper v2.3.0* (Francis 2017*)*. We used the –plink command in *POPULATIONS* to provide the files needed to run program ADMIXTURE v.1.3.0 (Alexander and Lange 2011). ADMIXTURE estimates individuals' ancestries by computing the maximum likelihood estimates in a parametric model. The cross-validation (CV) values can be used to assess the number of distinct genetic groups (lowest CV value represents *K*). We ran ADMIXTURE for *K*= 1-10, with the –cv parameter set to 10 and examined the CV values to determine the optimal *K*. ADMIXTURE plots were visualized in R. A principal component analysis (PCA) was used to independently evaluate the SNP genotypes used in STRUCTURE and ADMIXTURE analyses. PCAs were performed in the R package *pcadapt* (Luu et al. 2017).

We quantified the levels of genetic structure occurring between the genetic groups (*K* = 4) identified in PCA and STRUCTURE analysis using the *POPULATIONS* function in STACKS version 2.54. We used the –smooth parameter which enables kernel smoothed π, F_{IS}, F_{ST}, and ϕ_{ST} values. The number of private alleles for each genetic group was determined in the *POPULATIONS* function as well as a genetic diversity summary. To further examine the genetic structure in the largest group (GB), we ran POPULATIONS with the GB samples (n=75), with the criteria listed above (--write-single-snp, --ordered-export, --min-maf 0.05, and -r 0.7). Loci were examined in VCFTOOLS to ensure no more than 30% missing data per SNP occurred, and no samples had more than 50% missing data. Genotypes were run in STRUCTURE following the protocols described above.

Adaptive Loci

To evaluate potential locally adaptive loci, we ran POPULATIONS program without the –write-single-snp option, allowing for multiple SNPs per locus. The data set was then filtered in PLINK to remove any individuals with more than 50% missing data. We used two different programs to assess selection signatures on our SNP dataset. The first was the R package *pcadapt* which uses PCA and Mahalanobis distance to provide a better ranking of candidate SNPs under local selection pressures. Outlier loci were determined by running the *pcadapt* program where $K = 4$ as determined above. The p-values for each SNP were then converted in the R program *qvalues* (Storey et al. 2021). For a given α (real valued number between 0 and 1), SNPs with q-values less than α were considered as outliers with an expected false discovery rate bounded by α. For our analyses, α was set to 0.05 (5% false discovery rate) and we used the Benjamini-Hochberg correction option for SNP outlier discovery. Manhattan plots of q-values for each SNP were visualized in *pcadapt*.

The second program, BAYESCAN (Foll and Gaggiotti 2008), detects selection signatures using the F_{ST}-outlier approach. This approach identifies loci under selection because they show F_{ST} coefficients that are significantly more different than those expected under neutrality and a given demographic model. Standard PLINK files were converted to BAYESCAN format with PGDSpider v.2.0.7.3 (Lischer and Excoffier 2012). The analyses consisted of 20 pilot runs of 5,000 iterations, a burn-in of 50,000 iterations, a thinning interval of 10 (5,000 iterations were used for the estimation of posterior odds) resulting in a total number of 100,000 iterations, and a prior odds ratio of 10 (prior belief that a selection model us 1/10 as likely as the neutral model for a given SNP). For the loci identified in BAYESCAN, SNP positions (chromosome and bp locations) were compared to the annotated European rabbit genome in NCBI's genome data viewer, to determine if the SNP was positioned in a known gene. We then created a list of genes containing SNPs, and imported this into *panther* (Mi et al. 2017) to obtain gene ontology classifications for each gene based on which biological processes they are associated with.

Results

RADSeq Data Characterization

We obtained a total of 594,124,719 read pairs, each 150bp, across 232 individuals, representing the regional pygmy rabbit populations. Ninety-three samples were removed from the datasets because they contained fewer than 200,000 reads (Figure 2.1). Average alignment to the European rabbit genome was 62%. The catalogue created in Stacks2.54 contained a total of 132,932 loci. Further filtering in the POPULATIONS program of STACKS version 2.54 (single SNP per loci option, MAF >0.05, and the presence in at least 70% of all samples) removed 102,818 loci. From this

set of loci, 12,084 SNPs (variable sites) were identified, where missing data per individual ranged from 2.2% - 49.9% ($\bar{X} = 17.8\%$) and missing data per SNP ranged from 0.8% to 30.0% ($\bar{X} = 17.8\%$).

Genetic Structure

To assess the number of distinct genetic groups, we used the filtered, single population dataset, that contained 123 individuals (Figure 2.1), with STRUCTURE, ADMIXTURE and PCA analyses to determine the number of distinct genetic groups across the pygmy rabbit range. The cross-validation (CV) values from ADMIXTURE maximized at K=4 clusters for data (Figure 2.2a) as did ΔK per the Evanno method (Evanno et al. 2005) used on the STRUCTURE results (Figure 2.2b). At K=2, the Washington population was shown as a distinct group, and all other samples represented the 2nd group identified (Figure 2.3). At K=3, the northern Utah and Wyoming samples were identified as the third group (Figure 2.3). At K=4, the optimal model identified four distinct genetic groups: (1) Washington, (2) southern Utah, (3) northern Utah and Wyoming, and (4) Great Basin (includes Nevada, Oregon, Idaho, Montana, and California). Higher levels of admixture were observed in samples found in Nevada, Oregon, California, and southern Idaho (IDS-south of the Snake River) (Figure 2.4) compared to north of the Snake River (IDN), representing southeast Idaho and Montana. Each of the other identified regions showed little to no levels of admixture with the other groups. By K=5, Nevada, Oregon, and California started to break off as a distinct genetic group from Montana and the Idaho populations north of the Snake River, whereas those populations in Idaho, south of the Snake River, showed admixture between the two groups. At K=6, the new identified group is exhibited as admixture across most of the region. At K=7, samples within Idaho that were north of the Snake River but also were west of the Salmon River, separated out as a distinct genetic group, and continues with this grouping into K=8. Additionally, at K=8, the California population begins to separate from the rest of the groups, but still shows levels of admixture. The results of ADMIXTURE were congruent with STRUCTURE's results and are not presented here.

Analysis of genetic structure in the Great Basin samples used 75 samples genotyped at 12,284 SNPs. ΔK per the Evanno method (Evanno et al. 2005), indicated that K=2, among the Great Basin population. STRUCTURE results indicated that at K=2 (Figure 2.6), samples from IDN and MT form a single distinct genetic group, and samples from California, Nevada, and Oregon form a second distinct genetic group. Samples from north of the Snake River in Idaho and Montana showed higher levels of admixture between the two groups. At K=3, samples within Idaho that were north of the Snake River but also were west of the Salmon River, separated out as a distinct genetic group, and continues with this grouping into K=5. These results are similar to what was observed across pygmy rabbit range at K=7. Samples from the Idaho near Idaho National Laboratory begin to identify as a

distinct group at K=5 and continues through K=9. At K= 8, the California population separates from the remaining groups and continues into K=9, but still shows high levels of admixture with the Nevada and Oregon samples (Figure 2.6).

PCA (Figure 2.5) also supports four distinct genetic groups, and the results are congruent with the STRUCTURE assignments. PCA axes 1-2 highlight the distinctiveness of Washington individuals from all other regions since they break out on axis 1 which explains the greatest percentage of variation in the dataset (25%). The other 3 genetic groups become distinguishable on PCA axes 2-4. The south-east Idaho and Montana samples split from the southern Idaho and Nevada, Oregon, and California samples on PCA axes 3-4.

Pairwise F_{ST} values varied between 0.04 ($F_{ST-GB-UTS}$) and 0.27 ($F_{ST-WA-UTS}$) (Table 2.2a). Pairwise F_{ST} values reflected the greatest values of divergence between the Washington group and the southern Utah group, which was also reflected in the absolute divergence (D_{xy}) values (Table 2.2a). Divergence in haplotype values, θ_{ST}, for identified groups ranged from 0.09 ($\theta_{ST-GB-UTN}$) and 0.36 ($\theta_{ST-WA-UTS}$) (Table 2.2b).

Genetic Diversity and Adaptive Loci

We found moderate differences in genetic diversity of the four genetic groups identified (Table 2.2). Examining polymorphic loci only, nucleotide diversity (π) was as follows $\pi_{Washington} = 0.12$, $\pi_{Great\ Basin} = 0.20$, $\pi_{Northen\ Utah-Wyoming} = 0.18$, and $\pi_{Southern\ Utah} = 0.17$. Private alleles for each of the identified groups ranged from 9 (southern Utah) to 812 (Great Basin). The Great Basin group had greater levels of admixture and a larger sample size than the other identified groups possibly resulting in an increased number of private alleles compared to other groups. BAYESCAN and *pcadapt* identified outlier loci among the populations suggesting that the some of the loci identified in this study are under selective pressures in each of the regional groups (Figure 2.7). BAYESCAN identified 19 SNPs and *pcadapt* identified 516 SNPs with the more conservative, Bonferroni correction method, where nine of the SNPs identified in BAYESCAN overlapped with *pcadapt*. Five of the nine SNPs that overlapped *pcadapt* fell in regions of known genes (Table 2.3).

For the 19 SNPs identified in BAYESCAN, 14 (73.7%) were identified in genic regions of the annotated European rabbit genome; whereas 10 (52.6%) were identified in introns, 2 (10.5%) were identified in exons, and 2 (10.5%) were known to be in a gene but were could not be identified as intron or exon. Within the SNPs that were identified in introns, two SNPs were identified in intron regions overlapping two different genes. For the remaining 5 SNPs that were not identified in genic regions, one SNP (pyra_1709) was greater than 100,000bp and pyra_9805 was 65,000bp from the

nearest known genic region; the three other SNPs were less than 8,000bp away (Table 2.3). The SNPs identified were located on 11 of the 22 chromosomes, with no more than 3 SNPs on a single chromosome (10), and two SNPs were located on scaffolds with unknown chromosome placement. Gene ontology revealed that the outlier SNPs were associated with 8 biological processes (1) 33.8% of SNPs were associated with cellular processes, (2) 14.2 % with biological regulation, (3) 11.6% with response to stimuli, (4) 10.88% with localization, (5) multicellular organismal processes, (6) 8.2% with metabolic processes, (7) 7.9% with signaling, and (8) 4.5% with developmental processes (Figure 2.8).

Discussion

Here, we conducted a genome wide survey of the genetic variation in pygmy rabbits using RADSeq, to provide the first dataset of SNPs for this vulnerable species. Using 12,084 SNPs, we identified four distinct genetic groups, and as hypothesized the Washington group was the most distinctive, and highly divergent, separating from the other groups at K=2. We identified 10,172 SNPs (a subset of the 12,084 SNPs), represented in 70% of each of the populations, that demonstrated moderate to significant levels of genetic structure between each of the populations (F_{ST}=0.04-0.27, θ_{ST}=0.09-0.36), with the greatest occurring between Washington and the other regions. This finding supported our hypothesis of low to moderate levels of genetic structure between the other identified genetic groups. Great Basin populations had the most identified private alleles, followed by the Washington population. Potential locally adaptive loci were identified in which gene ontology was attributed to mostly cellular processes (33.8%), but 8.2% of the loci were responsible for metabolic processes. Identifying the distinct genetic groups across the pygmy rabbit range using thousands of SNP loci can help guide future management actions for pygmy rabbits, especially the endangered, reintroduced, admixed population within the Columbia Basin of Washington.

Distinct Groups and Genetic Structure

Our data suggest that four distinct genetic groups occur across the pygmy rabbit range, (1) Washington, (2) Great Basin (California, Nevada, Oregon, Idaho, and Montana), (3) northern Utah and Wyoming, and (4) southern Utah. These results confirm the findings of Warheit (2001) who used nine microsatellite loci and mitochondrial DNA *cytb* sequence data to identify the genetic distinctiveness of the Washington population compared to populations in Idaho, Oregon, and Montana. DeMay et al. (2015, 2017) identified 19 microsatellites that were used to determine the number of genetically distinct groups that represented the founding population for the reintroduced pygmy rabbit populations but were unable to separate the Washington and Idaho ancestry due to the lack of pure (non-admixed) Washington samples. Our expanded sampling and genomic data results

provide further resolution of the distinct genetic groups across the pygmy rabbit range. Although the Evanno method identified K=4 as the optimal grouping, it is important to note that this is just one interpretation of the data. Evidence of K=5, can also be observed in our data, where the Snake River acts as a barrier to gene flow, separating the Great Basin population. The levels of genetic differentiation (F_{ST} and θ_{ST} values) between the Washington population and other groups exhibited similar results to microsatellite and mitochondrial DNA studies (Warheit 2001; DeMay et al. 2015, 2017), reinforcing the distinct population status that warranted the Washington pygmy rabbit its initial protection under the Endangered Species Act in 2003.

The pronounced genetic differentiation of the Washington population is consistent with patterns of genetic divergence observed among diverse taxa in the Pacific Northwest, United States (Miller et al. 2006), where Pleistocene glacial refugia and vicariance are hypothesized to have shaped genetic structure for co-distributed species (Brunsfield et al. 2001; Espíndola et al. 2016). Oh et al. (2019) suggested increasing geographic isolation and restriction of gene flow, accompanied by declines in effective population sizes during the last glacial period led to the divergence in the sagebrush obligates, greater sage grouse (*Centrocercus urophasianus*) and Gunnison sage-grouse (*Centrocercus minimus*). Population differentiation by genetic drift and substantial range contraction during the late Pleistocene has been inferred for many bird species (Halley et al. 2014; Oh et al. 2019), and is consistent with the patterns of genetic structure observed in this study. The Columbia Basin in Washington where pygmy rabbits and other sagebrush obligates reside, occurs in sagebrush steppe habitat that is largely surrounded by mesic coniferous forests that represent significant barriers to gene flow and dispersal (Row et al. 2018), and likely contribute to the elevated degree of distinctiveness observed here and in other sagebrush obligate species (e.g., Miller et al. 2006; Oh et al. 2019).

The levels of genetic structure (F_{ST} and θ_{ST} values) between the Great Basin, southern Utah, and northern Utah and Wyoming pygmy rabbit populations were moderate and consistent with expectations. These three other groups represented a historically more continuous and connected population that only recently have become more isolated from one another due to loss of habitat and fragmentation in the sagebrush steppe ecosystem (Grayson 1987). Others have suggested that pygmy rabbit populations began declining at the end of the Pleistocene, reducing the connectivity of the populations ~10,000 years ago (Grayson 1987). The California population of pygmy rabbits has recently been identified as a genetically distinct population (Larrucea et al. 2018). In our study, the California population did not separate as a distinct genetic group when comparing populations across the pygmy rabbit range. Furthermore, it did not form a distinct genetic group among the Great Basin

samples until K=8, suggesting that cessation of gene flow between the Nevada and California populations is more recent.

Within the Great Basin samples, separation between populations north of the Snake River and Montana samples and the remaining regional populations (Nevada, Oregon, and California) occurred at K=2. South of the Snake River samples exhibited admixture between these two regions, suggesting that environmental factors near the Snake River act as a major barrier to gene flow and dispersal. Estes-Zumpf and Rachlow (2009) demonstrated how landscape features such as rivers, creeks and roads can act as filter or barriers to dispersal. They documented pygmy rabbits crossing the perennial streams, but the occurrences were rare. Additionally, in southern Idaho, one large interstate highway crosses most of the southern end possibly limiting dispersal and gene flow between populations. Interstate 80, in Wyoming, was suggested to be a semi-permeable barrier to gene flow among the Wyoming populations of pygmy rabbits (Thimmayya and Buskirk 2012) but is too recent to be reflected in our data. Possible evidence of rivers acting as a semi-permeable barrier to dispersal and gene flow can be seen at K=4 within the GB samples. Samples from populations north of the Snake River and west of the Salmon River, are the only samples to show admixture of the 4th group identified.

The current distribution of pygmy rabbits is highly patchy within their geographic range. Based on the spatial distribution of the four genetic groups, we hypothesize that Pleistocene lakes (Missoula, Bonneville, and Lahontan), were barriers to gene flow and dispersal, producing the distinct genetic groups identified in this study. Pleistocene lakes and glaciers covered most of the Pacific Northwest and Great Basin regions (Figure 2.9), limiting dispersal to plants and animals (Mehringer Jr. 1996; Smith et al. 2019). The climatic fluctuations of glacial lakes would rapidly and intensely alter the Pleistocene landscape through plant colonization, ultimately affecting animal distribution. Lake floors, such as glacial lake Missoula, were rapidly colonized by grasses and sagebrush, whereas pluvial lake Bonneville remained sparsely vegetated (Mehringer Jr. 1996). Gaps in habitat in northern to central Utah and western Nevada coincide with Pleistocene lakes Bonneville and Lahontan (Smith et al. 2019). The presence of Pleistocene lake Bonneville, but also the draining, would result in the persistent lack of vegetation, limiting dispersal of pygmy rabbits across Utah. This could result in the distinct genetic northern and southern Utah groups observed in this study. Further investigation using a landscape genomic approach could provide additional insight into how Pleistocene lakes influenced divergence and distribution of pygmy rabbits and other sagebrush obligate species across the western United States.

Genetic Diversity and Adaptive Loci

The Washington population of pygmy rabbits showed the lowest levels of diversity and was significantly less diverse than the Great Basin, Utah, and Wyoming populations, although the Washington population had the second highest value for private alleles. This finding is consistent with our hypotheses that the Washington population is highly divergent from other pygmy rabbit populations. The Washington population was a single, small population by 2001, and exhibited high levels of inbreeding (U.S. Fish and Wildlife Service 2003, 2012). The reductions in nucleotide diversity observed in Washington were similar to results from previous genetic studies (Warheit 2001) and likely reflect long-term geographic isolation and small population size. Reduction in nucleotide diversity was also identified in the Washington population of greater sage grouse, and was attributed to the same factors (Oh et al. 2019). The samples from Washington were collected from pygmy rabbits within the captive breeding program, which showed decreased pregnancy success, juvenile growth, and juvenile survival with increased Washington ancestry within one or more of the parents, suggesting inbreeding depression among the captive population (Elias et al. 2013). The genetic rescue from individuals within the Idaho region provided the breeding program with the increased genetic diversity and increasing pregnancy rates, juvenile growth, and juvenile survival were documented (Elias et al. 2013).

The Great Basin populations exhibited the highest levels of genetic diversity, nearly double the Washington population, which is congruent with other studies done in Nevada and Idaho (Estes-Zumpf et al. 2010; Larrucea et al. 2018). These two regions are more centrally located to the overall distribution of pygmy rabbits, which generally are less isolated and typically experience higher rates of gene flow (Lewontin 1974). The large number of private alleles within the Great Basin population reflects the overall diversity within the region. The Utah and Wyoming populations exhibited slightly lower diversity estimates compared to the Great Basin population but more than the Washington. These populations are more isolated, especially the Washington and southern Utah populations, and are located on the peripheral of pygmy rabbit distribution.

Isolated populations have an increased tendency to lose genetic variation, which increases the risk of extinction due to a reduced ability to adapt to environmental change (Lacy 1997; Willi et al. 2006; Jump et al. 2009). Compared to populations near the core, populations at the edges of geographic ranges may experience reductions in effective population size (Vucetich and Waite 2003) and genetic diversity creating increased genetic differentiation (Eckert et al. 2008). Like in our genomic study, microsatellite studies showed reductions in genetic diversity with increasing distance from the Great Basin core of the geographic range for Wyoming and Idaho populations (Estes-Zumpf

et al. 2008; Thimmayya and Buskirk 2012). The greatest diversity among our samples was found within samples from the south of the Snake River region, at the core of the Great Basin range with decreasing diversity near the margins of the geographic range suggesting more recent expansions, on an evolutionary time scale, into marginal regions such as Wyoming and Northern Utah compared to the Great Basin core.

Lack of connectivity favors local adaptations by reducing the homogenizing effects of gene flow (García-Ramos and Kirkpatrick 1997). Our analysis showed a lack of connectivity among the four genetic groups across the pygmy rabbit range and identified potential locally adapted loci. Oh et al. (2019) found locally adaptive loci in sage-grouse. Pygmy rabbits are similar in that they consume seasonal diets consisting almost exclusively of toxic sagebrush leaves, that contained genes responsible for dietary adaptation and detoxification of plant secondary metabolites, suggesting a potential genetic basis to local adaptation to different sagebrush varieties. Their results raise the possibility of distinct ecotypes specialized to each region's local sagebrush variety. These dietary adaptations might also apply to pygmy rabbits, further restricting their dispersal across the geographic range. Further investigation into the locally adaptive loci and their gene ontology should be conducted to determine if pygmy rabbits share similar dietary adaptations.

Conservation Implications

Identifying distinct genetic groups across the pygmy rabbit range using thousands of SNP loci can help guide future management actions for pygmy rabbits, especially the endangered, reintroduced, admixed population within Washington. SNPs that we identified could be used to design a GT-Seq SNP panel (Campbell et al. 2015) to assess individual identity, ancestry, parentage, and adaptive loci. The private alleles and ancestry informative markers identified for each region can help guide management strategies for additional augmentation into the Washington population. Because the Great Basin population had the greatest amount of genetic diversity and the lowest divergence values to Washington, this information already has been used in March 2020 to prioritize trapping sites for augmentation as part of demographic rescue. Additionally, the admixed population in Washington has exhibited a decline in the northern Utah/Wyoming ancestries and almost complete loss of the southern Utah ancestry (Gallie and Zinke 2019), while maintaining Washington ancestry in the wild populations. These changes suggest that selective pressures might be favoring the adaptive potential from local Washington rabbits in the region, as has been seen in other Washington sagebrush obligates (Oh et al. 2019). As for the other distinct genetic groups identified in this study, further investigation with SNPs and population surveys needs to be performed in the northern Utah/Wyoming region,

especially the southern Utah range, to better understand the fine-scale genetic structure occurring within these regions.

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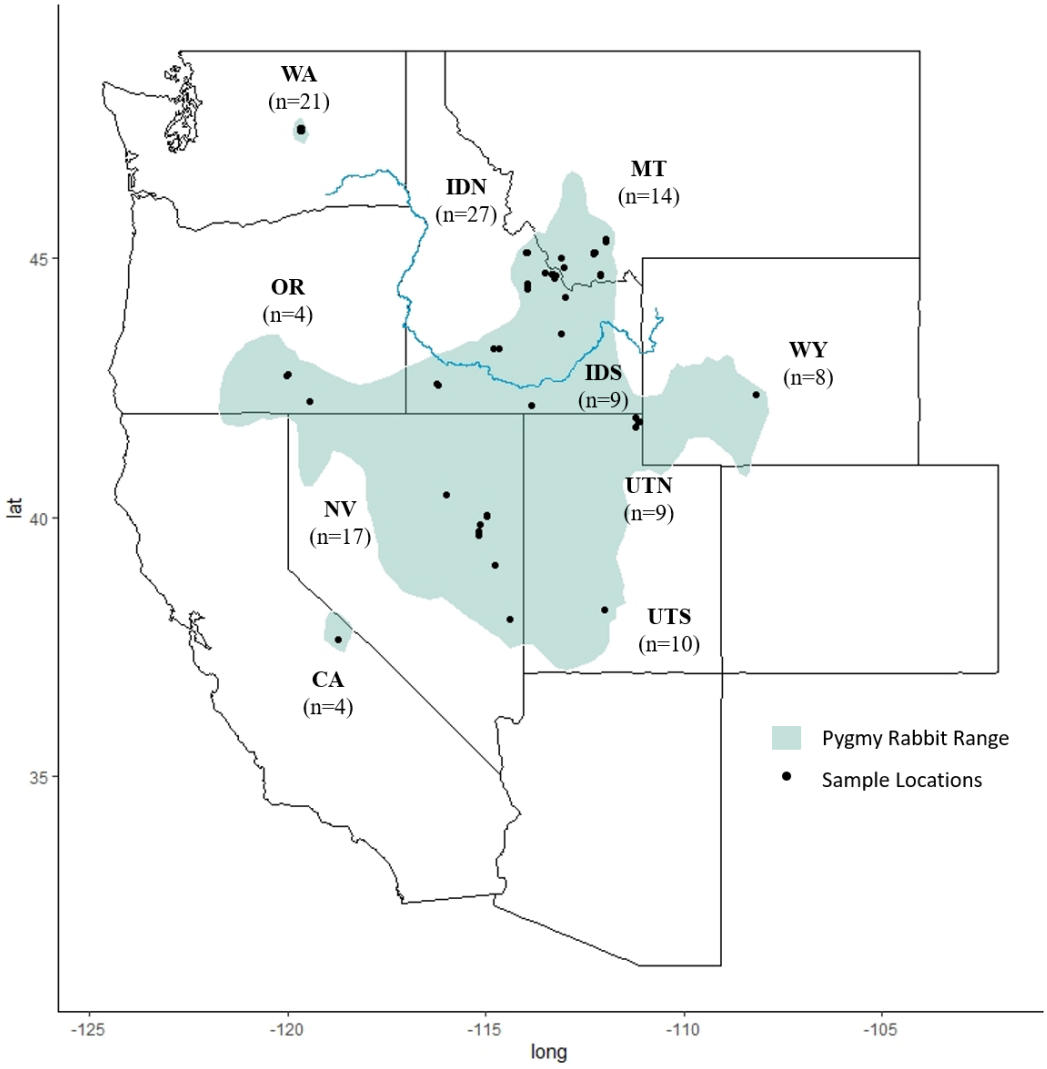

Figure 2.1 . Pygmy rabbit (*Brachylagus idahoensis*) range outline (Rachlow et al. 2016; green) and sample locations for 123 pygmy rabbit samples that passed all filtering protocols from each of the regional populations. Samples were collected during 2001-2018. Blue line represents the Snake River. Total sample sizes are represented for each region (pre-filtering sample size: post-filtering sample size). California (CA, n=10:4), Washington (WA, n=34:21), Nevada (NV, n=56:17), Oregon (OR, n=6:4), Idaho north of the Snake River (IDN, n=45:27), Idaho south of the Snake River (IDS, n=13:9), northern Utah (UTN, n=11:9), southern Utah (UTS, n=12:10), Wyoming (WY, n=30:8), and Montana (MT, n=15:14).

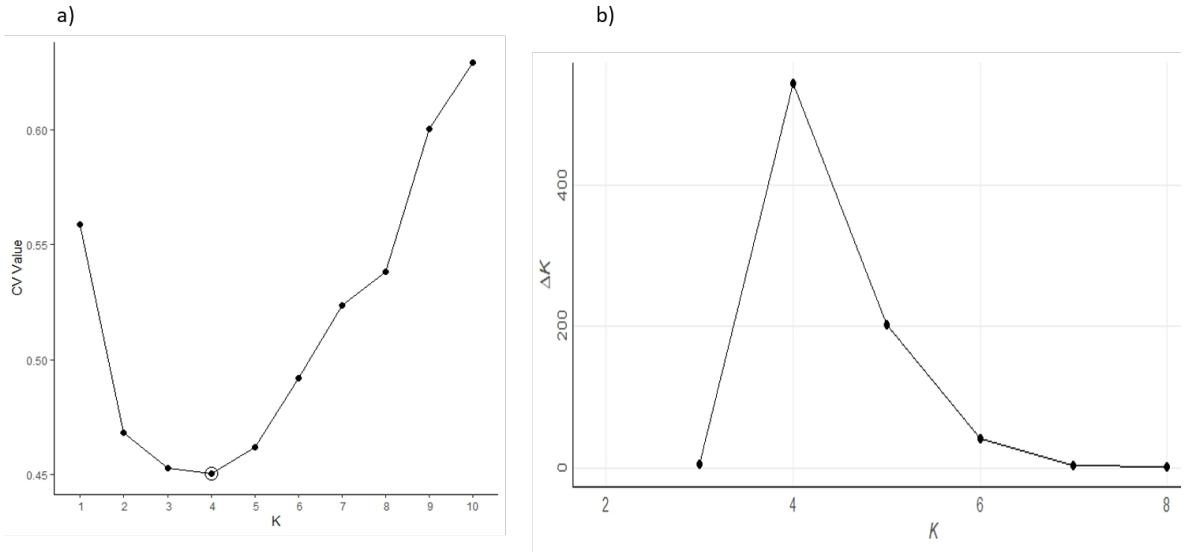

Figure 2.2. (a) Cross-validation (CV) values for genetic clustering of 123 pygmy rabbit (*Brachylagus idahoensis*) samples from across the pygmy rabbit range (sampled during 2001-2018) genotyped at 12084 SNPs using the program ADMIXTURE. The circled point represents the lowest CV value and the optimal *K* value for the most supported number of genetic groups. (b) Evanno's method (Evanno et al. 2005) showing the number of K groups that best represent the population structure among the same samples and SNPs using program STRUCTURE.

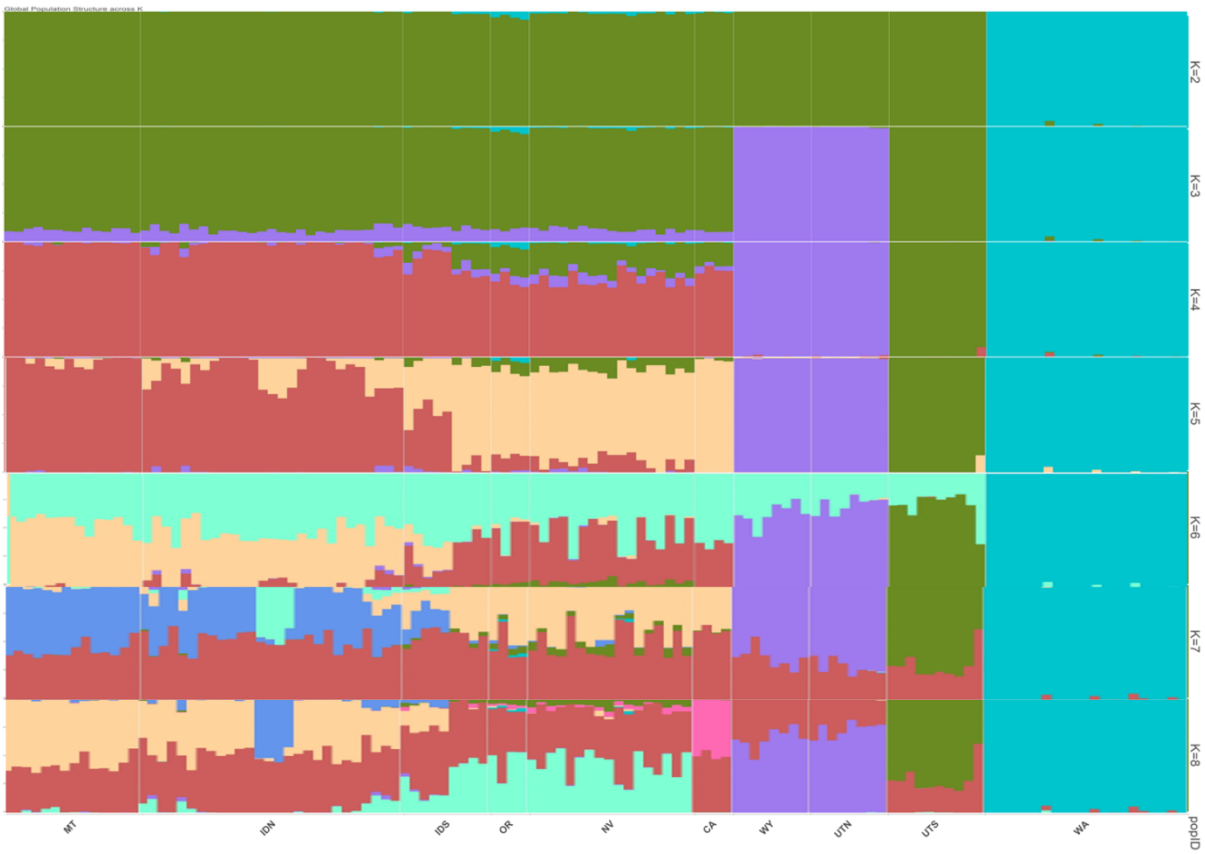

Figure 2.3. Graphical representation of the ancestry assignment in program STRUCTURE, obtained from 123 pygmy rabbit (Brachylagus idahoensis) samples, genotyped at 12,084 SNPs, and sampled across the pygmy rabbit range during 2001-2018. Each bar represents an individual, and each color, its inferred ancestry in each of the of the K (2-8) genetic groups identified. K=4 is the best supported value (Figure 2.3). The four identified groups at K=4 are: (1) Washington (WA), (2) Wyoming (WY) and northern Utah (UTN), (3) Great Basin populations including Montana (MT), Idaho north of the Snake River (IDN), Idaho south of the Snake River (IDS), Oregon (OR), Nevada (NV), and California (CA), and (4) southern Utah (UTS). After K=5, high levels of admixture occur with coloring.

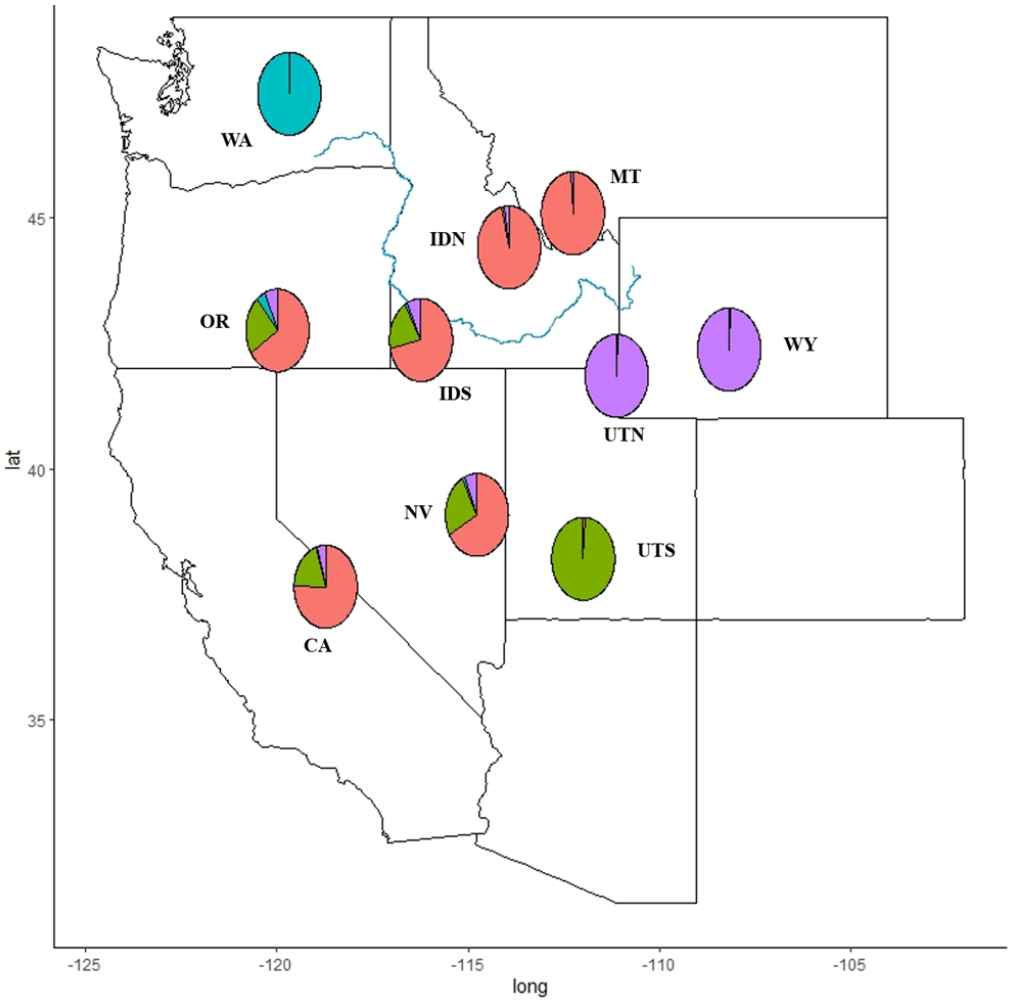

Figure 2.4. Map of the average ancestry proportions for pygmy rabbits (Brachylagus idahoensis) in each region based on the four genetic groups identified in program STRUCTURE using 12084 SNPs (sampled during 2001-2018). Blue line represents Snake River.

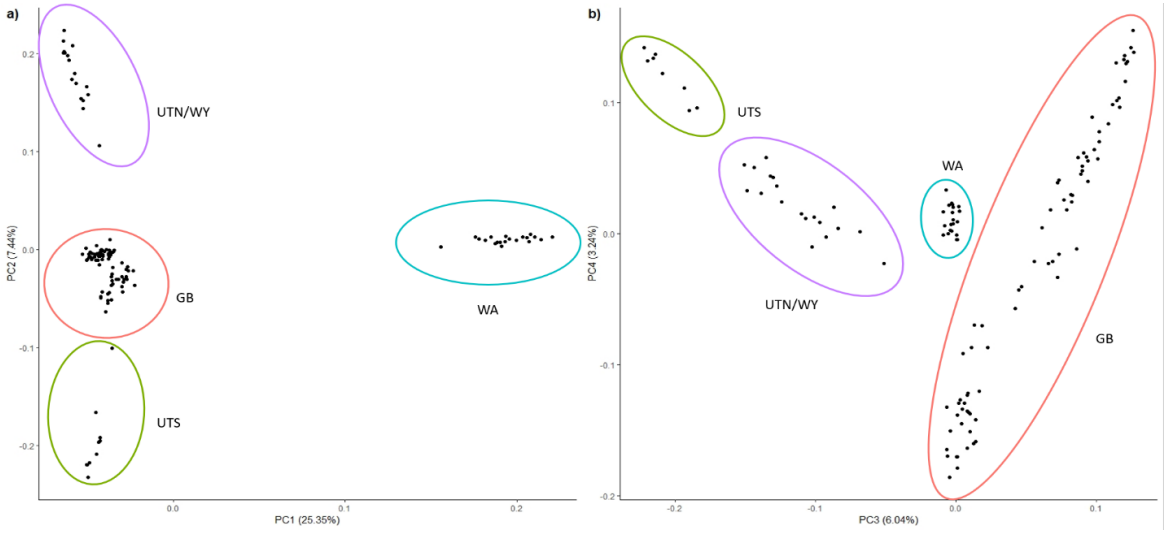

Figure 2.5. Principal components analysis based on genotypes of 123 pygmy rabbit (Brachylagus idahoensis) samples at 12084 SNPs, collected during 2001-2018. Circles represent the distinct genetic clades identified using program STRUCTURE (Figure 2.4).

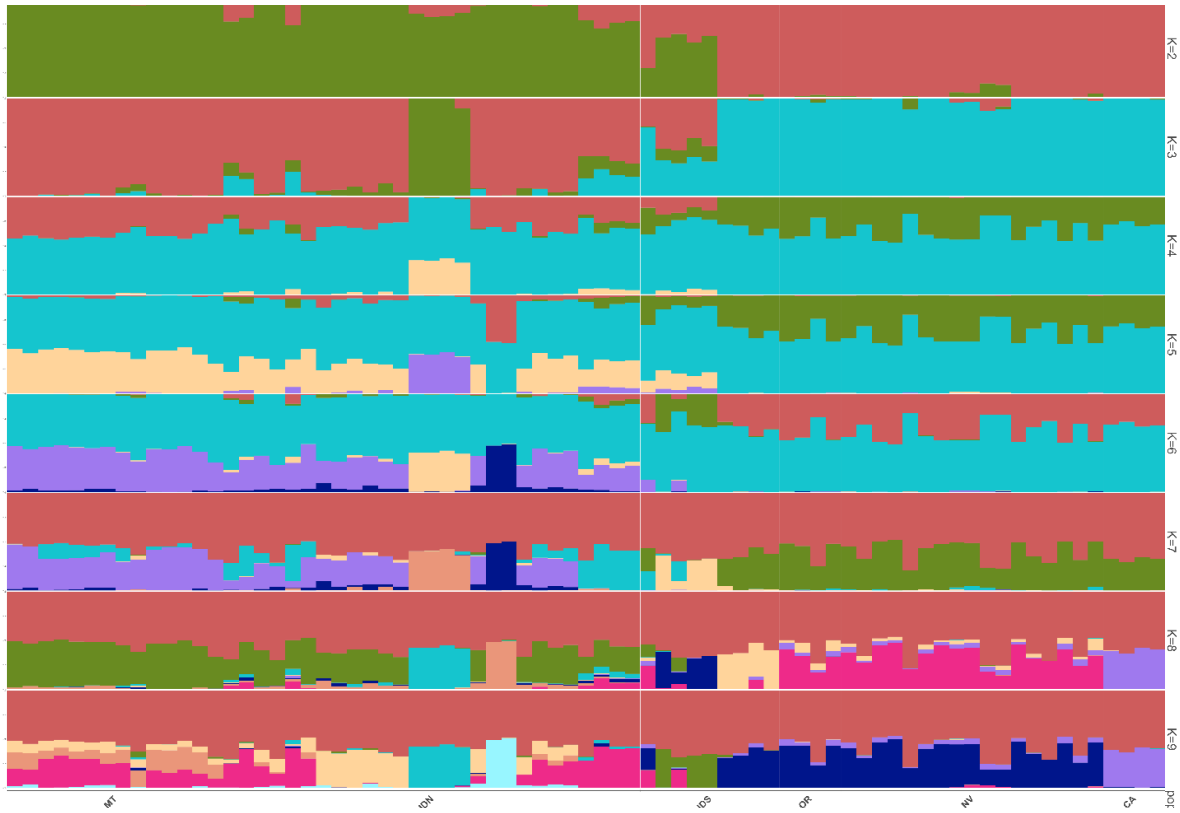

Figure 2.6. Graphical representation of the Bayesian clustering approach, in program STRUCTURE, obtained from 75 pygmy rabbit (*Brachylagus idahoensis*) samples from the Great Basin group, collected during 2001-2018 (Figures 2.3,2.5), and genotyped at 12,284 SNPs. Samples are oriented North-South within each region and across all regional populations. After K=2, high levels of admixture occur with coloring.

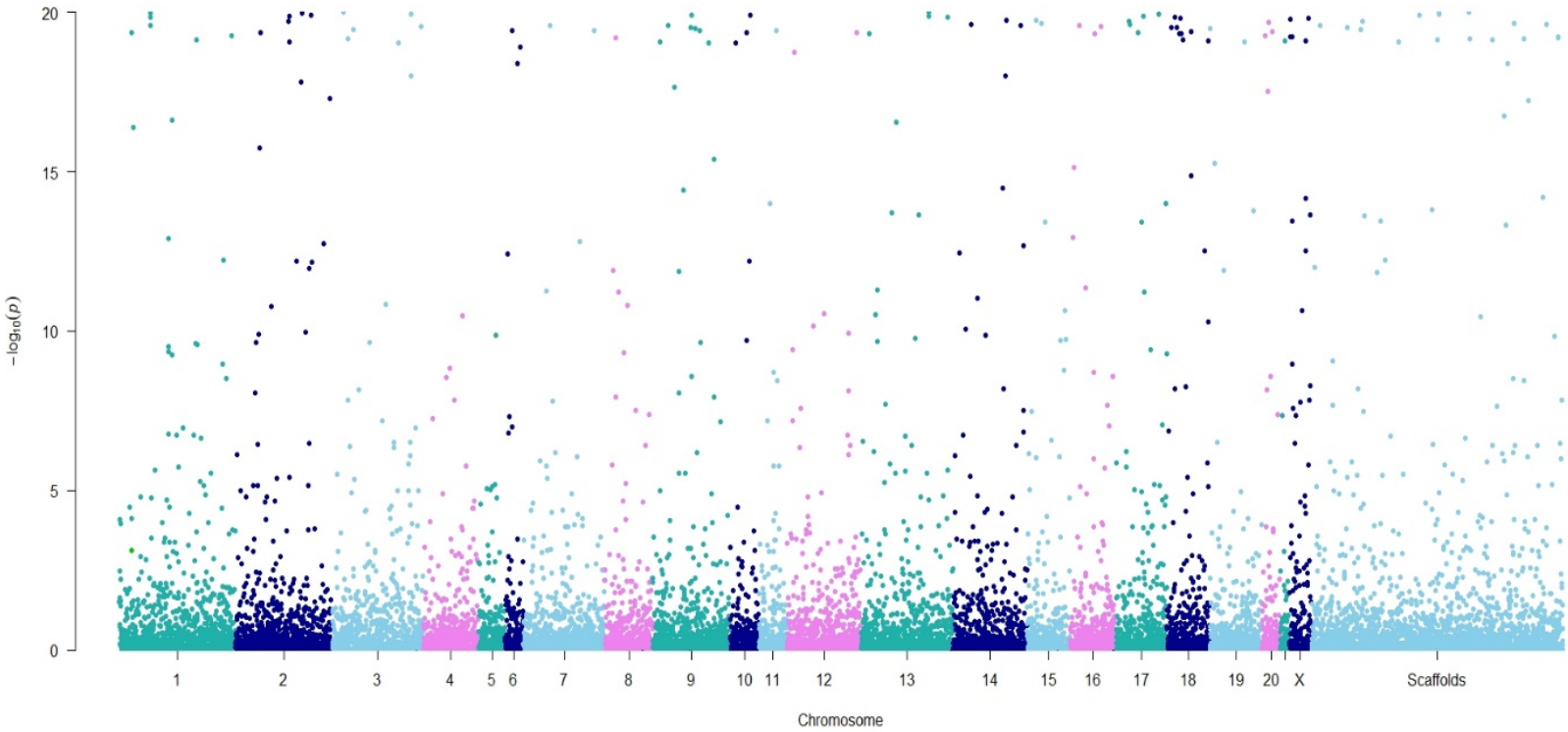

Figure 2.7. Genome scan for local adaptation using pcadapt showing significance values across 12,084 SNPs for pygmy rabbits (*Brachylagus idahoensis*). Samples represent 123 pygmy rabbits across the current species range and collected during 2001-2018. Colors represent chromosomes from the European rabbit reference genome assembly.

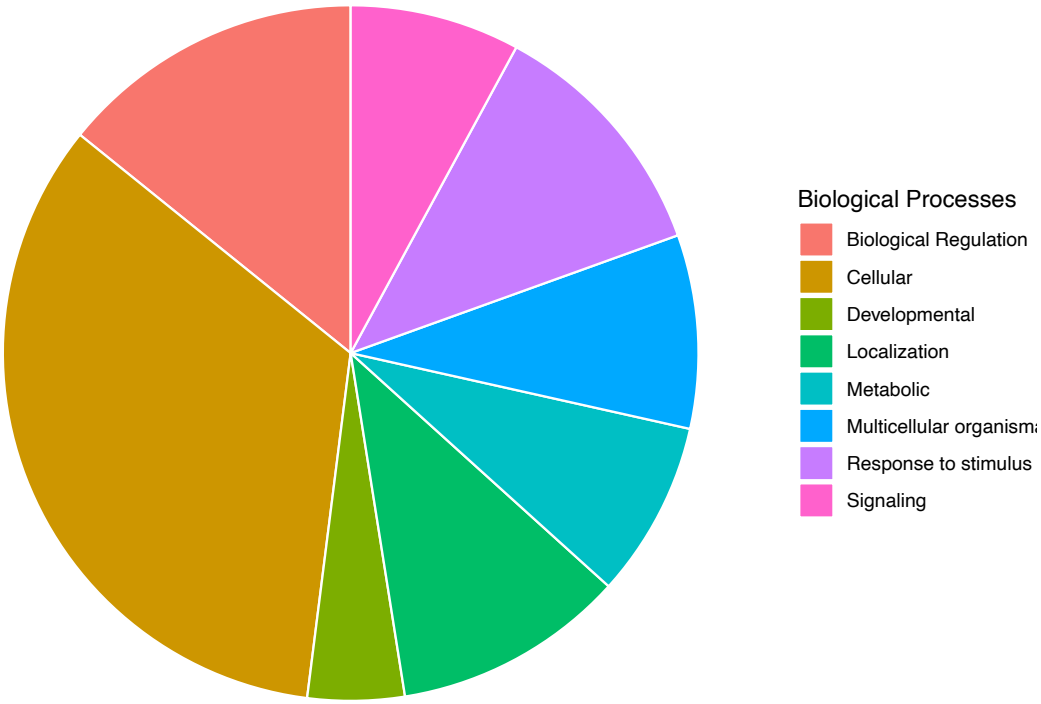

Figure 2.8. The proportion of biological processes associated with 19 outlier SNPs of pygmy rabbits (*Brachylagus idahoensis*), sampled from 2001-2018, identified in BAYESCAN. Gene ontology and biological processes were identified in PANTHER (Mi et al. 2017).

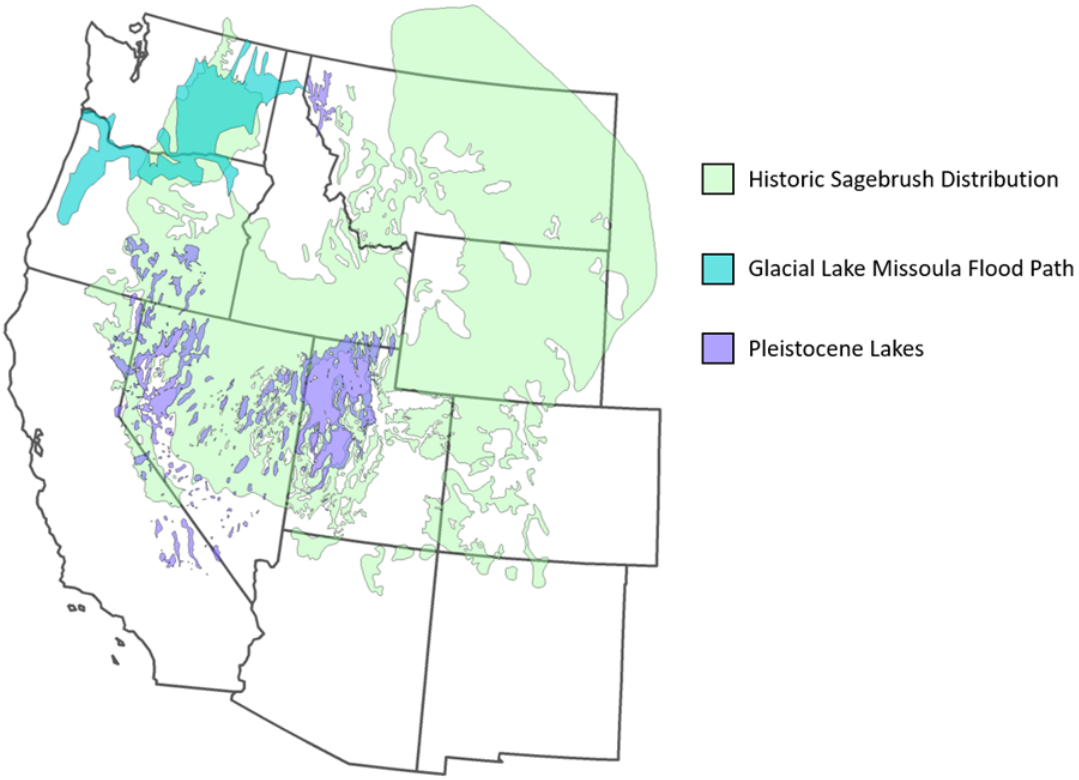

Figure 2.9. Historic distribution of sagebrush steppe habitat. Geographic range and location of glacial Lake Missoula, pluvial Lake Bonneville, and pluvial Lake Lahontan during the Late Pleistocene. The flood path is the areas affected by flooding from glacial Lake Missoula.

Table 2.1. Genetic diversity statistics for pygmy rabbit (Brachylagus idahoensis) populations in the Great Basin (GB), Washington (WA), northern Utah and Wyoming (UTN_WY), and southern Utah (UTS) of the USA from samples collected between 2001 and 2018. Statistics were performed in STACKS version 2.54, in which population assignments were given – 10,172 SNPs. The 10,172 SNPs represent a subset of the 12,084 SNPs that had to be present in 70% of each population. All represents both polymorphic and fixed sites, and variant considers only sites that were polymorphic. π represents nucleotide diversity.

	POPULATIONS			
GENETIC DIVERSITY STATISTICS	**GB**	**WA**	**UTN_WY**	**UTS**
Average Number of Individuals per population across loci	59.89	18.79	14.82	9.07
Number of polymorphic sites	9,599	3,590	6,014	5,176
Number of variant sites	10,172	10,172	10,172	10,172
Observed Heterozygosity (all)	0.00005	0.00003	0.00005	0.00005
Expected Heterozygosity (all)	0.00007	0.00004	0.00006	0.00005
Observed Heterozygosity (variant)	0.14	0.10	0.14	0.15
Expected Heterozygosity (variant)	0.20	0.11	0.17	0.16
Π (all)	0.00007	0.00004	0.00006	0.00006
Π (variant)	0.20	0.12	0.18	0.17
Private Alleles	812	492	31	9

Table 2.2. (a) D_{xy}, absolute divergence (above diagonal) and F_{ST} (below diagonal) values based on 10,172 SNPs for each of the four genetic groups of identified for pygmy rabbits (*Brachylagus idahoensis*) sampled across the species' range and collected between 2001 and 2018. (b) θ_{ST} values, representing the divergence in haplotypes, for each of the four identified groups (1) Washington (WA), (2) Great Basin (GB), (3) northern Utah and Wyoming (UTN_WY), and (4) southern Utah (UTS).

a)

	WA	**GB**	**UTN_WY**	**UTS**
WA	-	0.0007	0.0008	0.0008
GB	0.13	-	0.0007	0.0006
UTN_WY	0.24	0.04	-	0.0007
UTS	0.27	0.04	0.14	-

b)

	WA	**GB**	**UTN_WY**	**UTS**
WA	-			
GB	0.21	-		
UTN_WY	0.32	0.09	-	
UTS	0.36	0.10	0.19	-

Table 2.3. Locus position, chromosome, known annotated gene, location within gene (- denotes within gene but not in an exon or intron position), and protein information for 19 SNPs from pygmy rabbits (*Brachylagus idahoensis*) identified by BAYESCAN and compared to the annotated European rabbit (*Oryctolagus cuniculus*) genome. Intron and exon information is given if SNP could be identified in the region. For SNPs not located in a genic region, the closest known gene was provided and bp distance from gene. Loci that are bolded had SNPs identified as outliers in Bayescan as well as *pcadapt.*

LOCUS	CHROMOSOME	GENE	LOCATION	PROTEIN
PYRA_105	1	CD72	Intron 2-5	C-type lectin domain-containing
		Tpm2	Intron 7-8	protein
				Tropomyosin beta chain
PYRA_1709	2	ENSOCUG00000031382	132,531bp away	Uncharacterized protein
		TRIB2	134,873bp away	Tribbles pseudokinase 2
PYRA_2263	3	SULF1	-	Sulfatase 1
PYRA_2330	3	VIRMA	Exon 9	Vir like m6A methyltransferase
				associated
PYRA_3022	5	CTNNBL1	-	Catenin beta like 1
PYRA_3256	6	RYR1	Exon 35	Ryanodine receptor 1
PYRA_3263	6	SHISA9	Intron 2-3	Shisa family member 9
PYRA_5211	10	MINDY4	Intron 17-18	MINDY lysine 48 deubiquitinase 4
PYRA_5252	10	ANLN	Intron 25-26	Anillin actin binding protein
		AOAH	Intron 22-23	
PYRA_5349	10	ENSOCUG00000023828	Intron 1-2	CS domain-containing protein
PYRA_6093	12	OLIG3	3617bp away from	Oligodendrocyte transcription factor 3
PYRA_6254	13	ABCB8	Intron 8-9	ATP binding cassette subfamily B
				member 8
PYRA_8580	17	ENSOCUG00000001058	Intron 1-2	Uncharacterized Protein
PYRA_8819	18	LRMDA	Exon 5	Leucine rich melanocyte
				differentiation associated
PYRA_9114	18	ENSOCUG00000017933PLPP4	Intron 6-7	acidPPc domain-containing protein
PYRA_9805	X	RPS6KA3	65,901bp away from	Ribosomal protein S6 kinase
PYRA_9890	X	OTUD6A	4938bp away from	OTU deubiquitinase 6A
PYRA_10155	Scaffold	UTP11	6443bp away from	UTP11 small subunit processome
		FHL3	7909bp away from	component
				Four and a half LIM domains 3
PYRA_11044	Scaffold	SHROOM4	Intron 1-2	Shroom family member 4

Chapter 3: Habitat selection and occupancy of Conservation Reserve Program (CRP) land by the endangered Columbia Basin pygmy rabbit (*Brachylagus idahoensis*)

Abstract

Loss and fragmentation of native shrub-steppe habitat has led to the decline and near extirpation of the isolated population of the Columbia Basin pygmy rabbit in Washington. Therefore, Columbia Basin pygmy rabbits were part of a captive breeding program from 2001-2011, which transitioned to an onsite breeding program with the goal of reestablishing wild populations within central Washington. From 2011 – 2020, nearly 2000 rabbits were released onto Sagebrush Flats Wildlife Area (SBF), native sagebrush-steppe habitat for pygmy rabbits with deep soils and mima mound topography. Over that period, winter monitoring efforts identified a spatial shift of pygmy rabbit burrows, where 70% of all active burrows were found near the release areas in SBF before 2015 to ~95% of active burrows in nearby Conservation Reserve Program (CRP) restored cropland by 2020. Therefore, we examined factors that have might have led to the transition of active burrows from SBF to CRP. We hypothesized pygmy rabbits would create burrows in areas with (1) taller and denser sagebrush, because sagebrush is the main food source for pygmy rabbits and is also used as protection from predators, (2) sagebrush with higher levels of digestible content and crude protein to meet the nutritional requirements of pygmy rabbits, and (3) loam-like soil types for burrow development and integrity. Ninety-seven sites were stratified into four categories: (1) currently occupied SBF (n=19), (2) previously occupied SBF (n=27), (3) never occupied SBF (n=20), and (4) currently occupied CRP (n=31). At each site, we measured vegetation characteristics including shrub canopy cover and concealment. We also assessed the nutritional characteristics of sagebrush (sequential fiber and crude protein) and soil properties (type and pH). Aerial and terrestrial concealment were higher in currently and previously occupied sites than in never occupied sites. Currently occupied sites had greater distance to the first living branch of sagebrush, most likely because of continual browsing from pygmy rabbits. Canopy cover, living canopy cover, and composition of the canopy (living sagebrush) were significantly higher in occupied sites. All sites were predominantly located in silt loam soil types, and soil pH was significantly higher in occupied sites. Sagebrush was more nutritious in CRP compared to SBF. These findings can help guide management strategies and provide the necessary tools to identify suitable habitat for future release efforts for the endangered Columbia Basin pygmy rabbit and have demonstrated the value of habitat restoration efforts like CRP.

Introduction

As global human populations increase, the demand for food production continues to grow, intensifying land-use across the much of the globe. Humans have altered the landscape in ways that affect the spatial density, diversity, and quality of wildlife habitat, resulting in highly fragmented and isolated habitat patches (Radeloff et al. 2005; Donald and Evans 2006). The spread and intensification of agriculture are recognized as two of the most important threats to wildlife biodiversity (Foley et al. 2005; Donald and Evans 2006). Intact, native habitats have been replaced by monoculture crops, resulting in declining native plant diversity that act as food and cover for wildlife species. Reductions and fragmentation of habitat can reduce the overall quality of the habitat, affecting the availability of food and shelter for wildlife species (Menge and Sutherland 1976; Tilman and Lehman 1998). This change can ultimately affect reproduction and survival rates, immigration and emigration, and result in a smaller overall population size (Brockelman 1975; Fahrig 1997; Cushman 2006). Smaller populations are more vulnerable to stochastic events and have greater risk of extinction (Shaffer 1981; Shaffer and Samson 1985; Lande 1993).

Habitat loss and fragmentation are associated with the decline and extinction of numerous species (Fahrig 1997; Franken and Hik 2004). In particular, conversion of natural habitat to agriculture has been attributed to the decline in species such as the lesser prairie-chicken (*Tympanuchus pallidicinctus*; Haukos and Boal 2016), and honey bee (*Apis mellifera*; Otto et al. 2018). Habitat restoration is an essential component of recovery plans for such species (U.S. Fish and Wildlife Service 2012; Stinson and Schroeder 2014; Haukos and Boal 2016; Otto et al. 2018). In an attempt to restore and protect wildlife habitat across agricultural landscapes, the Food Security Act of 1985, required farm bills to include provisions for placing marginal cropland into long-term conservation covers under the Conservation Reserve Program (CRP). CRP is a land conservation program administered by the Farm Service Agency within the United States Department of Agriculture. In exchange for a yearly rental payment, farmers enrolled in the program agree to remove environmentally sensitive land from agricultural production and plant species that will improve environmental health and quality. Contracts with CRP are typically 10-15 years (https://www.fsa.usda.gov/programs-and-services/conservation-programs/conservation-reserve-program/). At its peak in 2006, over 14.6 million ha of land in the U.S. was enrolled in CRP, but the maximum permitted enrollment was reduced in 2008 and 2014 Farm Bills and is currently set to 9.6 million ha. Much of the land enrolled within CRP has been returned to grasslands especially within the mid-west (Otto et al. 2018), but more recently, CRP has been re-vegetated with other natural

habitats including the sagebrush steppe (Schroeder and Vander Haegen 2006; Stinson and Schroeder 2014).

The landscape of the intermountain west has changed dramatically over the last 150 years, particularly within semiarid sagebrush (*Artemisia* spp.) steppe ecosystems (Quigley and Arbelbide 1997). (Wisdom et al. 2005). The extent and functionality of the shrub steppe habitat have declined in recent decades creating conservation challenges for a variety of species. The loss of the shrub steppe communities in the western United States, has greatly reduced and affected the available habitat for wildlife that inhabit this ecosystem (Rotenberry and Wiens 1980; Knick and Rotenberry 1997; Morano et al. 2019). As many as 170 vertebrate wildlife species throughout the western United States and Canada are native to and somewhat dependent on sagebrush habitats, including ungulates, small mammals, and a diversity of bird species (Miller et al. 2011; Beck et al. 2012). The shrub steppe ecosystem historically dominated Eastern Washington (Daubenmire 1970) but anthropogenic changes have resulted in a loss 80% of Washington's historical shrub steppe, and much of the remaining ecosystem is fragmented, isolated from similar habitat, and highly degraded (Dobler et al. 1996; Vander Haegen et al. 2000; WDFW 2020). Conversion to cropland has resulted in the greatest loss of shrub steppe in Washington, leading to a fragmented landscape and a differentially high loss of deep-soil communities (Dobler et al. 1996; Vander Haegen et al. 2000). Loss of shrub steppe communities has occurred not only occurred in Washington but across the shrub steppe biome; north central Oregon, eastern Montana, southern Idaho (Paige and Ritter 1999), and the Great Basin. The shrub steppe of Washington is considered one of the most diverse ecosystems, and provides habitat to such sagebrush obligate species at the greater sage-grouse (*Centrocercus urophasianus*), sage sparrow (*Amphispiza belli*), Brewer's sparrows (*Spizella breweri*), sage thrashers (*Oreoscoptes montanus*) and the pygmy rabbit (*Brachylagus idahoensis*; Dobler et al. 1996). These species are at the greatest risk from the loss and decline of the shrub steppe habitat (Dobler et al. 1996; Vander Haegen et al. 2000; Oyler-McCance et al. 2001; Brooks et al. 2004; Ingelfinger and Anderson 2004; Schroeder and Vander Haegen 2006).

In Washington, ~10.3% of the agricultural fields have been enrolled in CRP (Schroeder and Vander Haegen 2006). Historically, CRP habitat within Washington was seeded with non-native grasses, but beginning in the mid-1990's, seeding transitioned to native grasses, forbs, and shrubs (Schroeder and Vander Haegen 2006; Stinson and Schroeder 2014), returning the land to its native habitat type. CRP fields may help connect fragmented patches of shrub-steppe habitat, creating a relatively continuous vegetative community for the dispersal of sagebrush obligates (e.g. Lupis et al.

2006). Many sagebrush steppe species including the greater sage-grouse (Schroeder and Vander Haegen 2011) , mule deer (*Odocoileus hemionus*) and jackrabbits (*Lepus californicus, Lepus townsendii*; Schroeder and Vander Haegen 2006) have chosen to occupy CRP habitat containing sagebrush, over adjacent, undisturbed sagebrush habitat. Additionally, increased nest survival has been documented in CRP habitat in Brewer's sparrows and sage thrashers (Vander Haegen et al. 2000). Numerous studies have examined the use of CRP by sagebrush obligate bird species, but no study has examined the use of CRP habitat by pygmy rabbits (*Brachylagus idahoensis*).

Pygmy rabbits occupy sagebrush steppe habitat in the Great Basin and Columbia Basin of the western United States (Figure 3.1; U.S. Fish and Wildlife Service 2012). Pygmy rabbits are a semi-fossorial species that uses burrows for shelter and thus require soils suitable for digging burrows in conjunction with sagebrush (Dobler et al. 1996; Sanchez and Rachlow 2009). Sagebrush provides both protective and thermal cover for pygmy rabbits, and they select for increased sagebrush height and cover (Green and Flinders 1980; Katzner and Parker 1997; Camp et al. 2012). Big sagebrush (*Artemisia tridentata*) makes up 50% of pygmy rabbits' summer diet and up to 99% of their winter (Green and Flinders 1980; Shipley et al. 2006). In the spring and summer, sagebrush is supplemented with grasses and forbs (Green and Flinders 1980). The Columbia Basin pygmy rabbit located within central Washington was listed as a threatened species because of its decline in population size and distribution (1990's), primarily due to the excessive loss of habitat due to agricultural conversion. It was federally recognized as a distinct population segment in 2003 (U.S. Fish and Wildlife Service 2003, 2010, 2012). In 2001, the last 16 rabbits were trapped from the last known population in Sagebrush Flat Wildlife Area (SBF) and placed into a captive breeding program and the Columbia Basin population was determined to be extirpated in 2003 (U.S. Fish and Wildlife Service 2003, 2012). Reintroduction efforts into SBF began in 2011 and continued until 2017, where nearly 2000 rabbits had been released by 2020 (DeMay et al. 2017; Gallie and Zinke 2018). Annual winter burrow surveys have been conducted to monitor the released and wild born rabbits, and to assess the spatial distribution of burrow systems across the landscape (DeMay et al. 2017; Gallie and Zinke 2018, 2019). From 2012-2015, most of the pygmy rabbit burrows identified were within the native shrub steppe habitat in SBF, but beginning in 2015, burrows identified were predominantly located in the CRP habitat surrounding SBF (Gallie and Zinke 2018, 2019). These CRP fields were seeded in the mid-1990's with native grasses, forbs and shrubs. Native shrubs (particularly big sagebrush) frequently seed-in from adjacent shrub steppe, making some fields potentially usable by pygmy rabbits. Therefore, the objective of this study was to compare characteristics of cover, food, and soil within native shrub steppe habitat at SBF that were currently, previously, and never occupied by

pygmy rabbits and adjacent currently occupied CRP habitat to test hypotheses about factors driving pygmy rabbit habitat selection. Previous habitat suitability models for Columbia Basin pygmy rabbits were based on habitat information acquired from studies conducted with Great Basin populations (Larrucea and Brussard 2008b; Parsons et al. 2016) and SBF (Thines et al. 2004), but no fine scale habitat data for CRP exists for the Columbia Basin pygmy rabbit population.

Because pygmy rabbits rely on sagebrush for security and thermal cover (McMahon et al. 2017; Milling et al. 2017), we predicted that occupied sites would have taller sagebrush with greater shrub canopy cover, with higher aerial and terrestrial cover than never occupied sites, and would also be greater in SBF compared to CRP. SBF is old growth sagebrush with minimal fire and other major disturbances for the last 50 years or more, whereas the shrubs of CRP were seeded in the mid-1990s or naturally seeded from shrubs in adjacent SBF, thus having less time to mature. Sagebrush height and cover were important in burrow establishment in Idaho (Parsons et al. 2016), Nevada and California pygmy rabbit populations (Larrucea and Brussard 2008a). Additionally, increased canopy cover will provide greater concealment from terrestrial and aerial predators.

Secondly, we predicted that pygmy rabbits would occupy sites with sagebrush that had higher crude protein concentrations and lower content of plant fiber, which reduces the amount of the plant that can be digested and used for nutritional requirements. We also predict that CRP sagebrush will have greater crude protein because the sagebrush is younger than SBF. Sagebrush is an evergreen plant, which, despite its high levels of toxic monoterpenes concentration, has a relatively high digestible energy and protein content year-round (Shipley et al. 2006). Pygmy rabbits are known to select for diets containing lower neutral detergent fiber (NDF), and higher crude proteins levels (Camp et al. 2015; Crowell et al. 2018). Increasing fiber intake decreases protein digestibility (Baer et al. 1997; Zhang et al. 2013). Energy expenditure relative to mass increases with decreasing body size (Kleiber 1932; Mcnab 1983), obtaining high quality forages is especially important for a small mammal like pygmy rabbits.

Finally, we predicted that burrows would be predominantly located in loamy soils. In other regions, pygmy rabbits occupy sites with friable soils, mostly in loamy-type soils (Larrucea and Brussard 2008a; Edgel 2013; Parsons et al. 2016). Loamy soils are comprised of approximate equal proportions of sand, silt, and clay and are ideal for digging burrows and maintaining burrow integrity (Larrucea and Brussard 2008a; Schmalz et al. 2014; Parsons et al. 2016). Additionally, pygmy rabbits can alter soil properties, such as pH, as a function of occupancy (Parsons et al. 2016). Soil pH among big sagebrush typically ranges from 5.9-10, but big sagebrush is most commonly found in neutral soil

(pH 7; Welch 2005). Habitat studies of pygmy rabbits have documented soil pH averaging 7.71 (Idaho; Parsons et al. 2016) whereas big sagebrush studies, within the Columbia Basin, have documented average soil pH around 6.16 (Meinke et al. 2009). We predicted that sites that were occupied at one point, previous or current, will have higher soil pH due to the alkaline nature of feces and urine.

Methods and Materials

Study Site

The study area included SBF (1514 ha) and the surrounding CRP habitat (~266ha), in Douglas County in central Washington within the Columbia Plateau Province (Crab Creek sub-basin). SBF is managed by Washington Department of Fish and Wildlife (WDFW) and is one of two sites within the state that contains pygmy rabbits. The Sagebrush Flat Wildlife Area is managed specifically for endangered and threatened species, including pygmy rabbits, sage grouse, and sharp-tailed grouse (WDFW 2006). SBF is surrounded by state, federal, and private lands, with a land cover mosaic of sagebrush steppe and wheat fields. CRP habitat was once agricultural fields that were revegetated with native flora in the mid-1990s (WDFW 2006) and naturally seeded by native sagebrush in adjacent SBF. SBF is characterized by mima mounds; natural mounds composed of loose, unstratified sediment that is overthickened with sagebrush and other grasses and forbs. The CRP site is also characterized by dense sagebrush and deep soils but lack the mima mound topography when the fields were plowed during agricultural use (WDFW 2006).

Soils at SBF are deep with a predominantly sandy loam texture. Big sagebrush (*A. tridentata sp.*) communities in our study area were occupied by numerous species including other lagomorphs (black-tailed jackrabbits (*Lepus californicus*), white-tailed jackrabbits (*Lepus townsendii*), and cottontail rabbits (*Sylvilagus* sp.). Predators of pygmy rabbits within SBF and CRP included badgers (*Taxidea taxus*), long-tailed weasels (*Mustela frenata*), coyotes (*Canis latrans*), short-eared owls (*Asio flammeus*) and several other raptor species. Temperatures range from an average minimum of -6℃ in December to an average maximum of 31.2℃ in July (Western Regional Climate Center 2020). This semi-arid environment averages about 20.3 cm of annual precipitation, over half of which is from snow (WDFW 2006; Western Regional Climate Center 2020).

Study Design and Site Selection

To compare habitat use by pygmy rabbits based on habitat (SBF vs. CRP) and occupancy status (currently, previously, and never), we selected at least 20 sites in each category. SBF sites were in the managed wildlife area and were representative of native shrub steppe habitat that had not been

agriculturally converted. CRP sites were located on land to the north, east and south of SBF that represented the newly restored habitat (Figure 3.1, 3.2). Burrow locations considered for site selection were identified in winter monitoring surveys performed on SBF and CRP using the protocols described in DeMay et al. (2017) from 2012-2018, where pygmy rabbit presence was confirmed through genetic analysis of fecal pellets (Adams et al. 2011; DeMay et al. 2017) . A random subset of burrows identified during winter monitoring surveys were chosen for each category and assessed during summer months for habitat variables.

Habitat surveys (n= 91 sites) were collected during May – August 2017, with a few surveys (n=6 sites) occurring from May – August 2018. Burrows classified as currently occupied at SBF and CRP had confirmed pygmy rabbit presence at an active burrow location during 2016-2017 and 2017-2018 winter surveys and were still occupied during surveys performed in the spring/summer of 2017 and 2018. We used signs of freshly excavated soil, fresh pygmy rabbit pellets, and an absence of cobwebs to determine if a burrow system was active (Rachlow et al. 2005; Sanchez et al. 2009). If the selected currently occupied sites did not contain an active burrow system, it was replaced by an alternative, randomly selected currently occupied site. We defined previously occupied burrow systems at SBF as an active burrow with a confirmed pygmy rabbit during winter surveys performed from 2012-2018 but were no longer active during habitat surveys. We defined never occupied sites at SBF as random mima mound locations across SBF that did not have an active burrow during any of the winter surveys from 2012-2018. Random locations were chosen by creating random points within polygons in ArcMap 10.1 (ESRI, Redlands, CA) for regions of SBF that were never occupied by pygmy rabbit burrows systems. If the random point did not fall on a mima mound, the nearest mima mound to the random point was then chosen.

Composition and Structure of Vegetation

To compare vegetation characteristics between CRP and SBF at each selected site, the burrow system for occupied sites (current or previous) was used as the center of the transect, and for never occupied sites, a random point near the center of the mima mound was used. At each selected site, two perpendicular 15-m transects were established that crossed at the center of the site, and the first was set by a random direction. On each of the four resulting half transects, we placed one 1-m × 1-m quadrat at a random location and sampled vegetative characteristics within it including plant species diversity.

In each quadrat, we measured concealment available to rabbits using a 15 × 15 cm cover board placed at the center of the quadrat and viewed from the perspective of both terrestrial and aerial

predators using the methods described by Camp et al. (2012). To examine terrestrial concealment, we measured in four cardinal directions from 4 m and for aerial concealment, we measured 1.5 m above the board. Within each quadrat, we estimated aerial cover of grasses, forbs, litter, moss/lichen, and bare ground, using vegetation cover classes (0 = 0%, 1 ≥ 0–5%, 2 ≥ 5–25%, 3 ≥ 25–50%, 4 ≥ 50–75%, 5 ≥ 75–95%, 6 ≥ 95%; Bonham 1989). Litter was defined as dead plant material that was detached and laying on the ground.

To sample characteristics of sagebrush, we selected a focal shrub (sagebrush >15cm) in each quadrat. If the quadrat contained no focal shrubs, we selected the nearest rooted sagebrush plant. For each focal shrub, we measured overall height and width, and the distance from the ground to the first living branch as a measure of change in potential cover resulting from rabbit browsing. Leaf and stem samples of ~10g/clipping (three clippings per plant) were also collected from the focal shrub and two additional sagebrush within the quadrant for nutritional analysis. Leaf and stem samples were placed on ice while in the field and then stored at -20°C until further analysis could be performed. We estimated live and dead canopy cover of shrubs for each site using the line–intercept method (Canfield 1941) on each of the two transects (*A. tridentata* ssp., stiff sagebrush - *A. rigida*, or *A. tripartita ssp. tripartite*), dead sagebrush, live rabbitbrush (grey rabbitbrush – *Chrysothamnus nauseosus ssp* or green rabbitbrush -*Chrysothamnus viscidiflorus ssp.*), and dead rabbitbrush.

Soil Properties

Soil samples were collected from the center of each quadrat for a total of four soil samples per site. Samples were collected using a hand trowel at a depth of surface to 10 cm. All soil samples were taken to the University of Idaho for laboratory analysis. Samples were air dried and stored in a cool, dry area. Samples were passed through a 2-mm sieve and then composited by site location. Soil pH was measured in duplicate, in a 1:2 soil: solution ratio using type 1 ultrapure 18.2 megohm cm water using a glass electrode following the protocol described in Robertson and VanderWulp (2019). The average soil pH for a site was determined as the average of the duplicates. Soil type for each site was determined from the Web Soil Survey (WSS) managed by the United States Department of Agriculture (USDA) Natural Resources Conservation Service (NRCS) in collaboration with National Cooperative Soil Survey.

Nutritional Quality of Sagebrush

To examine the nutritional quality of sagebrush among sites based on SBF, CRP and occupation categories, sagebrush samples were composited by site. Samples were kept at -20°C, until

freeze dried and ground for further analysis. Fiber composition of sagebrush was determined using a sequential detergent analysis (Goering and Van Soest 1970) with filter bags, sodium sulfite, and alpha-amylase in an Ankom Fiber Analyzer (Ankom Fiber Analyzer 200/220®; Ankom Technology, Fairport, NY, USA) to examine neutral detergent fiber (NDF, %), acid detergent fiber (ADF, %), acid detergent lignin (ADL, %), and acid insoluble ash (AIA, %). Crude protein was determined from nitrogen concentration measured by combustion using a TruSpec CN (LECO, St. Joseph, MI, USA at the Soil Plant Waste Analytical Lab at Washington State University, Pullman, WA.

Data Analysis

We calculated each site's vegetation, nutritional and soil values from site averages across all sampled sites within each of the categories. We used multivariate analysis (MANOVA; Stevens 1992) to examine differences among the 19 continuous habitat variables and one discrete dependent variables (soil type), with site category as the independent variable. Variables were assessed for collinearity and assumptions of normality. Following the MANOVA analysis, we used one-way ANOVA to examine differences among each category for each of the habitat variables. We conducted multiple pairwise-comparisons among the means of each category using Tukey's Honest Significant Differences (Tukey HSD). All analyses were conducted in R (R Core Team 2020), and figures were produced using *ggplot2* (Wickham 2016).

Results

Sample locations

sampled 97 sites (Figure 3.2) in the SBF and CRP area in central Washington from May 2017 through August 2018 for each of our four stratified categories: (1) currently occupied CRP (n=31), (2) currently occupied SBF (n=19), (3) previously occupied SBF (n=27), and (4) never occupied SBF (n=20). Sampling was completed for categories 2-4 in 2017 but currently occupied SBF category sites were sampled across two years (n=13, 2017, n=6, 2018) because we were unable to find the targeted number of sites in a single year.

Vegetation properties

Vegetation composition and structure differed little among sites for shrubs, but greatly differed for grass and forb composition (Table 3.2). The overstory vegetation at SBF was dominated by big sagebrush with an understory grass cover dominated by cheat grass (*Bromus tectorum*), rip-gut (*Hesperostipa comata*) and blue bunchgrass (*Festuca idahoensis*), and forb cover of shaggy fleabane (*Erigeron pumilus*), cushion fleabane (*Erigeron poliospermus*), Jim Hill mustard (*Sisymbrium*

altissimum), and twin arnica (*Arnica sororia*). Previously occupied and never occupied SBF understory was also composed of forbs, Grand Coulee owl clover (*Orthocarpus barbatus*) and Stolonous pussy-toes (*Antennaria flagellaris*), and grasses, foxtail fescue (*Vulpia microstachys*). Two additional sagebrush species, rigid sagebrush (*A. rigidia*) and three-tip sagebrush (*A. tripartita ssp. tripartite*) were found only in SBF. CRP's overstory vegetation was also dominated by big sagebrush and understory composition was dominated by cheat grass, and slender wheatgrass (*Elymus trachycaulus*), and forb species, Scouler's popcorn-flower (*Plagiobothrys scouleri*), Jim Hill mustard, yarrow (*Achillea millefolium*). Cheat grass, an invasive species, was the dominant grass species detected in both SBF and CRP (Table 3.1).

We detected strong differences among site categories (MANOVA, $F=4.10$, $p < 0.0001$) for vegetation, soil, and nutritional parameters. Moss and lichen coverage was higher in the never occupied sites (41.5%) compared to other categories within SBF ($F = 6.61$, $p < 0.003$; Table 3.2) and CRP ($p < 0.003$). Litter coverage was 1.5x higher in current and previously occupied SBF sites than in CRP ($F = 4.59$, $p < 0.007$-0.03; Table 3.2). Grass, forb, and bare ground coverage did not differ significantly among the categories (Table 3.2).

Both terrestrial and aerial concealment differed among the occupation categories, especially within SBF. Aerial concealment was 2.5x (20.0%) greater in previously occupied sites, and 3x (23.6%) greater in currently occupied sites, compared to mounds that had never been occupied (8.1%) by pygmy rabbits since 2012 (Table 3.2). Similarly, terrestrial concealment was lower in never occupied sites in SBF (69.2%, $p < 0.0001$) compared to any previous or currently occupied site in SBF and CRP (91.4-93.8%). Focal shrub height and width did not differ among site categories, but height to the first living branch did ($F = 15.9$, $p < 0.0001$). Never occupied SBF sites had the lowest distance to the first living branch (12.2 cm) and was significantly lower compared to occupied sites in CRP (18.4 cm, $p < 0.04$) and SBF (28.8 cm, $p < 0.001$). Previously occupied (15.2 cm) and CRP sites were also significantly lower compared to occupied sites in SBF ($p < 0.001$, Table 3.2).

Canopy cover, % living canopy cover, and % living sagebrush comprising the canopy cover differed among sites that were previously or currently occupied compared to never occupied sites in SBF. Canopy cover was lowest in never occupied sites within SBF (412.4cm). Canopy cover in occupied sites in SBF was 2x higher (843.1cm, $p < 0.001$), and previously occupied sites were 1.7x greater (708.7cm, $p < 0.001$). CRP canopy cover (596.3cm) was significantly less than occupied SBF ($p < 0.001$) but 1.4x greater than never occupied sites in SBF ($p < 0.02$). The % canopy cover that was living, and the % canopy cover comprised of living sagebrush significantly differed between the never

occupied sites (36.3%, 34.9%, respectively) and previously (53.0%, 51.4%) and currently occupied sites in SBF (62.4%, 60.9%) and CRP (58.7%, 56.6%; $p < 0.001$-0.003; Table 3.2).

Soil properties

All site locations were found in loam soil types, of which 45.3% (n=44) were in silt loam, 45.3% (n=44) were in ashy, silt loam soils, 8.2% (n=8) in cobbly loam, and 2.3% (n=1) in cobbly, sandy loam. We found no differences in the 15 soil types among site categories (Figure 3.3). Ground slope ranged from 0-30%, where sites were predominantly located in habitat with 0-8% slope (91.8%, n=89). Soil pH ranged from 6.13 – 8.15 across all sites and differed between never occupied SBF and currently occupied sites in CRP and SBF ($p < 0.002$-0.003; Table 3.2). Never occupied sites were more acidic (6.62) than occupied sites (7.16-7.19) with nearly a 5-fold difference.

Nutritional quality of sagebrush

The nutritional quality of sagebrush differed among sites (Table 3.3). Although NDF ($F = 0.1$, $p = 0.97$) and ADF ($F = 0.5$, $p = 0.70$) were similar among categories, ADL differed among all categories ($F = 25.1$, $p < 0.001$) except currently occupied CRP and SBF, and previously occupied and never occupied SBF. ADL concentrations were lowest in CRP (6.72%), followed by occupied SBF (7.44%) and greatest in the never occupied sites (9.32%, Table 3.3). AIA differed between occupied (current and previous SBF and CRP) and never occupied sites in SBF ($F = 16.5$, $p < 0.001$; Table 3.3). AIA was lowest in CRP (0.29%), followed by occupied SBF (0.30%), and previously occupied SBF (0.33%). Never occupied sites were nearly 1.5x higher in AIA (0.43%). Crude protein levels differed among all categories except currently occupied SBF and CRP ($F = 38.7$, $p < 0.001$-0.01; Table 3.3). Protein levels were highest in currently occupied CRP (13.3%) and SBF (13.2%) sites. Protein levels were nearly 1.4x lower (9.8%) in never occupied SBF sites. Previously occupied SBF sites were approximately halfway (11.0%) between the occupied and never occupied levels.

Discussion

We identified characteristics of land use (undisturbed native in SBF vs. restored croplands in CRP) associated with burrow occupancy of endangered Columbia Basin pygmy rabbits after reintroduction from captive breeding and translocation. Currently occupied sites, especially those within CRP, generally had higher levels of security cover as evidenced by increased canopy cover, aerial, and terrestrial concealment. They were also more nutritious, as evidenced by higher crude protein and lower levels of indigestible components of sagebrush. As we hypothesized, all occupied burrow systems were found on loamy soil types. We also found that occupied sites in CRP had the lowest levels of ADL and AIA, which compose the indigestible portion of plant cell walls, and never

occupied sites had the highest levels of ADL and AIA. Soil pH was higher in occupied sites (past and present) compared to non-occupied sites, possibly as a function of occupancy by pygmy rabbits. Our findings explain, in part, patterns of occupancy by pygmy rabbits and contribute to understanding an observed shift to CRP from SBF over the reintroduction period. These results also underscore the potential use of CRP habitat as a restoration tool for degraded sagebrush landscapes in the Columbia Basin.

Litter coverage differed between CRP and occupied (past and current) SBF sites, where litter was ~2x higher in SBF (Table 3.2). Measurements of "normal" annua1 litter production from big sagebrush in the Great Basin desert indicate that between 5.8% (West and Gunn 1974) and 13.4% (Mack 1977) of total aboveground standing crop biomass is converted to litter each year through senescence, drought, winterkill, insects, pathogens, etc. Leaves and inflorescences comprise a vast majority of the litter. Sagebrush is required by pygmy rabbits during all phases of their life cycle and provides both food and cover for this obligate species (White et al. 1982; Thines et al. 2004; Shipley et al. 2006). Bouts of prolonged foraging near burrow establishment could lead to an increased abundance of litter coverage over time. Rodents have also been attributed to substantial influences on rates of litter production in the sagebrush steppe ecosystem (Parmenter et al. 1987).

Moss and lichen coverage differed between never occupied sites and sites that had some type of occupancy (past or current) by pygmy rabbits (Table 3.2). The biotic crust is important in ecosystem function but is threatened by invasion of invasive grasses (i.e., cheatgrass) and mechanical soil disturbances usually caused by livestock trampling and human activities (Eldridge 1998; Ponzetti and McCune 2001; Belnap and Lange 2013). Various sections within SBF have been grazed historically (Thines et al. 2004; WDFW 2006), which could lead to differences in biotic layer coverage. Additionally, pygmy rabbits are known as the ecosystem engineers of the shrubs steppe environment because they dig their own burrows for both temperature regulation and protection (Dobler et al. 1996; Sanchez and Rachlow 2009; Milling et al. 2017, 2018). This mechanical soil disturbance may affect the biotic layer abundance near pygmy rabbit burrow systems.

As we expected, canopy cover differed between CRP and occupied sites (past and present) in SBF. The living composition of canopy cover, which provides concealment, was comprised of shrub cover from sagebrush (Table 3.2). These findings are consistent with others from California, Idaho, Nevada, Oregon, and Wyoming which have identified shrub cover as an important variable in site selection for pygmy rabbits (Green and Flinders 1980; Weiss and Verts 1984; Katzner and Parker 1997; Larrucea and Brussard 2008a; Parsons et al. 2016; McMahon et al. 2017). Living sagebrush

canopy cover within occupied (past and present) SBF and occupied CRP was greater (51.36-60.91%) compared to results found in Idaho (18.1-27.2%; Parsons et al. 2016). Yet, SBF sites in the never occupied category had comparable live sagebrush canopy and terrestrial concealment values to those found in Idaho. This may be due height and width differences between sagebrush in Idaho and Nevada compared to the Columbia Basin of Washington state, where focal shrub height measurements are nearly double (89.22-98.64cm) those found in Idaho (78.6cm; Heady and Laundré 2005 and 52.2cm; McMahon et al. 2017), but were comparable to Nevada (92.4-98.4cm; Larrucea and Brussard 2008a) and Oregon (84.4cm; (Weiss and Verts 1984). Concealment from canopy cover can decrease perceived risk of predation for pygmy rabbits (Camp et al. 2012), possibly resulting in pygmy rabbits selecting sites with greater coverage.

Terrestrial concealment significantly differed between CRP and occupied sites (past and present) in SBF whereas aerial concealment differed among the SBF categories only. Terrestrial concealment levels were much greater in occupied sites (past and current) in our study (91.4-93.8%) compared to those identified in Idaho (62.1-77.8%, Parsons et al. 2016; and 76.4%, McMahon et al. 2017). Pygmy rabbits have demonstrated strong selection for habitat patches that have reduced risk of predation by selecting for taller shrubs in summer habitat association studies (Heady et al. 2001; Heady and Laundré 2005; Schmalz et al. 2014; McMahon et al. 2017). Our currently occupied sites in both CRP and SBF represent burrow activity across winter and summer seasons. During winter, pygmy rabbits are thought to select habitat patches that have taller sagebrush, since this is the only structure that would provide concealment on snow surfaces (Katzner and Parker 1997). Contrary to the results of other studies, shrub height did not differ among our categories, suggesting that other factors such as the living composition of shrub cover may be more important in burrow establishment in the SBF and CRP sites.

The distance to the first living branch is much greater in currently occupied sites suggesting pygmy rabbits have a negative effect of browsing on the understory of sagebrush. The height to first living branch on the focal shrub differed among the categories, with the currently occupied SBF having the greatest distance (28.83cm; Table 3.2). Previously occupied and never occupied sites (7.4-7.8 cm) were comparable to sites in Idaho (9.8-12.6 cm). Browsing has been shown to open the sagebrush canopy and reduce live sagebrush in the understory (Parsons et al. 2016). The distance to the first living branch is much greater in currently occupied sites suggesting pygmy rabbits have a negative effect of browsing on the understory of sagebrush. This has been also documented in Idaho pygmy rabbit populations (Parsons et al. 2016). Declining quality and availability of forage due to

browsing likely reduces habitat quality for pygmy rabbits. Burrow survey studies suggest that pygmy rabbits shift areas of use over time, usually every 5-10 years (Parsons et al. 2016). The pygmy rabbit burrow systems within SBF and CRP, are typically occupied for no more than 1-2 years, based on monitoring data (DeMay et al. 2017; Gallie and Zinke 2018, 2019; Hayes 2018). Declining habitat quality is attributed to longer durations in which burrow systems are occupied (Parsons et al. 2016), but our durations are short compared to those identified in other studies. As pygmy rabbits transitioned to other locations, it appears that sagebrush understory returns to non-browsed levels quickly, as seen with the similar values between previously occupied and never occupied sites (Table 3.2). The shortened occupancy at our sites may be attributed to newly released, naïve rabbits searching for more ideal, higher quality habitat across the landscape. As rabbits assess the landscape and transition to higher quality habitats, the habitat degradation at the sites will be minimal compared to other studies that assessed sites with longer occupancy durations.

Soil pH differed significantly between occupied (past and present) and non-occupied sites. Occupancy of mima mounds by pygmy rabbits have resulted in cumulative changes to vegetative communities and soil properties on occupied mounds over time (Parsons et al. 2016). Meinke et al. (2009) evaluated soil pH associated with Wyoming big sagebrush within the Columbia Basin (6.19) and across various shrub steppe habitats of the Great Basin (6.06-6.67). The pH values identified in this study are much higher, but non-occupied sites (6.62) are comparable to the Great Basin. Sites with any type of occupancy exhibited more alkaline levels (6.96-7.19). Duration of occupancy of mima mounds by rabbits in Idaho influenced soil chemistry by decreasing NH_4-N and increasing NO_3-N concentrations around the site but the greatest effect was observed near burrow entrances where NH_4-N increased (Parsons et al. 2016). Increased accumulation of litter, urine, and pellets at burrow system sites, can result in an increase in NH_4-N and NO_3-N, resulting in the elevated pH levels. It has been suggested though, that soil and vegetative properties should return to non-occupied levels after five years of absence of pygmy rabbits (Parsons et al. 2016), but this was not observed in our data set. Our previously occupied samples covered a range of 1-5 years of absence at site locations, yet pH ranges were comparable from year to year among the previously occupied category with large amounts of overlap between the years. The pH levels of previously occupied sites did not differ significantly from the occupied sites, suggesting two potential explanations (1) that rabbits are occupying sites with higher pH levels or (2) the effects of pygmy rabbits on the landscape are longer lasting than previous noted in other studies. In general big sagebrush will grow in soils with a pH of 5.9 to 10.0, but their tolerance to alkalinity and acidity varies by subspecies (Welch 2005). Preliminary analysis of the sagebrush subspecies composition in a subset of the sites has revealed the

presence of two different subspecies present among the current and previously occupied sites: *Artemisia tridentata subsp. tridentata* and *Artemisia tridentata subsp. wyomingensis*. Further analysis of sagebrush subspecies composition is needed.

Nutritional quality of sagebrush seemed to be an important driver of burrow occupancy by pygmy rabbits and might explain the shift from SBF to CRP. Currently occupied CRP and SBF had higher crude protein levels compared to previous and never occupied sites. Similarly, crude protein in sagebrush was a strong indicator of pygmy rabbit burrow establishment in Idaho (Olsoy et al. in press). Crude protein levels in CRP and SBF occupied sites (13.3% and 13.2%, repsectively) higher compared to levels found in SBF nearly two decades prior (11.2-11.4%; Thines et al. 2004) but comparable to levels in Idaho (9.4 – 16.5%; Ulappa et al. 2014). Crude protein levels are consitent with known protein level variations in sagebrush. Shipley et al. (2006) suggest that pygmy rabbits require a diet consisting of a minimum of 7% crude protein. Sagebrush in our study area were nearly double the required amount, thus meeting the minimum dietary requirements of pygmy rabbits. Although NDF, a measure of all cell wall constituents, did not across the sites, our NDF values were comparable to those found in SBF in 2000-2004 (38.5-41.4%; Siegel Thines et al. 2004). However, ADL and AIA was nearly 2x lower in the occupied sites (past and present) than never occupied sites and sites measured in SBF nearly a decade previously (Thines et al. 2004). This finding indicates that sagebrush in occupied sites is were more nutritous because ADL and AIA represents the portion of plant cell wall that cannot be digested by vertebrate herbivores like pygmy rabbits. By foraging more efficiently on plants with higher protein, pygmy rabbits may spend less time exposed to predation, which can account for up to 88.9% of mortality. Additionally, pygmy rabbits are constrained by high energy needs and strict energy budgets (Katzner and Parker 1997; Shipley et al. 2006; Ulappa et al. 2014; Camp et al. 2015; Crowell et al. 2018), by minimzing the frequency and duration of foraging energy expenitures spent on foraging and associated energetically expensive activities such as evading predators while active could be minimized (Camp et al. 2012).

The fact sagebrush plants were more nutritious (higher protein, lower ADL/AIS) in occupied sites in restored cropland in CRP than in native habitat in SBF is particular notable. In eastern Washington, restored fields may be successful in supporting wildlife such as pygmy rabbits, in part, because croplands within shrub-steppe are typically found in patches of deeper, more productive soils (Schroeder and Vander Haegen 2011; Stonehouse et al. 2015). CRP fields may support more nutritious forage and taller and greater live sagebrush canopy cover within the shrub-steppe ecosystem. Management and treatment of old-growth native habitats, such as SBF, may be necessary

to produce newer, more nutrious sagebrush as seen in CRP but should be cautisuly applied. Burning treatments within the sagebrush steppe would not only remove structural cover but would reduce or eliminate forage provided by sagebrush for pygmy rabbits. Restored fields provide the greatest benefits to shrub-steppe obligates, such as pygmy rabbits and sage grouse, when they contain sagebrush, native forbs, and are situated within a shrub-steppe landscape (Schroeder and Vander Haegen 2006, 2011).

Sagebrush is the primary component in pygmy rabbit diets (Green and Flinders 1980; Thines et al. 2004). Although sagebrush is high in protein (Table 3.3), it contains high levels of toxins that inhibit the growth of gut microbiomes (White et al. 1982; Kohl et al. 2016). Sagebrush is heavily chemically defended with high concentrations of several classes of plant secondary metabolites, especially monoterpenes (Shafizadeh et al. 1974; Wilt and Miller 1992; Wilt et al. 1992), phenolics (Wilt et al. 1992) and sesquiterpene lactones (Kelsey et al. 1976). For herbivores, selecting a diet is often a tradeoff between toxicity and nutrients. Pygmy rabbits are one of the few animal species that can consume large amounts of sagebrush without noticeable toxicity (White et al. 1982). Further investigation into the monoterpene concentrations in each of our sites, may reveal differences among site selection by pygmy rabbits.

Our study has shown that CRP has more nutritional sagebrush compared to SBF (native shrub steppe habitat), demonstrating the value of habitat restoration efforts. It is apparent from our study and others that the future of pygmy rabbits is directly connected with the loss, degradation, and fragmentation of sagebrush habitats (Thines et al. 2004; Shipley et al. 2006; Larrucea and Brussard 2008b; Pierce et al. 2011). Given the multitude of threats to sagebrush habitats, it is essential that management decisions be made to mitigate impacts to pygmy rabbits and promote long-term conservation of shrub steppe habitats (Thines et al. 2004; Bradley 2010). A habitat model for Columbia Basin pygmy rabbits was designed in the 1990s but had two significant shortcomings: 1) it did not incorporate CRP as suitable habitat, and 2) it was limited by the quality of soil data (Gallie and Zinke 2018). CRP habitat was seeded in the mid-1990's and was not yet mature enough to be considered quality habitat. The soil data has since been updated for the county and provides a much more heterogenous landscape than previously recorded. In 2017, elevation, slope, aspect, and topographic position indexes were used to update the model, but no vegetative characteristics were available and implemented (Gallie and Zinke 2018). Our fine-scale data can now be incorporated into habitat models to provide more accurate modeling for recovery planning efforts for Columbia Basin pygmy rabbits. Our results indicate that restoration through CRP is a powerful tool for increasing

habitat for pygmy rabbits and potentially other sagebrush-dependent wildlife, and that such restoration efforts can connect fragmented remnant patches of shrub-steppe.

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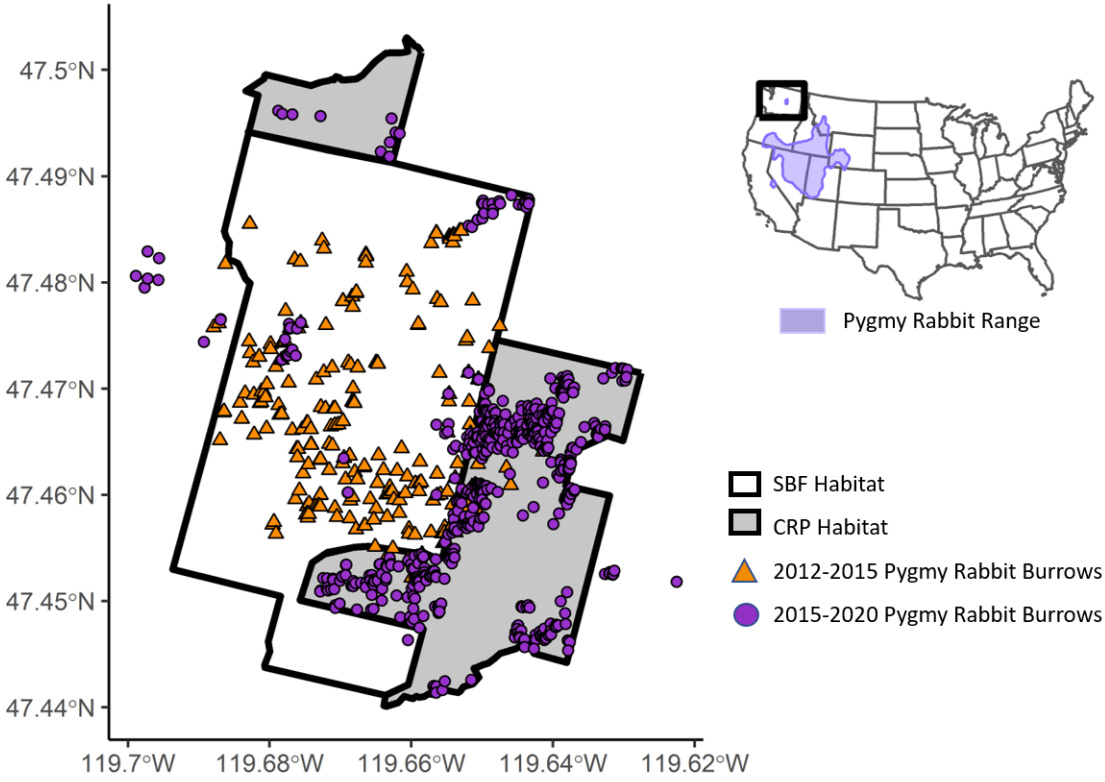

Figure 3.1. Current pygmy rabbit (*Brachylagus idahoensis*) range (purple) in the United States, with square representing Washington's population of Columbia Basin pygmy rabbits. Inlaid map represents the Sagebrush Flat (SBF) Wildlife Area and surrounding Conservation Reserve Program (CRP) land where pygmy rabbit burrows transitioned from native shrub steppe habitat in SBF to CRP habitat. ▲ represent active pygmy rabbit burrow locations identified during winter monitoring surveys from 2012-2015. ● represent active pygmy rabbit burrow locations identified during winter monitoring surveys from 2015-2020.

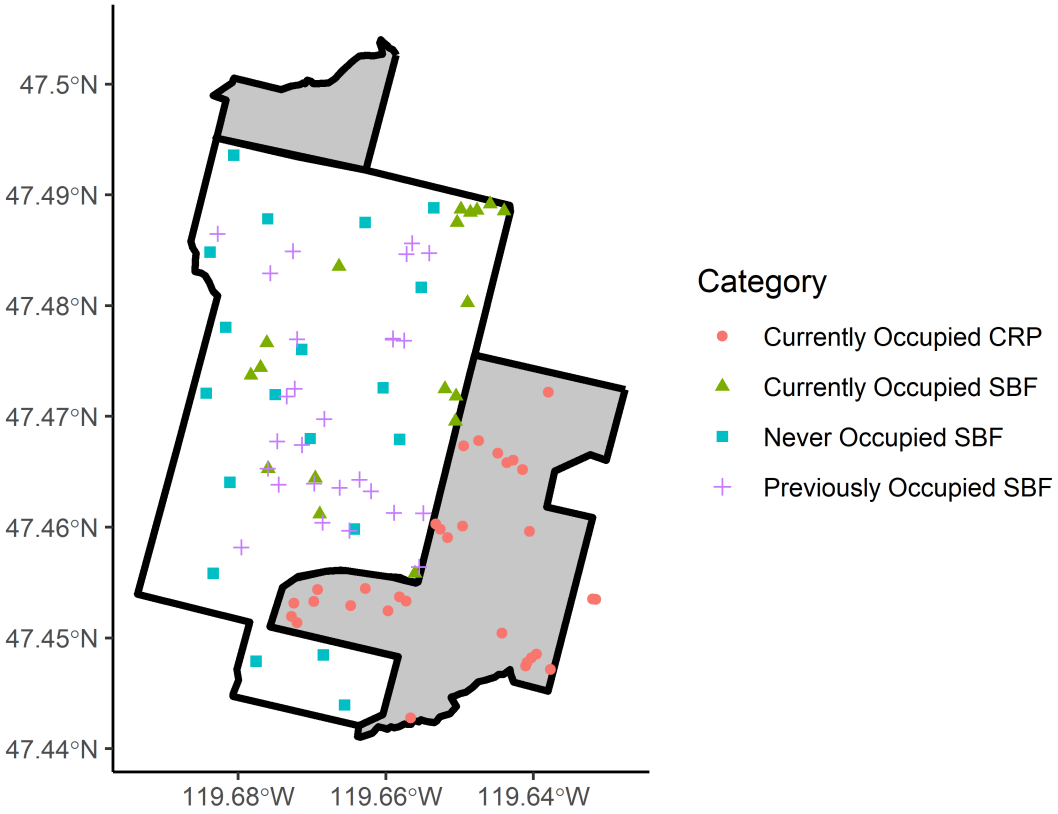

Figure 3.2. Site locations at Sagebrush Flat Wildlife Area (SBF - white), Washington and surrounding Conservation Reserve Program (CRP - grey) for each of the stratified categories where vegetative and soil samples were taken from May-August 2017 and 2018. Samples size for each stratified category are (1) Currently occupied CRP (n=31), (2) currently occupied SBF (n=19), (3) Previously occupied SBF (n=27), and (4) never occupied SBF (n=20).

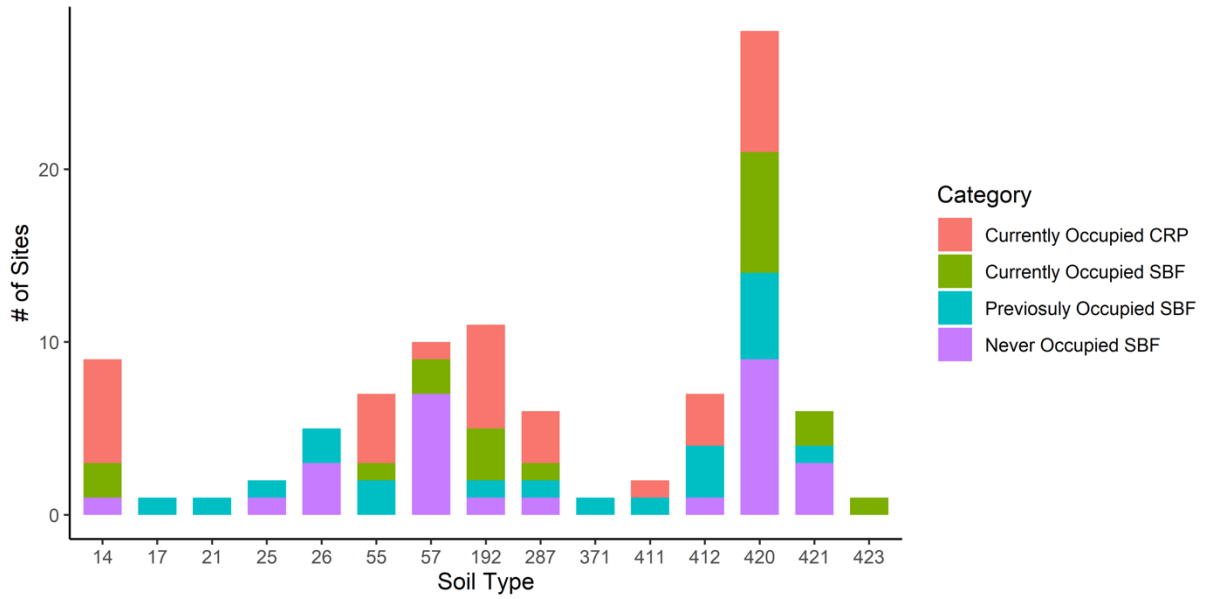

Figure 3.3. Stacked bar-chart illustrating the number of sites in each soil type identified using US Department of Agriculture's WebSoil survey (https://websoilsurvey.sc.egov.usda.gov/App/WebSoilSurvey.aspx). Soil types are 14 - Alstown silt loam, 0 to 8 percent slopes, 17 - Alstown-Renslow complex, 0 to 8 percent slopes, 21 - Argabak very cobbly loam, 0 to 20 percent slopes, 25 - Argabak-Horseflat complex, 0 to 30 percent slopes, 26 - Argabak-Horseflat-Toler complex, 0 to 20 percent slopes, 55 - Benwy-Alstown complex, 0 to 8 percent slopes, 57 - Benwy-Selah-Alstown complex, 0 to 8 percent slopes, 192 - Haploxerolls, nearly level to gently sloping, 287 - Renslow silt loam, cemented substratum, 0 to 8 percent slopes, 371 - Strat-Tubspring complex, 0 to 8 percent slopes, 411 - Toler ashy silt loam, 0 to 3 percent slopes, 412 - Toler ashy silt loam, 3 to 8 percent slopes, 420 - Toler-Horseflat complex, 3 to 8 percent slopes, 421 - Toler-Horseflat complex, 8 to 15 percent slopes , and 423 - Toler-Horseflat-Benwy complex, 3 to 8 percent slopes.

Table 3.1. Plant species composition at Sagebrush Flat Wildlife Area (SBF) and surrounding Conservation Reserve Program (CRP) habitat for each of our categories; (1) Currently occupied SBF (n=19), (2) currently occupied CRP (n=31), (3) previously occupied SBF (n=27), and (4) never occupied SBF (n=20). Value represents the number of sites within a category that the species was identified. Unknown forbs and grasses were samples that could not be identified.

Species	Currently Occupied SBF (n=19)	Previously Occupied SBF (n=27)	Never Occupied SBF (n=20)	Currently Occupied CRP (n=31)
Achnatherum sp.	-	-	1	-
Achillea millefolium	2	-	-	1 2
Achnatherum thurberianum	3	-	-	4
Agropyron cristatum	-	2	4	-
Agrostis exarata	-	1	1	-
Allium scilloides	-	-	-	1
Amsinckia menziesii	2	-	1	2
Amsinckia tessellata var. tessellata	-	-	-	2
Antennaria flagellaris	-	12	14	-
Aristida purpurea var. longiseta	-	-	-	1
Arnica sororia	6	1	-	2
Artemisia rigida	3	8	9	-
Artemisia tridentata ssp	13	24	8	24
Artemisia tripartita ssp. tripartita	-	-	1	-
Astragalus filipes	-	-	1	1
Bromus hordeaceus	-	-	-	3
Bromus japonicus	-	3	-	-
Bromus tectorum	16	26	18	31
Carex filifolia var. filifolia	-	-	-	1
Castilleja thompsonii	3	-	2	5
Chrysothamnus viscidiflorus ssp.	2	7	1	4
Crepis atribarba	4	1	-	2
Descurainia sophia	-	7	5	-
Distichlis spicata	-	1	-	-
Elymus elymoides ssp.	3	2	2	-
Elymus trachycaulus	-	-	-	11
Ericameria nauseosa ssp.	-	2	2	1
Erigeron corymbosus	-	-	-	2
Erigeron filifolius	-	1	-	-
Erigeron linearis	-	1	-	-
Erigeron poliospermus	1	3	2	-
Erigeron pumilus ssp.	6	11	4	20
Eriogonum sp.	1	1	-	-
Festuca idahoensis	14	21	19	4

Helianthus annuus	1	-	-	-
Hesperostipa comata	9	13	10	-
Leymus cinereus	4	-	-	3
Lupinus lepidus	3	1	-	-
Madia sp.	-	-	-	1
Orthocarpus barbatus	2	15	9	1
Penstemon sp.	-	1	-	-
Phlox hoodii ssp. canescens	-	2	-	-
Phlox longifolia	-	-	-	1
Plagiobothrys scouleri	1	6	3	18
Plantago patagonica	4	-	-	-
Poa sp.	-	-	1	-
Pseudoroegneria spicata	-	12	7	1
Puccinellia rupestris	2	-	1	-
Purshia tridentata var. tridentata	-	1	2	-
Salsola tragus	1	5	-	-
Silene douglasii	3	1	2	3
Sisymbrium altissimum	7	6	2	14
Sonchus asper ssp. asper	1	2	-	6
Thinopyrum intermedium	-	-	4	1
Tragopogon dubius	-	2	-	1
Unknown Forb 1	1	-	-	1
Unknown Forb 2	3	-	-	4
Unknown Forb 3	2	-	-	1
Unknown Forb 4	1	-	-	-
Unknown Forb 5	1	-	-	-
Unknown Forb 6	5	-	-	-
Unknown Forb 7	5	-	-	-
Unknown Forb 8	2	-	-	1
Unknown Grass 1	5	-	-	-
Unknown Grass 2	5	-	-	1
Unknown Grass 3	2	-	-	-
Unknown Grass 4	-	-	-	3
Unknown Grass 5	-	-	5	-
Unknown Grass 6	3	3	-	3
Vulpia microstachys	2	13	11	1

Table 3.2. Vegetative and soil characteristics measured at Sagebrush Flat Wildlife Area (SBF) and surrounding Conservation Reserve Program (CRP) habitat for each of our categories. Values are calculated from site averages across all sampled sites within each of the categories, (1) Currently occupied SBF (n=19), (2) currently occupied CRP (n=31), (3) previously occupied SBF (n=27), and (4) never occupied SBF (n=20). Bolded parameters represent parameters that showed significant difference between categories.

Vegetation Parameter	Currently Occupied SBF			Previously Occupied SBF			Never Occupied SBF			Currently Occupied CRP		
	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
Grass Cover (%)	41.88	17.35	4.50-70.62	36.02	16.3	9.00-67.50	30.36	13.52	2.50-50.00	33.84	17.51	5.75-67.62
Forb Cover (%)	4.33	3.03	1.25-14.5	2.31	2.11	0-9.00	5.06	10.76	0-50.00	5.19	3.85	1.25-17.75
Litter Cover (%)	41.06[a]	23.25	5.75-91.88	41.42[b]	16.7	12.25-91.25	33.32	14.76	2.50-56.25	26.44[a,b]	15.03	2.5-61.88
Moss/Lichen Cover (%)	21.51[a]	17.68	0.63-67.50	25.24[b]	15.49	1.25-61.25	41.54[a,b,c]	17.26	17.12-76.88	25.37[b]	13.25	1.88-50
Bare Ground (%)	9.46	9.28	0.63-30.13	5.87	6.25	0-22.5	8.26	7.47	0.63-24.00	7.42	6.54	0-26.38
Aerial Concealment (%)	23.61[a]	19	0-51.00	20.04[b]	12.71	0-52.00	8.1[a,b]	10.22	0-36.00	18.13	14.51	0-45
Terrestrial Concealment (%)	91.42[a]	8.68	67.75-100	91.59[b]	7.36	75.50-99.25	69.24[a,b,c]	24.25	8.25-95.75	93.83[a]	5.23	80.75-100
Focal Shrub Height (cm)	98.64	17.06	72.75-136.25	90.65	20.22	52.5-136.75	89.22	38.09	50.50-236.75	89.66	20.59	52.75-123.25
Focal Shrub Width (cm)	101.1	19.84	53.5-128.2	100.99	33.55	47.50-184.00	97.29	31.33	9.33-151.00	86.99	25.77	37.50-143.50
Height to First Living Branch (cm)	28.83[a,c]	8.08	14.5-41.75	15.23[d]	7.41	4.50-38.25	12.22[b,c,d]	7.83	0-30.50	18.44[a,b]	8.27	3.75-48.75
Live Sagebrush Cover (%)	60.91[a]	14.5	25.72-83.65	51.36[b]	12.1	26.51-78.34	34.94[a,b,c]	10.35	18.08-57.62	56.62c	18.65	27.21-71.5
Live Rabbitbrush Cover (%)	1.09	15.89	0-7.24	1.65	12.46	0-7.96	1.29	10.11	0-11.85	1.97	19.42	0-31.73
Live Canopy Cover (%)	62.41[a]	2.3	38.66-83.65	53.02[b]	2.52	30.61-81.07	36.25[a,b,c]	3.06	18.45-59.04	58.68[c]	6.09	27.21-100
Soil pH	7.19[a]	0.48	6.44-7.96	6.96	0.58	6.14-8.13	6.62[a,b]	0.2	6.33-7.11	7.16[b]	0.59	6.13-8.15

Different letters represent significant differences between categories on a row.

Table 3.3. Nutritional results of site composited sagebrush samples in Sagebrush Flat (SBF) and surrounding Conservation Reserve Program (CRP) habitat. Values are calculated from site averages across all sampled sites within each of the categories, (1) Currently occupied SBF (n=19), (2) currently occupied CRP (n=31), (3) previously occupied SBF (n=27), and (4) never occupied SBF (n=20). Bolded parameters represent parameters that showed significant difference between categories.

Nutritional Parameter	Currently Occupied SBF			Previously Occupied SBF			Never Occupied SBF			Currently Occupied CRP		
	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range	Mean	SD	Range
Dry Matter (%)	86.04	1.09	82.76-87.45	86.63	0.71	85.26-87.84	86.78	0.73	85.27-87.63	86.25	0.77	84.99-87.63
Neutral Detergent Fiber (%)	35.21	2.29	30.98-38.92	35.22	3.05	29.68-40.39	34.86	5.21	31.38-53.45	35.45	3.58	29.45-42.86
Acid Detergent Fiber (%)	23.97	2.23	20.20-29.14	23.59	2.78	20.05-29.53	24.75	3.82	21.21-37.36	24.16	3.38	19.3-32.95
Acid Detergent Lignin (%)	7.44[a,b]	1.05	5.82-9.58	8.5[a,b,d]	0.95	6.97-10.01	9.32[a,b,c]	1.11	8.01-9.53	6.72[d,e]	1	4.79-8.55
Acid Insoluble Ash (%)	0.3[a]	0.09	0.12-0.52	0.33[b]	0.05	0.21-0.43	0.43[a,b,c]	0.07	0.33-0.57	0.29[c]	0.06	0.21-0.44
Crude Protein (%)	13.23[a,b]	1.46	10.98-16.24	11.04[a,c,d]	1.12	9.13-13.00	9.81[b,c,e]	0.75	8.28-10.90	13.33[d,e]	1.36	10.02-15.50

Different letters represent significant differences between categories on a row.

Appendix A - PCR Protocols

Species Identification Protocols

RBT2020 – Species ID (2018-2020)

	PCR:		
	Initial Denature	95°C	15 min
Touchdown	# of cycles:	**15**	
	Denature:	94°c	30 sec
	Annealing:	**63°C - 0.5°C**	90 sec
	Extension:	72°C	1 min
Cycling	# of cycles:	**30**	
	Denature:	94°c	30 sec
	Annealing:	**55°c**	90 sec
	Extension:	72°C	1 min
	Final Extension	60°C	30 min
	Cooldown	4°C	10 min

RBBT65 – Species ID (2012-2018)

	PCR:		
	Initial Denature	95°C	10 min
Touchdown	# of cycles:	**15**	
	Denature:	95°c	30 sec
	Annealing:	**63°C - 0.5°C**	30 sec
	Extension:	72°C	1 min
Cycling	# of cycles:	**30**	
	Denature:	95°c	30 sec
	Annealing:	**55°c**	30 sec
	Extension:	72°C	1 min
	Final Exten	72°C	3 min
	Cooldown	4°C	10 min

Microsatellite Protocols

	PyRbM1P – Multiplex1 – Pellet Samples (2012-2020)		
	Initial Denature	94°C	15 min
Touchdown	# of cycles:	**10**	
	Denature:	94°c	30 sec
	Annealing:	**65°C - 0.5°C**	90 sec
	Extension:	72°C	1 min
Cycling	# of cycles:	**35**	
	Denature:	94°c	30 sec
	Annealing:	**60°c**	90 sec
	Extension:	72°C	1 min
	Final Extension	60°C	30 min
	Cooldown	4°C	10 min

PCR length: 4 hrs

	PyRbM1T – Multiplex1 – Tissue Samples (2012-2020)		
	Initial Denature	94°C	15 min
Touchdown	# of cycles:	**10**	
	Denature:	94°c	30 sec
	Annealing:	**65°C - 0.5°C**	90 sec
	Extension:	72°C	1 min
Cycling	# of cycles:	**21**	
	Denature:	94°c	30 sec
	Annealing:	**60°c**	90 sec
	Extension:	72°C	1 min
	Final Extension	60°C	30 min
	Cooldown	4°C	10 min

PCR length: 3 hrs

	PyRbM2T (Tissue) PyRbM2P (Pellets) – Multiplex 2 – Tissue and Pellets (2012-2020)		
	Initial Denature	94°C	15 min
Touchdown	# of cycles:	**6**	
	Denature:	94°c	30 sec
	Annealing:	**62°C - 0.5°C**	90 sec
	Extension:	72°C	1 min
Cycling	# of cycles:	**34pellets, 26 tissue**	
	Denature:	94°c	30 sec
	Annealing:	**59°c**	90 sec
	Extension:	72°C	1 min
	Final Extension	60°C	30 min
	Cooldown	4°C	10 min

PCR length: 3 hrs tissue, 4 hrs pellets

	PyRbM3T (Tissue) PyRbM3P (Pellets) – Multiplex 3 – Tissue and Pellets (2012-2020)		
	Initial Denature	94°C	15 min
Touchdown	# of cycles:	**10**	
	Denature:	94°c	30 sec
	Annealing:	**56°C - 0.5°C**	90 sec
	Extension:	72°C	1 min
Cycling	# of cycles:	**35pellets, 22 tissue**	
	Denature:	94°c	30 sec
	Annealing:	**50°c**	90 sec
	Extension:	72°C	1 min
	Final Extension	60°C	30 min
	Cooldown	4°C	10 min

PCR length: 3 hrs tissue, 4 hrs pellets

RAD Seq Protocols

Restriction Enzyme Ligation

Temperature	Time
37°C	60 min
80°C	20 min
-3°C/45 sec X 20	20 cycles
4°C	∞

BestRAD Adapter Ligation

Temperature	Time
20°C	12 hours
65°C	20 min
-3°C/min X 20	20 cycles
4°C	∞

NEBNext End Prep

Temperature	Time
20°C	30 min
65°C	30 min
4°C	∞

Sequencing Adapter Ligation

Temperature	Time
20°C	15 min
4°C	∞

"Test" PCR

Temperature	Time	
98°C	30 sec	
98°C	10 sec	
65°C	75 sec	**19 cycles**
72°C	5 min	
4C	hold	

"Real" PCR

Temperature	Time	
98°C	30 sec	
98°C	10 sec	
65°C	75 sec	**12-13 cycles**
72°C	5 min	
4C	hold	

Appendix B - Form and Permits

DEPARTMENT OF THE INTERIOR
U.S. FISH AND WILDLIFE SERVICE

3-201
(1/97)

FEDERAL FISH AND WILDLIFE PERMIT

1. PERMITTEE

WASHINGTON DEPARTMENT OF FISH AND WILDLIFE
1111 WASHINGTON ST SE
600 CAPITOL WAY N
OLYMPIA, WA 98501-1091
U.S.A.

2. AUTHORITY-STATUTES
16 USC 1539(a)

REGULATIONS
50 CFR 17.22

50 CFR 13

3. NUMBER
TE050644-5
AMENDMENT

4. RENEWABLE
☒ YES
☐ NO

5. MAY COPY
☒ YES
☐ NO

6. EFFECTIVE
04/19/2011

7. EXPIRES
04/18/2015

8. NAME AND TITLE OF PRINCIPAL OFFICER *(If #1 is a business)*
NATHAN PAMPLIN
ASSISTANT DIRECTOR, WILDLIFE MANAGEMENT PROGRAM

9. TYPE OF PERMIT
NATIVE ENDANGERED SP. RECOVERY - E WILDLIFE

10. LOCATION WHERE AUTHORIZED ACTIVITY MAY BE CONDUCTED
ON LANDS SPECIFIED WITHIN THE ATTACHED SPECIAL TERMS AND CONDITIONS

11. CONDITIONS AND AUTHORIZATIONS:

A. GENERAL CONDITIONS SET OUT IN SUBPART D OF 50 CFR 13, AND SPECIFIC CONDITIONS CONTAINED IN FEDERAL REGULATIONS CITED IN BLOCK #2 ABOVE, ARE HEREBY MADE A PART OF THIS PERMIT. ALL ACTIVITIES AUTHORIZED HEREIN MUST BE CARRIED OUT IN ACCORD WITH AND FOR THE PURPOSES DESCRIBED IN THE APPLICATION SUBMITTED. CONTINUED VALIDITY, OR RENEWAL, OF THIS PERMIT IS SUBJECT TO COMPLETE AND TIMELY COMPLIANCE WITH ALL APPLICABLE CONDITIONS, INCLUDING THE FILING OF ALL REQUIRED INFORMATION AND REPORTS.

B. THE VALIDITY OF THIS PERMIT IS ALSO CONDITIONED UPON STRICT OBSERVANCE OF ALL APPLICABLE FOREIGN, STATE, LOCAL OR OTHER FEDERAL LAW.

C. VALID FOR USE BY PERMITTEE NAMED ABOVE.

D. Further conditions of authorization are contained in the attached Special Terms and Conditions.

RECEIVED
MAY 16 2011
U.S. FISH & WILDLIFE SERVICE
ECOLOGICAL SERVICES
SPOKANE, WA

☒ ADDITIONAL CONDITIONS AND AUTHORIZATIONS ALSO APPLY

12. REPORTING REQUIREMENTS
ANNUAL REPORT DUE: 01/31

ISSUED BY: Harriet L. Ziebler

TITLE
PROGRAM MANAGER - ENDANGERED SPECIES

DATE
05/11/2011

United States Department of the Interior

FISH AND WILDLIFE SERVICE
911 NE 11th Avenue
Portland, Oregon 97232-4181

In Reply Refer to:
FWS/IR9/IR12/AES/Recovery Permits

Dear Permittee:

Enclosed is your U.S. Fish and Wildlife Service recovery permit issued under section 10(a)(1)(A) of the Endangered Species Act (ESA), 16 U.S.C. 1531 *et seq.*, and its implementing regulations.

Please refer to the permit number in all correspondence and reports concerning permit activities. Engagement in any activity pursuant to this permit constitutes understanding and acceptance of the Special Terms and Conditions attached to your permit.

By accepting this permit and conducting activities authorized by it, you are agreeing to adhere to the attached Special Terms and Conditions. Failure to comply with the permit Special Terms and Conditions could result in ESA section 9 take violations, or suspension/revocation of this permit.

Please be aware that some species named in your recovery permit may also be listed under various State Endangered Species Acts or otherwise be of special concern to the States. As such, activities affecting those species may not be conducted without first obtaining the appropriate State permits. Possession of a Federal permit does not obviate the need for State authorization.

If you have any questions regarding this matter, please contact Colleen Henson, Regional Recovery Permit Coordinator, at 503-231-6283 or Colleen_Henson@fws.gov. Thank you.

Sincerely,

Acting Sarah B. Hall Digitally signed by SARAH HALL Date: 2020.02.24 16:45:44 -08'00'

Program Manager for Restoration and
Endangered Species Classification

Enclosures

INTERIOR REGION 9
COLUMBIA–PACIFIC NORTHWEST
IDAHO, MONTANA*, OREGON*, WASHINGTON
*PARTIAL

INTERIOR REGION 12
PACIFIC ISLANDS
AMERICAN SAMOA, GUAM, HAWAII, NORTHERN MARIANA ISLANDS

University of Idaho
Office of Research Assurances
Institutional Animal Care and Use Committee
875 Perimeter Drive, MS 3010
Moscow, ID 83844-3010
Phone: 208-885-6162
Fax: 208-885-6014
Email: iacuc@uidaho.edu

Date: March 24, 2021

To: Lisette P. Waits

From: University of Idaho Institutional Animal Care and Use Committee

Re: Protocol IACUC-2020-13 *COLUMBIA BASIN PYGMY RABBIT POPULATION RECOVERY 2020-2023*

Your requested renewal of the animal care and use protocol listed above was reviewed and approved by the Institutional Animal Care and Use Committee on 03/24/2021.

This renewal was originally submitted for review on: 03/10/2021 02:51:37 PM PST

The original approval date for this protocol was: 04/27/2020
This approval will remain in effect until: 03/23/2022
The protocol may be continued by annual updates until: 04/26/2023

Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of the protocol you will be asked to submit an annual update form describing any changes in procedures or personnel. The committee may, at its discretion, extend approval for the project in yearly increments until the third anniversary of the original approval of the project. At that time, the protocol must be replaced by an entirely new submission.

Janet Rachlow

Janet Rachlow, IACUC Chair