

POPULATION STRUCTURE, GENE FLOW, AND GENETIC DIVERSITY OF  
ROCKY MOUNTAIN BIGHORN SHEEP INFORMED BY GENOMIC ANALYSIS

by

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of

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DEDICATION

To my mother, who has always provided unwavering support and love.

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## ABSTRACT

This dissertation evaluated the genomics of bighorn sheep (*Ovis canadensis*) herds across the Rocky Mountain West to determine optimal sample size for estimating kinship within and between populations (Chapter Two), to detect gene flow due to natural dispersal and translocations (Chapter Three), and to evaluate the correlation between genetic diversity and influences on population size (Chapter Four). To date, wildlife managers have moved many bighorn sheep across the Rocky Mountain West in an effort to provide new genetic diversity to isolated herds. However, little is known about the genetics of these herds and the real impacts of translocations. To learn how populations have been impacted by these management actions, we genotyped 511 bighorn sheep from multiple populations using a new cutting-edge genomic research technique, the Illumina Ovine High Density array, which contained about 24,000 to 30,000 single nucleotide polymorphisms informative for Rocky Mountain bighorn sheep. First, we determined that a sample size of 20 to 25 bighorn sheep was adequate for assessment of intra- and interpopulation kinship. In addition, we concluded that a universal sample size rule for all wild populations or genetic marker types may not be able to sufficiently address the complexities that impact genomic kinship estimates. Secondly, we synthesized genomic evidence across multiple analyses to evaluate 24 different translocation events; we detected eight successful reintroductions and five successful augmentations. One native population founded most of the examined reintroduced herds, suggesting that environmental conditions did not need to match for populations to persist following reintroduction. Finally, we determined that influences on population size over time were correlated with genetic diversity. Gene flow variables, including unassisted connectivity and animals contributed in augmentations, were more important predictors than historic minimum population size and origin (i.e. native vs. reintroduced). This hypothesis-based research approach will give wildlife managers additional biological insight to help inform various management options for bighorn sheep restoration and conservation.

## CHAPTER ONE

## GENERAL INTRODUCTION

Bighorn sheep conservation and management is currently a challenge. About one hundred years ago, most populations of native ungulates in the western United States decreased in number and distribution due to habitat modifications, overexploitation, and absence of wildlife management based on science (Picton and Lonner 2008). Over the past eighty years, wildlife managers have implemented harvest regulations, habitat protections, animal translocations, and science-based management, resulting in the recovery of most ungulate species in Montana (Picton and Lonner 2008). Despite efforts to restore bighorn sheep, most populations in Montana are still patchily distributed, small in number, and periodically declining, even though adequate habitat appears to be available (Montana Department of Fish, Wildlife and Parks 2010). Bighorn sheep populations can experience all-age die-offs due to respiratory disease, which is a significant problem that may limit population growth (Monello et al. 2001, Cassirer and Sinclair 2007, Besser et al. 2008, 2012, Miller 2008, Cassirer et al. 2013).

Genetic research may be a useful approach to improve understanding of factors that may limit productivity and long-term viability of bighorn sheep herds. Because many populations were founded by a small number of animals and currently have fewer than one hundred individuals, genetic factors could limit bighorn sheep restoration due to inbreeding and lack of genetic diversity (Ramey et al. 2000). Inbreeding in small herds can negatively impact reproduction, but translocations of animals from other areas have the potential to improve herd demography through increasing genetic diversity (Ralls et

al. 1979, Hogg et al. 2006). Thus, genetic investigations could provide an assessment of the current genetic composition of different bighorn sheep herds to inform future decisions regarding bighorn sheep management.

There are many different approaches to evaluate DNA, and critical differences exist among their respective inferences when applied to genetic management of populations (Frankham et al. 2017). Over the past few decades, researchers commonly employed microsatellite genotyping to study population genetics of bighorn sheep (Boyce et al. 1997, Hedrick et al. 2001, Epps et al. 2006, Hogg et al. 2006, Poissant et al. 2009, Buchalski et al. 2015, 2016, Driscoll et al. 2015). However, microsatellite panels have some limitations, such lower resolution in discerning differences in genetic relationships among individuals and populations (Tokarska et al. 2009, Fernández et al. 2013). Thus, a genomic approach that employs markers across the genome is likely to advance bighorn sheep population genetic research to help inform management. To evaluate bighorn sheep genomics, we genotyped samples using the Ovine High Density Single Nucleotide Polymorphism (SNP) microarray. The Ovine array (also called a SNP chip) is a new genomic analysis technique originally developed for domestic sheep, but an estimated 24,000 SNPs are informative for Rocky Mountain bighorn sheep (Miller et al. 2015).

Using this genomic approach, this dissertation addressed three general research questions to examine the population structure, gene flow, and genetic diversity of bighorn sheep populations. The first investigation (Chapter Two) evaluated the optimal number of bighorn sheep needed to be genetically sampled from each population to accurately discern differences in average kinship within and between populations. The second

investigation (Chapter Three) assessed the genomic consequences of translocations and population isolation to enhance understanding of evolutionary processes that affect population genetics and inform future restoration strategies. The third investigation (Chapter Four) assessed herds with different management histories, to determine if gene flow via translocations or dispersal, lowest historic population size, and population origin (native vs. reintroduced) were correlated with genetic diversity at the population level.

## CHAPTER TWO

### EVALUATING SAMPLE SIZE TO ESTIMATE GENETIC MANAGEMENT METRICS IN THE GENOMICS ERA

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Abstract

Inbreeding and relationship metrics among and within populations are useful measures for genetic management of wild populations, but accuracy and precision of estimates can be influenced by the number of individual genotypes analyzed. Biologists are confronted with varied advice regarding the sample size necessary for reliable estimates when using genomic tools. We developed a simulation framework to identify the optimal sample size for three widely used metrics to enable quantification of expected variance and relative bias of estimates and a comparison of results among populations. We applied this approach to analyze empirical genomic data for 30 individuals from each of four different free-ranging Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) populations in Montana and Wyoming, USA, through cross-species application of an Ovine array and analysis of approximately 14,000 single nucleotide polymorphisms (SNPs) after filtering. We examined intra- and interpopulation relationships using kinship and identity by state metrics, as well as  $F_{ST}$  between populations. By evaluating our simulation results, we concluded that a sample size of 25 was adequate for assessing these metrics using the Ovine array to genotype Rocky Mountain bighorn sheep herds. However, we conclude that a universal sample size rule may not be able to sufficiently address the complexities that impact genomic kinship and inbreeding estimates. Thus, we recommend that a pilot study and sample size simulation using R code we developed that includes empirical genotypes from a subset of populations of interest would be an effective approach to ensure rigor in estimating genomic kinship and population differentiation.

Key Words: *Ovis canadensis canadensis*, kinship, sampling, single nucleotide polymorphism

### Introduction

The composition of individual genomes can be an important influence on the health and long-term persistence of wildlife populations. Genomes can have profound impacts on individual fitness (Romanov et al. 2009, Kristensen et al. 2010), population-level demography (Hogg et al. 2006), resilience to environmental change (Manel et al. 2010), and response to novel pathogens or parasites (Coltman et al. 2001, Acevedo-Whitehouse et al. 2005, Siddle et al. 2007). Thus, genomic assessment of populations can be an important component of wildlife research and conservation efforts. Two important genetic attributes are inbreeding measured for individuals and kinship among individuals (Blouin 2003). At the population level, these attributes serve to evaluate gene flow among populations (Morin et al. 1994, Streiff et al. 1999), detect population differentiation (Funk et al. 2012), and evaluate demographic history (Li and Durbin 2011, Sheehan et al. 2013). On the individual level, inbreeding and relatedness metrics can indicate inbreeding depression effects (Grueber et al. 2010, Nielsen et al. 2012), heritability of observed phenotypes (Kruuk 2004, Daetwyler et al. 2014), and life history characteristics, such as propensity for dispersal (Shafer et al. 2011, Gueijman et al. 2013).

Researchers and wildlife managers often have limited resources and seek to maximize biological insight derived for the resources invested in wildlife capture and genomic analysis. To make informed decisions regarding study design, biologists require an approach to evaluate the expected level of uncertainty in inbreeding and kinship

results and decide on an acceptable sampling intensity. The level of biological insight and uncertainty derived from estimates of inbreeding and kinship can be influenced by many aspects of study design, including the metric employed, marker type, number of markers, number of individuals sampled per population, and composition of the populations and individuals under consideration (Csilléry et al. 2006, Frankham et al. 2017). Despite the potential for these study design decisions to impact biological inferences, few studies evaluate the reliability and precision of relatedness and inbreeding estimates prior to sampling, resulting in potential for imprecise estimates and results being interpreted out of context (Taylor 2015). Thus, guidelines are needed to promote robust conclusions regarding relatedness and inbreeding metric performance for each dataset (Taylor 2015). As a result, in this study we sought to conduct a rigorous simulation study to evaluate multiple inbreeding and kinship metrics while accounting for different influences on estimator precision.

There are many different metrics and alternative approaches for estimating inbreeding and kinship using molecular markers, and critical differences exist among their respective inferences when applied to genetic management of populations (Frankham et al. 2017). Three of the main metric types include identity by state, kinship coefficients, and F-statistics. In terms of single nucleotide polymorphisms (SNPs), identity by state (IBS) means that the same nucleotide is located at the same genomic position in both the maternal and paternal chromosomes (Toro, Villanueva, & Fernández, 2014). The probability of zero identity by state sharing is calculated pairwise between two individuals (dyads) and estimates the probability that two individuals share zero

alleles that are identical by state (Manichaikul et al. 2010a). Kinship coefficient ( $\phi$ ), also termed coancestry, is calculated between two individuals and estimates the probability that two randomly selected alleles, one from each individual, from any locus are identical by descent (Manichaikul et al. 2010a). A particularly common F-statistic is  $F_{ST}$ , which measures differentiation among subpopulations.

The type, number, and polymorphism of molecular markers used as inputs for kinship and inbreeding calculations impacts the accuracy of resulting estimates (Blouin 2003). Microsatellite markers, which are short tandem repeats of DNA motifs, have been applied in many wildlife genetic studies, but often include a limited number of markers, resulting in kinship and inbreeding estimates that may correlate poorly with those derived from pedigrees (Toro et al. 2002, Slate et al. 2004, Taylor et al. 2015). Thus, employing a small number of microsatellite markers may have limited utility to inform management decisions to maintain genetic diversity in conservation programs (Fernández et al. 2012). Consequently, multiple studies have recommended the use of genomic data over microsatellites for this purpose (Saura et al. 2013, Toro et al. 2014, Frankham et al. 2017). Genomic data are composed of many more markers across the genome and can be generated by RADseq (Thrasher et al. 2018), whole genome sequencing (Pool et al. 2010), and cross-species application of SNP chips (Miller et al. 2011, 2012, Haynes and Latch 2012). When SNP data were used instead of microsatellites, inbreeding and kinship estimates were more strongly correlated with genealogical data, and the addition of microsatellite data to SNP data did not improve accuracy (Santure et al. 2010). Mapped genomic data also enable evaluation and management of inbreeding and kinship

across specific genomic regions (Roughsedge et al. 2008). In general, genomic data have the potential to provide stronger inference on patterns of kinship and inbreeding than a limited number of microsatellites and may require 52% fewer samples per population (Jeffries et al. 2016).

Sample size is an important study design factor that influences study cost and inferential strength. There are generally two types of sampling that occur in inbreeding and kinship studies. First, there is process variance, also sometimes termed genetic sampling in the genetic literature, due to variations in allele frequencies caused by natural processes, such as genetic drift and local adaptation (Holsinger and Weir 2009). Thus, existing composition and demographic history of a considered population can impact precision of results, e.g., low variance in kinship can result in lower power to address research questions (Van de Casteele et al. 2001, Csilléry et al. 2006, Robinson et al. 2013, Taylor 2015). Secondly, there is sampling variance, caused by variation in allele frequencies when a subset of individuals (the sample) is drawn from the population (Holsinger and Weir 2009). This source of variation can be addressed by increasing the number of animals sampled from each population (Holsinger and Weir 2009). Despite the influence of sampling variance, actual and recommended sample sizes for evaluating a population has varied widely by study. Evaluations of simulated microsatellite datasets recommended a range of 20 to 100 individuals per population to evaluate  $F_{ST}$  (Kalinowski 2004), and 50 individuals per population to identify immigrants (Paetkau et al. 2004), while another study that used an empirical microsatellite dataset estimated that

25-30 individuals were necessary to accurately estimate allele frequencies (Hale et al. 2012).

Evaluations of sample size for genomic data have also varied in their approach and recommendations. Generally, studies using high-throughput sequencing have tended to use smaller sample sizes due to expense, in comparison to microsatellite genotyping studies. However, limited sampling can greatly impact population genetic inferences (Meirmans 2015). Hoban & Schlarbaum (2014) recommended 25-30 samples per plant population to capture spatially restricted alleles using a simulated microsatellite and SNP dataset. In contrast, a simulation using 10,000 bi-allelic loci found that a sample size of four to six could be sufficient for some but not all  $F_{ST}$  statistics (Willing et al. 2012). A study that simulated sequencing data of varying depth estimated that 40 samples with low sequencing depth had the highest accuracy to evaluate population structure (Fumagalli 2013). Empirical datasets may be even more useful for this evaluation because simulated datasets are unlikely to include all aspects of real systems (May 2004). A recent empirical study using SNPs from a tree species suggested that increasing sample sizes beyond eight individuals had little impact on estimates of genetic diversity within and among populations (Nazareno et al. 2017). The study was a step forward in contributing to the sample size literature by using an empirical genomic dataset but was limited to a small number of replicates per simulation (100) and a small number of SNPs (1000) from two populations for a non-model plant species. Another empirical simulation study employed 23,057 SNPs to evaluate precision of  $F_{ST}$  estimates between Galapagos tortoise populations and determined that 3 or 5 samples per population provided more precise

estimates than 2 samples (Gaughran et al. 2017). However, no empirical genomic simulation has been published for free-ranging mammals.

Due to the many factors that can impact population genetic metrics, it can be prudent to evaluate the precision and accuracy of estimators for each unique dataset (Van de Casteele et al. 2001, Wang 2011, Taylor 2015). This is especially relevant when evaluating populations of conservation concern with past bottlenecks and suspected low genetic diversity (Taylor 2015). Thus, multiple simulation software options have been developed to address the need to test how a particular method might perform for a given research question, molecular marker dataset, and study species (Hoban 2014). For example, the program “Coancestry” and its associated package “related” (Wang 2011, Pew et al. 2015) for use with the R statistical software environment (R Core Team 2017) were developed to allow users to select the best relatedness or inbreeding estimator for a given dataset. The software utilizes empirical allele frequencies to conduct *a priori* simulations and evaluate the reliability of moment and likelihood estimators. However, this tool is limited to seven metrics, all of which estimate relatedness and inbreeding relative to a reference population assumed to include unrelated and non-inbred animals (Taylor 2015). These metrics are based on comparing molecular markers of a specified homogeneous population to those found in individuals or dyads (Purcell et al. 2007). However, detecting population structure depends on correctly identifying individuals that are not related (Zhu et al. 2008), which violates the assumption of a homogeneous population and consequently results in less accurate relationship inferences (Manichaikul et al. 2010a).

There is a need for a more user-friendly and flexible approach to evaluate sample size for inbreeding and kinship metrics, given different molecular markers and study populations. If researchers implemented an analysis of sample size to derive relationship inferences, reporting of results would become more comparable among studies and allow for more generalizable insights. Thus, we sought to conduct a rigorous simulation study using an empirical genomic dataset for wild animals to evaluate inbreeding and kinship metrics, account for different influences on estimator precision, and provide sampling guidance for future similar efforts. Specifically, we wanted to determine how variance in the population average for metric values related to sample size across a gradient of sample sizes, ranging from inadequate to sufficient for reliable and informative insights. To accomplish this, we conducted simulations employing three selected metrics to evaluate genomic relationship inferences within and between populations of Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*). The genomics of Rocky Mountain bighorn sheep provide an excellent opportunity to evaluate and compare genetic management metrics across population sizes that range from small, isolated herds recovering from population bottlenecks to large metapopulations that can sustain human harvest. Therefore, the herds we examined consisted of different management and demographic histories that likely impacted inbreeding and kinship and are representative of many other wildlife populations of conservation concern. Since sampling guidelines might not apply equally well to all situations and species (Hoban and Schlarbaum 2014), we sought to develop a flexible and transparent approach that could be easily applied by other researchers and managers to other datasets. Thus, we developed well-annotated,

straightforward code for R (R Core Team 2017) that others can modify and implement to make informed sample size decisions and achieve desired biological insights for other populations. We seek to shift from a paradigm of a single sample size recommendation to a more adaptive framework, where researchers employ a similar method to evaluate sample size decisions for specific datasets and metrics, to enhance inference reliability and maximize comparability of studies that estimate inbreeding and kinship.

## Methods

### Genomic Dataset

Marker density of many agricultural animal SNP chips can provide informative molecular kinship estimates that are better than pedigrees when applied to related, non-model species of conservation concern (Gómez-Romano et al. 2013). Thus, we employed a SNP genotype dataset generated from the High Density (HD) Ovine array, which contains approximately 606,006 SNPs with a density of 1 SNP per 4.279 kb. The Ovine array (also called a SNP chip) is a new genomic analysis technique originally developed for domestic sheep, but its development included genotypes from 5 bighorn sheep and 4 Dall's sheep (*Ovis dalli*; Kijas et al., 2009, 2014). Species divergence between domestic sheep and bighorn sheep took place around 3 million years ago (Bunch et al. 2006), but domestic sheep and bighorn sheep can interbreed and produce viable hybrid offspring (Young and Manville 1960). In addition, the two species have the same number of chromosomes and are expected to have high genomic synteny (Poissant et al. 2010). An estimated 24,000 SNPs on the HD Ovine array are informative for evaluation of Rocky Mountain bighorn sheep (Miller et al. 2015). Furthermore, the domestic sheep

reference genome enables whole genome genotyping of bighorn sheep and the potential to map informative SNPs to genomic areas of known function (Kohn et al. 2006).

However, it is important to consider that the use of SNP chips can result in ascertainment bias, as only select individuals were assessed to construct the panel (Albrechtsen et al. 2010), and cross-species application could be biased toward highly conserved markers. Thus, we sought to address this issue by comparing results across sample sizes among herds with different known population attributes.

### Study Populations

We examined four wild populations of bighorn sheep that we expected to differ in kinship both within and between herds, due to a spectrum of population attributes and geographic isolation among herds (Figure 1). Bighorn sheep populations located in Glacier National Park, Montana, and across the Beartooth Absaroka Mountains in Wyoming served as baseline examples of large, native metapopulations with high anticipated connectivity and genetic diversity. The selected samples from Glacier National Park spanned the eastern front of the park inside Glacier and Flathead Counties, with approximately 16 from north of St. Mary Lake and 14 from the southern areas of the park (Flesch and Graves 2018). The samples from the Beartooth Absaroka metapopulation spanned the eastern front of the Greater Yellowstone Area, across Wyoming hunt units 1, 2, 3, 5, and 22 inside Park, Hot Springs, and Fremont Counties. The Fergus (Fergus County) and Taylor-Hilgard (Gallatin and Madison Counties) herds served as examples of herds with more complex management histories. The Fergus herd is a large population that was reintroduced (43 bighorn sheep reintroduced from 1958 to

1961), experienced a population bottleneck of a limited number of individuals, and was supplemented with additional augmentations. Thus, this population is representative of a herd with a successful reintroduction and a current population size of greater than 200 individuals, as well as a past bottlenecks and augmentations. The Taylor-Hilgard herd represents a native population that experienced multiple augmentations and catastrophic die-offs that reduced the population to several 10s of animals, but has recovered to a moderate size of about 280 individuals. In addition, this herd has been impacted by respiratory disease, which is a major limiting factor to bighorn sheep conservation and management throughout the western U.S. (Monello et al. 2001, Cassirer and Sinclair 2007, Besser et al. 2008, 2012, Miller 2008, Cassirer et al. 2013). Based on a synthesis of these herd history characteristics, we expected inbreeding and kinship to be lower within the Beartooth Absaroka and Glacier National Park herds, in comparison to the Fergus and Taylor-Hilgard herds.

### Sample Collection

Bighorn sheep samples were collected using chemical immobilization, helicopter net-gunning, or baited drop-nets. All capture and handling protocols complied with scientific guidelines and permits from the States of Montana and Wyoming, Yellowstone National Park, and Glacier National Park. Animal capture and handling protocols were approved by Institutional Care and Use Committees at Montana State University (Permit # 2011-17, 2014-32), Montana Department of Fish, Wildlife, and Parks (Permit # 2016-005), Wyoming Game and Fish Department (Permit # 854), or the U.S. Geological Survey (Permit #2004-01). Samples were collected at the Fergus, Taylor-Hilgard, and

Beartooth Absaroka populations from 2013 to 2016; Glacier samples were collected from 2004 to 2011. We collected multiple types of genetic samples, including gene cards, biopsy ear punches, whole blood, and tissue. Collection using gene cards involved placing 2-4 drops of whole blood directly onto an FTA Classic gene card. Biopsy punches were obtained from ear cartilage during ear tagging and stored frozen in 90% ethanol. We also collected whole blood samples for a limited number of animals and tissue samples from hunter-harvested animals. We performed the DNA extractions using the Maxwell 16 LEV Blood DNA Kit for blood and gene card samples and the SEV Tissue Kit for biopsy punch and tissue samples. Because of sample availability and the fact that a previous simulation study (Hoban and Schlarbaum 2014) suggested that 25-30 samples should be used per population to assess population structure, we evaluated 30 samples per herd. We employed stratified random sampling to select 6 samples from each of 5 hunt units across the Beartooth Absaroka population, which had a total of 86 samples. However, because one study suggested that more than 30 samples per population should be used (Fumagalli 2013), we also employed all 86 samples from the Beartooth Absaroka for a separate simulation with a greater sample size to evaluate within population kinship, compare how results might differ from analyses using a sample size of 30, and assess changes in variance when samples were drawn from a larger pool.

#### Data Quality Control and Analysis

We performed quality control of the 30-samples-per-herd dataset and all 86 samples from the Beartooth Absaroka dataset separately. The 86 sample Beartooth

Absaroka dataset included the 30 samples selected for 30-samples-per-herd analysis. We completed preliminary filtering for quality control using Golden Helix SNP & Variation Suite v8.6 software (*SNP & Variation Suite* 2016). First, we filtered for sample quality using a call-rate threshold of 0.85. We deactivated markers of unknown mappings and on the sex chromosomes. We filtered SNPs using a minor allele frequency of less than 0.0001 to remove monomorphic and extremely rare markers (de Cara et al. 2013). Finally, we removed markers with poor performance by requiring a SNP call rate of greater than 0.99 and this dataset was used for input in the simulations. We also generated a principal components analysis (PCA) of the 30-samples-per-herd dataset using Golden Helix after additional filtering using a minor allele frequency threshold of 0.01 and Hardy-Weinberg equilibrium p-value less than 0.00001 (*SNP & Variation Suite* 2016). We also performed linkage disequilibrium (LD) pruning of the dataset used for the PCA analysis, which removes non-independent SNPs that inform the presence of nearby variants, using a window size of 100, window increment of 25, LD statistic of  $r^2$ , LD threshold of 0.99, and LD computation method CHM (Huisman et al. 2016).

We conducted simulations of inbreeding and kinship estimates within and among bighorn sheep populations to determine optimal sample size for these analyses. We used program R (R Core Team 2017) for simulations, modifying the approach by Nazareno et al., (2017)). Our criteria for optimal sample size included an assessment of variance and precision. For variance, we used boxplots to evaluate differences among estimates with increasing sample size, as high variance in estimates would result in similar or potentially misrepresentative estimates. In addition, we evaluated differentiation among mean

estimates using the standard deviation of the mean for all replicates in each simulation. We also used boxplots to compare the distribution of mean estimates provided by the 10,000 replicates with the estimates of other populations with similar or different management histories. We compared the mean estimate for kinship, IBS, and  $F_{ST}$  generated from each simulation of a certain sample size to the 30-sample estimate, which for these evaluations we assumed represented ‘truth’ and calculated relative bias. We combined both variance and relative bias into root mean squared error (RMSE), which estimates the sample standard deviation of the differences between predicted and observed values. Thus, we looked for decreasing root mean squared error for each sample size simulation, which would indicate lower overall relative bias and variance.

For intrapopulation simulations, we estimated mean proportion of SNPs with zero IBS and mean kinship within each population using 30 samples to provide a benchmark for precision of the simulation replicates. Each simulation consisted of randomly selecting (without replacement) sample sizes of 5, 10, 15, 20, and 25 drawn from 30 possible genotypes for each population using R and PLINK v1.07 or v1.90 software (Purcell et al. 2007, R Core Team 2017), with 10,000 replicate simulations for each sample size. There was a large number of possibilities for unique combinations of samples for each selected sample size from each herd (ranging from 142,506 possible unique combinations for sample sizes of 5 and 25 to 155,117,520 unique combinations for the sample size of 15), so we used the “sample” command in R to select each of the 10,000 replicates (R Core Team 2017). Due to a maximum of 30 genotypes, replicates were generally not independent of one another. For example, for the sample size of 25,

pairs of replicate data sets shared on average about 20 of the same genotypes.

Independence among replicates increased at lower sample sizes, and a pair of replicates for a sample size of 20 shared an average of 13 genotypes in common. Despite these limitations, we were able to evaluate the decrease in variance due to sample size with more independent replicates using the 86-sample Beartooth Absaroka dataset, and for a sample size of 25, pairs of replicate datasets shared an average of 7 genotypes in common. Populations composed of unrelated individuals are expected to have mean kinship values that are normally distributed around 0 (Manichaikul et al. 2010a).

Negative mean kinship values can be produced but can be effectively truncated at 0 to indicate low kinship, but we did not truncate values to evaluate the distribution of simulation results (Manichaikul et al. 2010a). Identity by state and kinship simulations did not require filtering within the simulation or LD pruning (Manichaikul et al. 2010a). Sub-setting of samples was implemented in PLINK v1.90 for identity by state and v1.07 for kinship simulations (Purcell et al. 2007). Probability of zero identity by state and kinship estimates were calculated using KING software v2.0 (Manichaikul et al. 2010a). For all metrics, we calculated the mean of the estimates produced by each sampling group. We compared variance and bias of the 10,000 replicate estimates for each sample size simulation relative to values obtained when we used all available samples ( $n = 30$  or  $86$ ). To evaluate intrapopulation metric estimates across the spectrum of sample sizes, we determined the extent of overlap in the range of estimates provided by each sample size for populations with different management histories.

For interpopulation metrics, we conducted simulations to compare results by sample size for  $F_{ST}$ , probability of zero identity by state, and kinship between herds. We estimated mean kinship, IBS, and  $F_{ST}$  between populations using 30 samples from each population (60 total) to provide a benchmark for precision of the simulation replicates. For the simulations we randomly selected (without replacement) sample sizes of 5, 10, 15, 20, and 25 drawn from 30 possible genotypes for each population using R and PLINK v1.07 or v1.90 software (Purcell et al. 2007, R Core Team 2017) using 10,000 replicates. There was a large number of possibilities for unique combinations of samples for each selected sample size (ranging from 285,012 possible unique combinations for sample sizes of 5 and 25 to 310,235,040 unique combinations for the sample size of 15), so we used the “sample” command in R to select each of the 10,000 replicates (R Core Team 2017). For interpopulation metric estimates we evaluated if the range of estimates produced by the simulation provided different inferences regarding the relative comparisons of population differentiation among herds for various sample sizes. The  $F_{ST}$  simulation required filtering each random sampling group and analysis using PLINK v1.90 (Purcell et al. 2007) to emulate typical  $F_{ST}$  analysis procedures, which involved removal of SNPs with a minor allele frequency of less than 0.0001 and those that deviated from Hardy-Weinberg equilibrium using a threshold of  $p < 0.00001$ . In addition, we employed LD pruning of each sampling group to ensure independence of markers using a window size of 100, window increment of 25, LD statistic of  $r^2$ , LD threshold of 0.99, and LD computation method CHM (Huisman et al. 2016).

## Results

### Kinship of Bighorn Sheep Populations

Ovine HD array analysis produced a genotype of 605,898 SNPs from the forward strand. Applying a sample quality filter call rate threshold of 0.85 did not remove any samples from the 30-samples-per-herd dataset and removed one sample from the Beartooth Absaroka large dataset, leaving 86 samples. Deactivation of SNPs of unknown mappings and on sex chromosomes removed 29,401 SNPs from both datasets. Application of the minor allele frequency of less than 0.0001 removed 505,235 SNPs in the 30-samples-per-herd dataset and 530,050 SNPs from the Beartooth Absaroka 86 sample dataset. Requiring a SNP call rate of greater than 0.99 resulted in removal of 57,038 SNPs from the 30 samples per herd dataset and 35,665 SNPs from the Beartooth Absaroka 86 sample dataset. These quality control steps resulted in 14,224 SNPs remaining for the 30-samples-per-herd dataset and 10,782 SNPs remaining for the 86 sample Beartooth Absaroka dataset, with a SNP density of 1 SNP per 170.404 kb and 1 SNP per 224.002 kb, respectively. We generated a PCA of the 30-samples-per-herd dataset using 7,688 SNPs after filtering, which suggested four distinct populations (Figure 2).

Mean proportion of SNPs with zero IBS within populations was similar for the two metapopulations using all 30 samples, Glacier ( $0.023 \pm 0.005$  SD) and Beartooth Absaroka ( $0.024 \pm 0.003$  SD). Intrapopulation mean kinship was also comparable between Glacier ( $-0.002 \pm 0.067$  SD) and Beartooth Absaroka ( $0.003 \pm 0.033$  SD). As expected for a population composed of unrelated individuals, Glacier and the Beartooth

Absaroka had average population mean kinship values that were close to 0 (Manichaikul et al. 2010a). Estimates based on all 86 samples from the Beartooth Absaroka were  $0.032 \pm 0.004$  SD for probability of zero IBS and  $0.020 \pm 0.033$  SD for mean kinship, which were slightly higher than values obtained from the sub-sample of 30 for this herd. The two smaller populations with more complex herd histories had greater within-herd kinship than the metapopulations (Glacier or Beartooth Absaroka) for both mean proportion of SNPs with zero IBS using all 30 samples (Fergus x =  $0.019 \pm 0.005$  SD; Taylor-Hilgard x =  $0.018 \pm 0.005$  SD) and mean kinship (Fergus x =  $0.045 \pm 0.063$  SD; Taylor-Hilgard x =  $0.064 \pm 0.055$  SD).

Interpopulation metric estimates using 30 samples from each herd (60 total genotypes) resulted in differences in relative ranking of relationships between herds, depending on the metric employed. Application of  $F_{ST}$  resulted in the Fergus vs. Beartooth Absaroka comparison estimated to be most distantly related (0.086), followed by Fergus vs. Glacier (0.080), Fergus vs. Taylor-Hilgard (0.079), Glacier vs. Taylor-Hilgard (0.075), and Glacier vs. Beartooth Absaroka (0.071). Taylor-Hilgard and Beartooth Absaroka were the most closely related of the  $F_{ST}$  estimates (0.032). Mean proportion of SNPs with zero IBS resulted in slightly different ranking of interpopulation estimates, with Glacier vs. Beartooth estimated to be the most distantly related ( $0.0363 \pm 0.004$  SD), followed by Fergus vs. Beartooth Absaroka ( $0.0361 \pm 0.004$  SD), Fergus vs. Glacier ( $0.034 \pm 0.005$  SD), Glacier vs. Taylor-Hilgard ( $0.033 \pm 0.005$  SD), Taylor-Hilgard vs. Beartooth Absaroka ( $0.032 \pm 0.004$  SD). Fergus and Taylor-Hilgard were estimated to be most closely related using IBS ( $0.029 \pm 0.005$  SD). Mean kinship

estimates between populations resulted in similar relative ranking of estimates as the IBS approach, except Fergus vs. Beartooth Absaroka ( $-0.188 \pm 0.108$  SD) was estimated to be more distantly related than Glacier and Beartooth Absaroka ( $-0.182 \pm 0.088$  SD). All interpopulation mean kinship values were estimated to be negative, indicating relatively low kinship among the populations.

### Sample Size Simulations

For intrapopulation metric estimates, sample size influenced whether the range of estimates provided by each sample size overlapped for populations with different management histories (Figure 3). We calculated one minus mean kinship so that relative kinship estimates among herds would be comparable to IBS results. At smaller sample sizes of  $n \leq 15$ , the two metapopulation (Glacier and Beartooth Absaroka) distributions overlapped mean kinship and IBS distributions of herds with more complex management histories (Fergus and Taylor-Hilgard). Increasing differentiation among herds was noticeable at  $n = 20$  for mean proportion of SNPs with zero IBS, and the two different types of management histories were clearly differentiated at a sample size of 25 for IBS and mean kinship metrics. Increasing sample size also resulted in decreasing RMSE across all populations, regardless of management history. RMSE was primarily influenced by variance, as bias relative to the mean for  $n = 30$  was relatively low across all sample sizes. Evaluation of intrapopulation metrics using all 86 samples from the Beartooth Absaroka showed that RMSE continued to decrease at a slower rate beyond the smaller sample sizes of  $n = 5$  to  $n = 20$  and declined toward zero at a sample size greater than  $n = 25$ . In general, uncertainty in estimates indicated that we could not confidently

discern differences in IBS and mean kinship between herds of differing population histories at sample size 15.

Similar to our results for the intrapopulation simulation results, all metric estimates based on sample sizes of 5 to 15 had high variance and similar distributions across population comparisons (Figure 4). Furthermore, RMSE decreased with increasing sample size per herd and was generally not affected by the estimated relative kinship among herds. Increasing sample size changed the mean  $F_{ST}$  estimates the most of all three metrics across the 10,000 replicates, likely due to required filtering implemented for each individual replicate. As sample size increased, the number of SNPs removed at each filtering stage generally decreased. Thus, greater sample sizes included a disproportionately greater number of SNPs in the  $F_{ST}$  calculation than smaller sample sizes, which resulted in a greater change in the mean estimate for the 10,000 replicates than observed for the other metrics. Within the simulation we used a filtering process that typically is applied to calculate  $F_{ST}$  given a certain sample size. The differences in mean  $F_{ST}$  estimates by sample size due to the filtering process demonstrated that a comparison of  $F_{ST}$  estimates using different sample sizes has the potential to be especially problematic for this metric. For sample sizes 5 to 25, the distribution of  $F_{ST}$  estimates for many of the population comparisons overlapped, with greater differentiation among estimates at sample sizes of 20 and 25. Estimates of mean proportion of SNPs with zero IBS provided slightly different results and provided more non-overlapping distributions of estimates than  $F_{ST}$  estimates at sample sizes of 20 and 25. Mean kinship simulations resulted in similar overall results to IBS estimates, with decreases in overlap with

increasing sample size, but distributions overlapped only up to a sample size of 20. At sample size of 25, populations that were most related had distributions of mean kinship estimates that could be distinguished from populations that were estimated to be the least related.

By evaluating our simulation results, we concluded that a sample size of 25 was adequate for intrapopulation and interpopulation genetic management metric assessments of bighorn sheep populations using the HD Ovine array. Clear differences in estimates between herds for intrapopulation metrics and between herd comparisons for interpopulation metrics were apparent across metric types at a sample size of 25. In addition, RMSE consistently decreased with increasing sample size across all metrics and populations, regardless of management history. For the intrapopulation metrics, a sample size of 25 allowed for differentiation in estimate results among herds with different management histories, as the metapopulations (Glacier and Beartooth Absaroka) had lower mean intrapopulation kinship and IBS than the relatively small native herd (Taylor-Hilgard) and the reintroduced herd (Fergus; Figure 3). Interpopulation metric results also supported a sample size of 25 to discern meaningful differences among herd comparisons for all metrics (Figure 4). The relative ranking of herd comparisons differed slightly for  $F_{ST}$  estimates, in comparison to mean kinship and IBS, which is likely due to the metric's differing approach and filtering requirements.

Since the simulations drawn from a total sample size of 30 had less independence among replicates for larger sample sizes, the standard deviation estimates for the sample size of 25 may be slightly underestimated. To evaluate the extent of this issue, we

compared the standard deviation of the Beartooth Absaroka dataset at a sample size of 25 drawing from 30 samples and to that drawing from 86 samples. For the 86 sample size pool, pairs of replicate datasets will on average share about 7 out of 25 individuals. The standard error is roughly double for  $n=25$  (.00547) drawing from 86 samples in comparison to  $n=25$  using 30 samples (.00258). Even if the standard error estimates are actually double those presented in that figure, the distributions of estimates for each population would still have little overlap (Figure 3a). The results of intrapopulation simulations for the Beartooth Absaroka using 86 samples also suggested that a sample size greater than 25 can further reduce RMSE. Furthermore, the inclusion of additional Beartooth Absaroka samples resulted in slightly different within herd mean kinship and IBS estimates, in comparison to the 30-sample estimate. The slight change in estimates is likely due to the 30 samples being selected across a wide geographic area, whereas the 86-sample approach included more individuals from similar areas, which impacted overall estimated kinship and IBS. If a reduced RMSE is important to study objectives, these results indicate that increased sample size continues to improve RMSE beyond a sample size of 25 for within herd kinship estimates.

### Discussion

The framework outlined here provides an approach to identify the optimal sample size for 3 different common relationship metrics to facilitate comparing results among different populations, as well as quantify expected variance of estimates. As genomic metrics are estimates, rather than exact measures of quantities of interest, the inherent

uncertainty due to sampling is important to consider. Uncertainty can be introduced through selection of a limited number of individuals to represent the population of interest. For the evaluated bighorn sheep populations, a sample size of less than 20 to 25 would introduce an unacceptable level of uncertainty to estimates both within and between populations for the selected metrics. The sample size of 8-10 recommended by Nazareno et al., (2017) using plant genomic data would not provide clear results to compare any of our examined metrics among herds using our bighorn sheep genotype dataset. For example, transformed mean population kinship estimates at a sample size of 10 for Glacier ranged from 0.936 to 1.070 and for Fergus ranged from 0.918 to 1.012. However, differences in within herd kinship were detected at a sample size of 25, which provided estimates for Glacier ranging from 0.979 to 1.016 and for Fergus ranging from 0.933 to 0.966 (Figure 3a).

Thus, we suggest that a universal sample size rule is unlikely to exist given the complexities that impact genomic kinship and inbreeding estimates. These complexities include not only the molecular markers genotyped, but also the spatial distribution of samples and the genetic characteristics of the individuals and populations examined. The Beartooth Absaroka generally had lower RMSE for within herd mean kinship (Figure 3b and 3d), suggesting that a combination of population allele frequencies and the markers genotyped can result in slightly different variance and relative bias by population. Individual genetic samples are often non-randomly collected at low numbers, due to the cost and logistical difficulties of live animal capture, and a small sample of convenience may influence inferences from genetic management metric estimates. Uncertainty in

estimates provided by our bighorn sheep empirical simulations indicated that we cannot confidently discern differences in intrapopulation kinship and IBS between herds of differing population histories at commonly used sample sizes of 15 and lower (Figure 3).

Depending on the samples drawn using a sample size of 15 individuals for each herd, one might incorrectly infer the Glacier metapopulation had higher average within herd mean kinship than the Fergus and Taylor-Hilgard herds with more complex histories. However, when we increased sampling intensity by a modest amount (5-10 samples), the estimates provided more accurate inferences, such that we detected that the metapopulations had lower within population mean kinship and IBS than the smaller herds. Similarly, between population mean kinship, IBS, and  $F_{ST}$  estimates required higher sample sizes for precise estimates, and relative differences among the comparisons were not clear for sample sizes 15-20 or lower (Figure 4). Small sample sizes have the potential to result in a lack of clarity in the relative comparisons of estimates, which limits our ability to accrue reliable knowledge, effectively address ecological questions, and make management decisions. For example, mean kinship can be useful for making translocation decisions to maximize genetic diversity, by identifying the best source population for a translocation based on which candidate population is least related to the recipient population (Frankham et al., 2017). Thus, it would be beneficial for researchers to have a resource that can help with the process of deriving meaningful inferences based on comparing genomic estimates of mean kinship among populations within the same study area or among studies.

Researchers can add rigor by increasing sample size per population and using consistent sample sizes across compared populations to ensure comparable precision. To determine how many samples are adequate for study goals, a pilot study and simulations using our provided R code with actual genotype data from a select number of populations can help determine what sample size is required to detect differences among sampled herds that are deemed biologically meaningful. A challenging decision at the simulation stage is to determine the level of investment necessary to create a group of samples to use in a simulation context. One limitation of our work is the use of only 30 genotypes from which replicates were drawn, and this resulted in lack of independence among replicates. We sampled to the extent possible given the expense of captures and genotyping, and similar non-independence among replicates is a common issue among other empirical simulations (Nazareno et al. 2017, Gaughran et al. 2017). Employing a greater number of samples than 30 would allow us to generate more unique replicates. We can address this limitation by evaluating the intrapopulation simulations for the Beartooth Absaroka using 86 samples. The within population mean kinship and IBS simulations for the Beartooth Absaroka using 86 samples suggested that a sample size of 25 and greater can further reduce RMSE. Thus, if the largest sample size available in the simulation is unacceptably small, the relative bias and RMSE calculations can be misrepresented. The Beartooth Absaroka results indicated that researchers should look for a decrease in RMSE as sample size increases, which suggests that the maximum sample size available may be an acceptable reference point. If the RMSE decreases dramatically or the mean simulation estimate experiences large changes at all examined sample sizes, as seen from

sample sizes 5-15 in our simulation results, it may be necessary to examine a greater maximum sample size for effective sample size decisions. We suggest that the effect of non-independent replicates in empirical simulations with limited data is an important area for future research.

A simulation approach to evaluate sample size not only provides a procedure to standardize uncertainty as effectively as possible, but also provides more information to the scientific community to draw biological inferences. The standard deviation of the mean for all simulation replicates of a certain sample size serves as an appropriate estimate of the empirical standard error of the mean, as long as it is reasonable to assume that 30 sample estimates for our selected metrics are adequate to represent truth. The extent to which this is true depends on the proportion of the population that was sampled and the amount of variation in kinship and inbreeding found in the population. Thus, our standard errors should be viewed as approximate values for the range of standard error values that can be expected for metric estimates at a specified sample size. For example, the standard deviation or standard error of the mean for mean kinship simulation replicates for Fergus was 0.0211 for a sample size of 10, which could be interpreted to indicate that one could only expect to estimate the mean kinship for the population within  $\pm 0.0422$  for an estimate of 0.0448. In contrast, with a sample of 25 from the Fergus population, the standard deviation was reduced to 0.0065, so that the mean kinship estimate could be calculated within  $\pm 0.013$  for an estimate of 0.0445. The level of variance that is acceptable can vary by study goals and inherent differences among populations examined. For making management decisions, Frankham et al. (2017)

recommends detecting differences in mean kinship at a scale of 0.10 or finer. However, given our dataset and filtering protocols, we found that slightly greater precision was necessary to distinguish between different populations.

Bighorn sheep captures can be expensive and relatively difficult, so we promoted genetic sampling by collaborating agencies of all animals captured for management actions and non-genetic research objectives such as collaring or disease monitoring, given the low cost of collecting gene cards and biopsy punch samples. Our motivation to evaluate sample size was to inform managers as to the number of samples that should be genotyped per bighorn sheep herd for a future large scale genomic assessment of additional populations, by conducting simulations with a small number of herds with a range of possible management histories. In addition to providing sampling insights for our own study, we think that the herds used in our simulation may capture the range of attributes of bighorn herds throughout western North America, and our findings can inform sample size decisions for population genomic assessment of other bighorn sheep herds when the HD Ovine array is employed.

For other species, our approach can be applied to conduct sample size simulations specific to the examined species and molecular markers to provide information regarding the sampling required for evaluation of population genetic metrics. When animals are routinely captured for research and management purposes, it would be worthwhile for field personnel to collect genetic samples from all captured animals and build an archive of samples at a relatively low expense. In the event that the management agency or research entity has interest in research questions that can be addressed through population

genomics, this archive would enable geneticists to be much more efficient with resources and have samples available for future genomic techniques that may be developed. By conducting a small simulation pilot study on a subset of available samples, they can select an optimal number of samples per population to genotype for the larger study. An additional consideration that can be important in a pilot study may be the proportion of the population of interest captured within a sample. Specifically, when the sample includes  $\geq 5\%$  of the actual population with an accurate population size estimate, biologists can use a finite population correction factor to more accurately estimate the standard deviation of mean kinship. Our approach did not include a finite population correction factor, and thus our standard deviation estimates may be conservative for Taylor-Hilgard, our smallest population examined, with an estimated population size of 280. In regard to very large populations, spatial distribution of the selected sample size may become increasingly important and the reference sample size may need to be larger than 30 to successfully capture a representative sample that accurately describes mean kinship across the area of interest, particularly if the population may exhibit genetic spatial structure.

Selecting an appropriate sample size and making that decision consistent across populations of interest would enhance population genetic inferences and serve as an informative alternative to sampling based on convenience. Our suggested simulation method may not be possible for studies concerning species or populations that are extremely rare or difficult to capture, and in this case researchers are constrained to the available genetic samples. However, this method can be relatively easily implemented

with the use of other genomic marker types for species that are accessible or easily captured. Our annotated R code employs commonly used software, data formatting, and metrics for straightforward application to any genomic dataset. There are many more population genetic metrics than those included in this study, and we expect our workflow can be easily adapted to include almost any alternative genetic metric within the simulation script. When a sample size simulation is not feasible for a particular study, researchers could apply insight from other comparable species with similar marker sets to establish a reasonable sample size target. As genomics continues to become an increasingly important approach to address questions for both ecologists and wildlife managers, we recommend that sample size simulations be conducted to help standardize precision of results across evaluated populations to indicate the necessary sample size for research regarding relationship inferences and population structure. Rigorously evaluating uncertainty and adapting sample size decisions to each unique problem can serve to enhance inference reliability and maximize comparability of studies that estimate inbreeding and kinship.

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Tables and Figures

Figure 1. Bighorn sheep populations in Montana and Wyoming that were evaluated. Hunting districts are labelled for the Beartooth Absaroka population, but exact hunting district boundaries have been modified to more precisely show known species range.

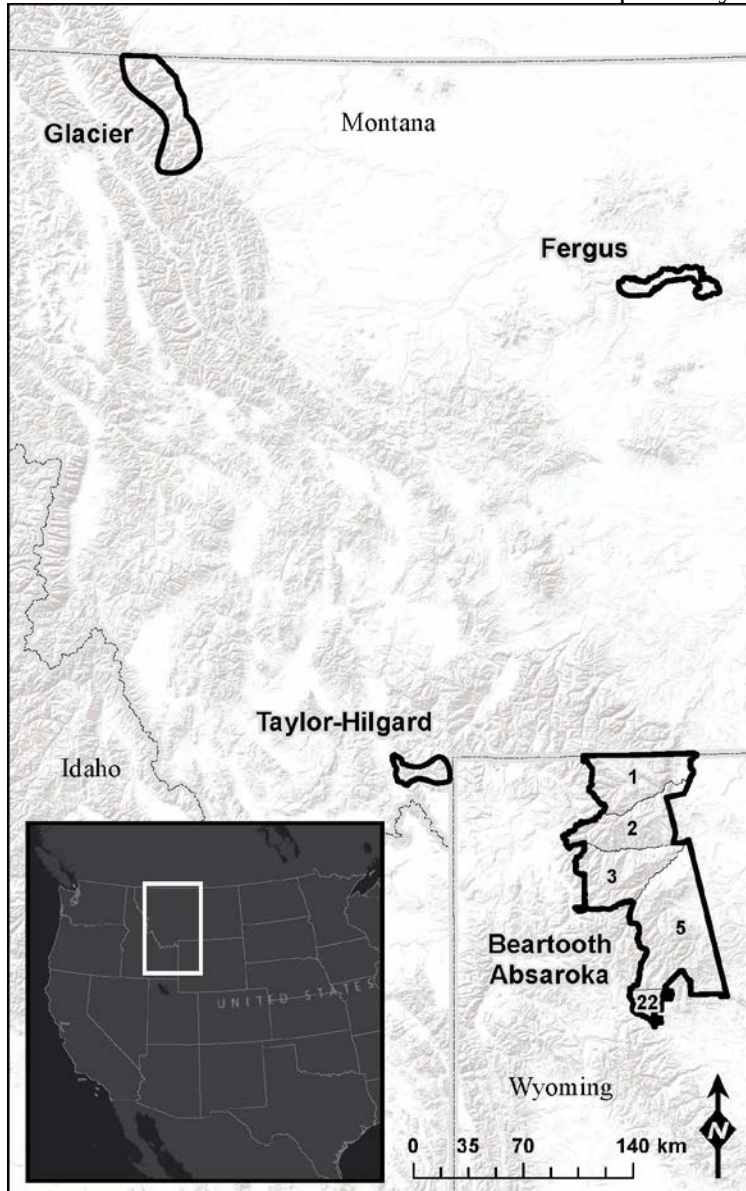


Figure 2. Principal component 1 (PC1; eigenvalue = 4.03) plotted against principal component 2 (PC2; eigenvalue = 3.40) for 30 Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) per population from four different herds. Different populations are indicated by color, including Beartooth Absaroka (light gray), Fergus (white), Glacier (black), and Taylor-Hilgard (dark gray). PCA analysis included 7,688 SNPs and was completed using Golden Helix SNP & Variation Suite v8.6 software (SNP & Variation Suite 2016).

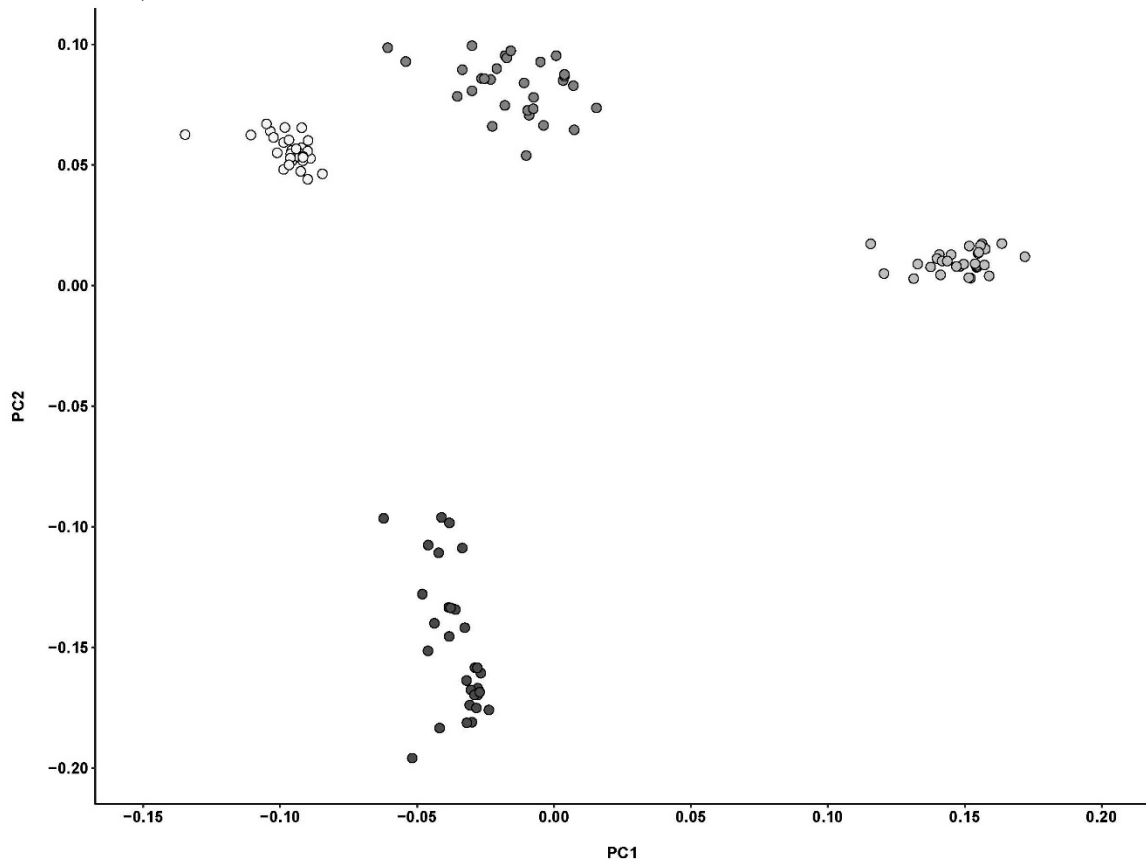


Figure 3. Boxplots of intrapopulation metric estimates based on 10,000 replicate simulations using empirical SNP genotypes from populations of Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*), including one minus mean kinship (A) and mean proportion of SNPs with zero identity by state (IBS; C) by increasing sample size. Center lines represent the median, box limits represent the 25<sup>th</sup> and 75<sup>th</sup> percentiles, whiskers indicate 1.5 multiplied by the interquartile range from the 25<sup>th</sup> and 75<sup>th</sup> percentiles, points represent outliers. Root mean squared error (RMSE) is plotted for mean kinship within herd (B) and mean proportion of SNPs with zero identity by state (IBS; D). Different populations are indicated by color, including Beartooth Absaroka (light gray), Fergus (white), Glacier (black), and Taylor-Hilgard (dark gray).

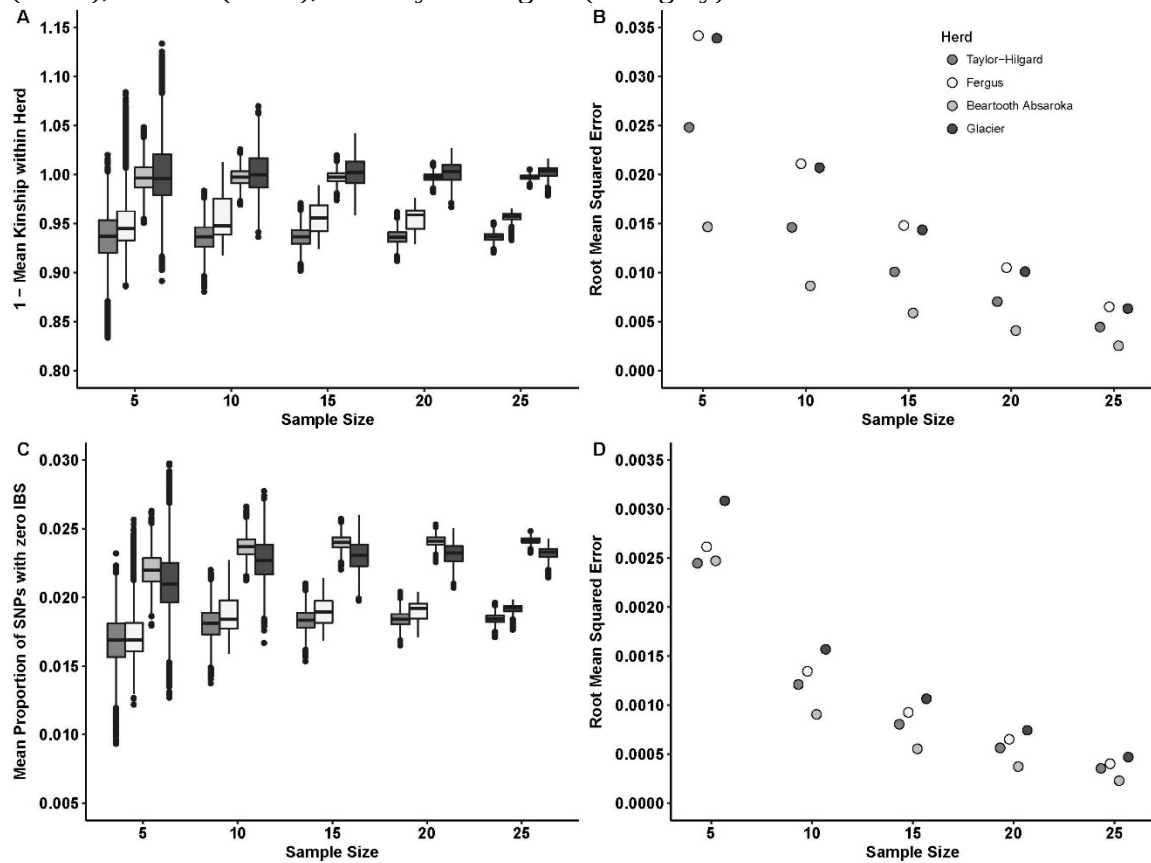
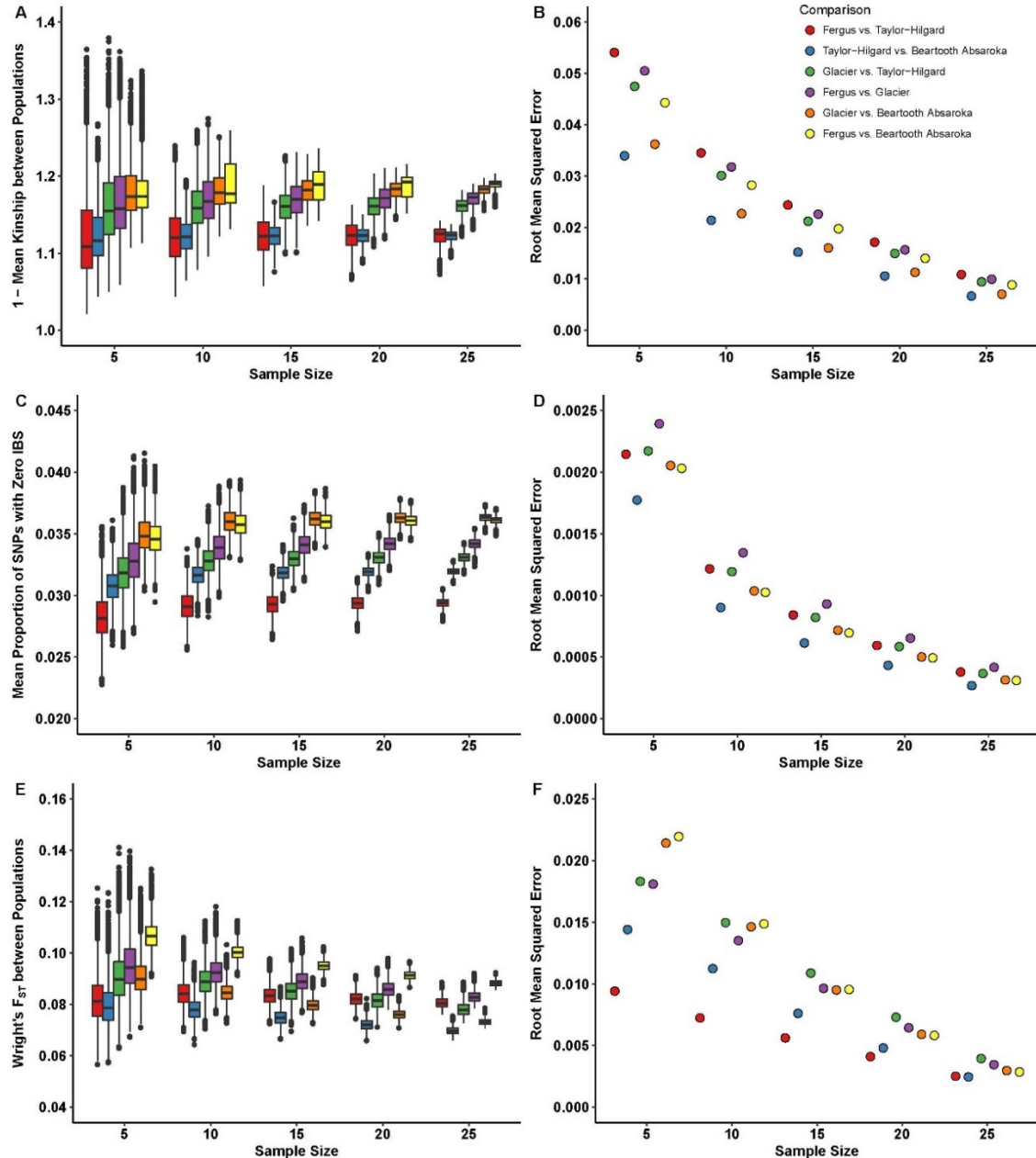


Figure 4. Boxplots of interpopulation metric estimates based on 10,000 replicate simulations using empirical SNP genotypes from populations of Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*), including one minus mean kinship (A), mean proportion of SNPs with zero identity by state (IBS; C), and Wright's  $F_{ST}$  (E) by increasing sample size per individual population included. Center lines represent the median, box limits represent the 25<sup>th</sup> and 75<sup>th</sup> percentiles, whiskers indicate 1.5 multiplied by the interquartile range from the 25<sup>th</sup> and 75<sup>th</sup> percentiles, points represent outliers. Root mean squared error (RMSE) is plotted for mean kinship within herd (B) and mean proportion of SNPs with zero identity by state (IBS; D), and Wright's  $F_{ST}$  (F). Different population comparisons are indicated by color.



CHAPTER THREE

EVALUATING WILDLIFE TRANSLOCATIONS USING GENOMICS: A BIGHORN  
SHEEP CASE STUDY

Contribution of Authors and Co-Authors

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Contributions: Led collaborations to obtain samples, collected samples in the field, completed laboratory work, analyzed data, wrote the manuscript, edited the manuscript and approved the final version

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Manuscript Information

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Abstract

Wildlife restoration often involves translocation efforts to reintroduce species and supplement small, fragmented populations. We examined the genomic consequences of bighorn sheep (*Ovis canadensis*) translocations and population isolation to enhance understanding of evolutionary processes that affect population genetics and inform future restoration strategies. We conducted a population genomic analysis of 511 bighorn sheep from 17 areas, including native and reintroduced populations that received 0-10 translocations. Using the Illumina High Density Ovine array, we generated datasets of 6,155 to 33,289 single nucleotide polymorphisms and completed clustering, population tree, and kinship analyses. Our analyses determined that natural gene flow did not occur between most populations, including two pairs of native herds that had past connectivity. We synthesized genomic evidence across analyses to evaluate 24 different translocation events and detected eight successful reintroductions (i.e. lack of signal for recolonization from nearby populations) and five successful augmentations (i.e. reproductive success of translocated individuals) based on genetic similarity with the source populations. A single native population founded six of the reintroduced herds, suggesting that environmental conditions did not need to match for populations to persist following reintroduction. Augmentations consisting of 18-49 animals including males and females succeeded, whereas augmentations of two males did not result in a detectable genetic signature. Our results provide insight on genomic distinctiveness of native and reintroduced herds, information on the relative success of reintroduction and

augmentation efforts and their associated attributes, and guidance to enhance genetic contribution of augmentations and reintroductions to aid in bighorn sheep restoration.

Key Words: bighorn sheep, genomics, kinship, *Ovis canadensis*, population genetics, translocation

### Introduction

Population restoration using translocation, which involves moving live individuals from one area to another, is an important tool for restoring biodiversity (Weeks et al. 2011, IUCN/SSC 2013). Reintroduction starts a new population within formerly occupied landscapes, whereas augmentation adds individuals to an indigenous or reintroduced population (IUCN/SSC 2013). The goal of augmenting a small, genetically isolated population is frequently to enhance viability by increasing the number of individuals in certain demographic groups or increasing genetic diversity to improve reproductive fitness, termed genetic rescue (Tallmon et al. 2004, IUCN/SSC 2013).

Successful survival and breeding of translocated animals can depend on many factors, including environmental similarity between source and release areas in case of local adaptation, reproductive attributes of the species, habitat quality of the target area, distance from other conspecific populations, number of individuals moved, and management program duration (Griffith et al. 1989, Magdalena Wolf et al. 1998, Groombridge et al. 2012, Bleich et al. 2018). Managers can control some of the factors that influence survival and breeding of translocated animals to enhance the probability of

translocation success. However, the relative survival and reproduction of translocated individuals in augmentations and reintroductions and the long-term genetic effects of translocation efforts may vary widely by species and population of interest. Thus, it is beneficial to enhance understanding of the multi-generational genetic effects of reintroduction and augmentation efforts in wild populations (Moraes et al. 2017, White et al. 2018).

While reintroductions can successfully establish a new wildlife population, previous studies have found that some populations thought to be the result of reintroduction efforts were in fact the result of recolonization (Kruckenhauser and Pinsker 2004, Statham et al. 2012, Stewart et al. 2017). In this case, animals from nearby areas naturally dispersed and established a population in previously occupied terrain, meaning that costly reintroduction efforts may not have been necessary. Further, some studies have suggested that matching environmental attributes between the source population and the reintroduction area is important to establishment of translocated animals, whereas others have found that sourcing from a native, rather than reintroduced, population is more important (Olson et al. 2013, Malaney et al. 2015). Thus, evaluating the genetic source of populations thought to be reintroduced can provide insight into whether similar translocation efforts would be productive in other areas, or if enhancement of habitat connectivity would be more important to encourage recolonization (Stewart et al. 2017). Further, evaluating the genetic differences between populations that were the result of reintroduction events and their founding source can teach us about the many influences on the reintroduced population's evolution (Jamieson

2011, White et al. 2018). For example, the literature frequently recommends large founder populations for reintroductions to represent the genetic profile of the source and minimize inbreeding (Jamieson and Lacy 2012). However, the necessary founder size for reintroductions may vary widely by species, population, and reintroduction area of interest. Genetic evaluation of reintroduced populations can help address these uncertainties in reintroduction planning.

In addition, augmentation of existing populations may not result in genetic contribution to the target recipient population. Previous studies have found that the sex of animals moved and species mating strategy can influence whether translocated animals breed or not with the resident population (Sigg et al. 2005, Miller et al. 2009, Mulder et al. 2017). For example, no translocated males contributed to a resident desert tortoise (*Gopherus agassizii*) population; resident and translocated females only produced progeny with resident males (Mulder et al. 2017). Thus, determining what factors influence animal breeding following augmentation can help biologists avoid implementation of costly augmentation efforts that fail to result in genetic contribution (Fischer and Lindenmayer 2000, Armstrong and Seddon 2008). In addition, evaluating unassisted genetic connectivity among populations can be used to assess whether a population needs a translocation. For example, dispersal was greater than expected among reintroduced elk (*Cervus elaphus*) populations originally thought to be genetically isolated (Hicks et al. 2007). If it is determined that dispersal provides sufficient genetic connectivity to certain populations, wildlife managers could devote resources to augmentation efforts for other populations that are genetically isolated.

After a translocation event, selection, genetic drift, unassisted gene flow, and mutation can influence population evolution on different timescales, making it difficult to identify relative influences on population viability (Frankham et al. 2017). Thus, assessing how translocations affected multiple isolated populations with contrasting translocation histories and situations could help us understand the variation in population evolution (Moraes et al. 2017, White et al. 2018). Genomic techniques can be used to evaluate the success of past translocations and plan future efforts (Bell et al. 2019). Specifically, genomic analyses can help evaluate the genetic effects of previous translocations and assess mean kinship to inform new translocation efforts (Frankham et al. 2017, Jahner et al. 2018). Additionally, comparing long-term genomic effects of translocations in multiple wild populations would further understanding of this conservation tool and inform future strategies for genetic management of species with fragmented distributions and genetically isolated populations.

Bighorn sheep (*Ovis canadensis*) in North America continue to face challenges found in many other fragmented wildlife populations, such as poor demographic rates in some populations and widespread disease issues. Prior to European expansion across the American west in the late 19<sup>th</sup> and early 20<sup>th</sup> centuries, there were an estimated 1.5 to two million bighorn sheep (Seton 1929). However, market hunting, competition with domestic sheep, and new respiratory diseases introduced by comingling domestic sheep and goats resulted in a decline by 1960 to fewer than 20,000 bighorn sheep in scattered patches across North America (Norris 1877, Buechner 1960, Valdez and Krausman 1999, Whittlesey et al. 2018a, 2018b, Cassirer et al. 2018). Continued concerns regarding

resident pathogens in bighorn sheep populations and disease spillover from domestic sheep that may cause epizootic events have resulted in management to keep populations small and isolated (Cassirer et al. 2016, Butler et al. 2018).

Translocation has been the primary management tool used to reintroduce bighorn sheep to previously occupied areas and augment the size and genetic diversity of existing populations. Over 1,460 translocations of 21,500 bighorn sheep have taken place across the indigenous range of this species, and some populations have been studied as examples for genetic rescue (Hogg et al. 2006, Olson et al. 2012, Wild Sheep Working Group 2015, Poirier et al. 2018). Despite these efforts, restoration of the species continues to be a challenge, as there are still large areas of unoccupied historic range and many small populations with poor demographic performance (Montana Department of Fish, Wildlife and Parks 2010).

To inform future translocations and restoration of bighorn sheep, we used a genomic approach to evaluate multiple hypotheses regarding reintroduction, augmentation, and unassisted gene flow of Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) populations with different origins and translocation histories (Figure 1). The origin of bighorn sheep populations included native (indigenous) herds and reintroduced herds with different founder sizes and number of generations since establishment. Some reintroduced and native herds received augmentations (translocations into an existing herd). Using this diverse set of populations, we evaluated unassisted genetic connectivity within and between herds (i.e. gene flow indicating natural movements of breeding individuals) to determine 1) if large, spatially-structured

populations expected to be genetically connected contain barriers to gene flow and 2) if populations managed and expected to be isolated have genetic contributions due to natural dispersal. Secondly, we evaluated genetic differences between reintroduced herds and their documented founding source to determine 1) if the population was established via translocation or recolonization and 2) what factors influenced reintroduction success (i.e. lack of signal for recolonization), such as environmental attributes or origin of the source herd. Thirdly, we evaluated genetic differences between reintroduced herds and their founding source to compare alternative hypotheses about which factors influenced the evolution of herds after reintroduction, including founder population size and unassisted/assisted gene flow between herds. Finally, we evaluated relative contributions of past augmentations (i.e. reproductive success of translocated individuals) to determine what factors influenced augmentation success, including the number and sex of translocated animals. We synthesized this information to inform risk and benefit assessments of future augmentations and reintroductions. The results of this study provide insights into long-term genomic consequences of different translocation approaches used for species restoration and may help inform future genetic management of bighorn sheep.

## Methods

### Study Populations

We evaluated sixteen Rocky Mountain bighorn sheep populations found in the United States (U.S.) and Canada, including the U.S. states of Montana, Wyoming, Colorado, and Utah, and the Canadian provinces of Alberta and British Columbia (Figure

2). We sought to sample at least 20-25 individuals per population, based on sample size simulations that determined sampling less than this number would introduce an unacceptable level of uncertainty to estimates of genomic kinship between populations (Flesch et al. 2018). We evaluated seven native herds, including one population of 80-90 animals with no augmentations (Galton), three small to moderately sized populations (80-200 animals) with augmentation attempts (Spanish Peaks, Taylor Hilgard, and Stillwater), and three large, continuous populations (380-3800 animals), including Beartooth-Absaroka, Castle Reef, and Glacier. Castle Reef is a geographic portion of a large, spatially-structured population (a collection of subpopulations that occupy distinct geographic areas but are linked by animal movement) and is expected to have connectivity across four administrative units (Figure 2; Montana Department of Fish, Wildlife and Parks, 2010). Beartooth-Absaroka and Glacier provided baseline genetic examples of large, spatially-structured populations prior to widespread fragmentation of the species' range without an extensive history of augmentations. Due to large population size and range, we had a greater sample size of 90–95 individuals to represent each of these two herds. The Beartooth-Absaroka population spans multiple management units along the eastern portion of the Greater Yellowstone Area, including Yellowstone National Park and Wyoming hunt units 1, 2, 3, 5, and 22 (Figure 2). Wyoming units 5 and 22 each received an augmentation from a nearby native population, Whiskey Mountain. The Glacier population spans the U.S.-Canada border, including Glacier and Waterton Lakes National Parks. A large lake and adjacent forest forms at least a partial geographic barrier to most bighorn sheep movement within Glacier, so in some analyses

we assessed the Glacier population in units north and south of this drainage (Figure 2; Tosa et al. In prep).

We also evaluated nine reintroduced herds. For eight of those herds, one or all of the initial founding sources were included in this study (Figure 2). We considered translocations within three years of the first reintroduction event to an unoccupied area to be part of the potential founding source. Founder size in reintroduced populations ranged from six to 53 bighorn sheep. Based on a generation length of six years, estimated using the mean age of reproductively active females, there were five to 11 generations since establishment of the reintroduced study populations (Hogg et al. 2006, Johnson et al. 2011). Montana Department of Fish, Wildlife and Parks (2010) defined five ecological regions that contain bighorn sheep herds within the state of Montana, including prairie/mountain foothills, prairie/breaks, northwest montane, mountain foothills, and southern mountains. We initially included the ninth reintroduced population, geographically distant Dinosaur National Monument in northern Colorado and Utah, to serve as a same-species outgroup, but later learned that some translocations from the previously mentioned Whiskey Mountain herd occurred in this region.

As a true outgroup, we evaluated a different subspecies, Sierra Nevada bighorn sheep (*Ovis canadensis sierrae*; Buchalski et al., 2016; Wehausen et al., 2005). We included five outgroup samples following a similar genotyping approach by Sim *et al.* (2016). Three Sierra Nevada samples originated from the native Sawmill population, and two samples came from the reintroduced Wheeler population, which was founded by Sawmill individuals. We gathered records for all known translocations received by study

populations, including translocations that originated from areas not included in the study. Where both the source and recipient populations were in this study, approximately zero to eight generations occurred between augmentation and genetic sampling.

#### Sample Collection and DNA Extraction

Bighorn sheep were live-captured for genetic sampling between 2002 and 2018. Each population was sampled over a time span of 1 day to 4 years (less than one generation), except for the Glacier population that was sampled over a period of 9 years (about 1.5 generations). Animal capture and handling protocols were approved by Institutional Care and Use Committees at Montana State University (Permit # 2011-17, 2014-32), Montana Department of Fish, Wildlife, and Parks (Permit # 2016-005), Parks Canada (Permit # WL-2005-638), Wyoming Game and Fish Department (Permit # 854), U.S. Geological Survey (Permit # 2004-01, DINO-2008-SCI-0010), and University of Montana (Permit # 024-07MHWB-071807 and 012-16MMMCWRU-022916). Genetic samples included FTA Classic gene cards, whole blood samples, biopsy punches from ear cartilage, and muscle or lung tissue from hunter-harvested or road killed animals. We extracted DNA with Maxwell 16 LEV Blood DNA and Tissue Kits using the Promega Maxwell 16 Magnetic Particle Automated Extractor per kit instructions. For gene cards, we used a modification of the Promega LEV Blood DNA kit. Specifically, we incubated two to three 5mm gene card punches with proteinase K and lysis buffer in a DNA IQ spin basket (Promega), spun at 3000 XG for five minutes, and loaded the flow-through into the Maxwell 16 LEV Blood DNA cartridge.

### Genomic Dataset and Quality Control

We genotyped bighorn sheep samples using the Illumina High Density (HD) Ovine array, also referred to as a SNP (single nucleotide polymorphism) chip. For genotyping, we used samples with a minimum of 300 ng of DNA, a minimum DNA concentration of 20 ng/ $\mu$ L, and a 260 nm/280 nm ratio of 1.0 to 1.5. The Ovine array is composed of 606,006 SNPs and was originally developed for domestic sheep with a density of one SNP per 4.279kb, but its development included five bighorn sheep and four Dall's sheep (*Ovis dalli*; (Kijas et al. 2009, 2014). Speciation between domestic and bighorn sheep occurred about three million years ago, but the two species can interbreed to produce viable hybrid offspring (Young and Manville 1960, Bunch et al. 2006). Domestic and bighorn sheep have the same number of chromosomes and are expected to have high genomic synteny (Poissant et al. 2010). An estimated 24,000 SNPs in the HD Ovine array are informative for Rocky Mountain bighorn sheep, and the domestic sheep reference genome enables mapping SNPs to chromosomes (Kohn et al. 2006, Miller et al. 2015). SNP chips can have ascertainment bias, as only a select number of individual samples were evaluated to create the panel and the chip can have an uneven distribution of informative SNPs (Albrechtsen et al. 2010), but Flesch *et al.* (2018) determined that Rocky Mountain bighorn sheep populations could be differentiated using the HD Ovine array with adequate sample size.

We imported all Illumina genotype data into Golden Helix SNP and Variation Suite v8.6.0 (SNP & Variation Suite 2016). Using Golden Helix software, we concatenated multiple datasets, mapped the dataset to the domestic sheep reference genome Oar v.4.0, removed samples with a call rate less than 0.85, removed markers on

sex chromosomes and with unknown mappings, and exported data into PLINK format for filtering (Purcell et al. 2007, *SNP & Variation Suite* 2016). We completed additional filtering and data analysis in the RStudio environment using GNU bash v4.3.48(1) and R v3.4.4 (*GNU bash, version 4.3.48(1)-release x86\_64-pc-linux-gnu*, 2013; R Core Team, 2017; RStudio Team, 2015). For kinship calculations, we filtered SNPs using a minor allele frequency of less than 0.0001 to remove monomorphic and extremely rare markers, and we removed markers with poor performance by requiring a SNP call rate greater than 0.99 (de Cara et al. 2013, Huisman et al. 2016). The dataset used for the kinship analysis did not require additional filtering of low frequency SNPs prior to analysis based on KING software guidelines (Manichaikul et al. 2010a). To infer population structure and ancestry, we further filtered the dataset using a minor allele frequency of 0.01 and a Hardy-Weinberg equilibrium p-value of less than 0.00001 (Huisman et al. 2016). We used linkage disequilibrium (LD) pruning to remove non-independent SNPs that informed the presence of nearby variants, using a window size of 100 SNPs, window increment of 25 SNPs, and an LD statistic of  $r^2$  (Huisman et al. 2016).

#### Population Structure, Ancestry, and Kinship Analyses

Because we did not know the degree of genetic similarity among evaluated individuals and populations, we addressed our research objectives using multiple complementary methods that employed differing approaches and assumptions regarding the categorization of individuals into genetic populations. To assess the number of populations (K clusters) in our dataset and estimate global ancestry (estimated ancestry proportions from each population for each individual), we used fastStructure software

(Pritchard et al. 2000, Raj et al. 2014, Pina-Martins et al. 2017). This analysis used clustering to estimate the number of distinct genetic groups and employed a probabilistic analysis to assign individuals to one or more genetic populations without reference to the sampling locations. The STRUCTURE approach assumes that there is a certain number of genetic populations that contain random mating and that these distinct groups have different allele frequencies. Thus, the fastStructure model can provide useful information regarding if animals moved in a translocation successfully bred with the resident population, as global ancestry results can detect hybrids of multiple genetic populations. However, the approach has lower accuracy with uneven sampling and assumes random mating, which is frequently inaccurate for wild populations (Alexander et al. 2009, Puechmaille 2016, Frankham et al. 2017). In addition, while STRUCTURE models can evaluate admixture, they lack a temporal assessment of fragmentation (Frankham et al. 2017).

Thus, we also completed a nested multidimensional scaling (MDS) analysis using KING v2.1.4 (Manichaikul et al. 2010a). In contrast to fastStructure, MDS can identify clusters of populations without assumptions regarding Hardy-Weinberg equilibrium, random mating, or the cause of population structure (i.e. isolation-by-distance). This is because MDS does not assign individuals to a genetic population before or after the analysis. Instead, MDS uses an unsupervised approach to reduce the dimensionality of the genomic dataset to an interpretable plot, which allows for visualization of patterns of genomic variation and identification of separation among individual samples to address

research questions regarding genetic similarity between individuals and populations. We used a nested approach to understand substructure across multiple levels of organization.

To determine the lineage of reintroduced populations in comparison to their hypothesized founding source, we estimated a bifurcating tree that described differentiation among populations. Because we did not expect most of the evaluated bighorn sheep populations to have dispersal of breeding animals between them, we assigned population identity of individuals based on sampling location. To model differentiation due to genetic drift among these populations defined by geography while still accounting for gene flow, we estimated a maximum likelihood bifurcating tree of populations using Treemix v1.13 (Pickrell and Pritchard 2012). Treemix uses allele frequencies from each population and Gaussian approximation for genetic drift to estimate a tree that represents each population on a branch (Pickrell and Pritchard 2012). The amount of genetic drift that occurred among populations is represented by a drift parameter calculated by the Treemix software (Pickrell and Pritchard 2012). Thus, we could use this analysis to determine the lineage of reintroduced populations, in comparison to their hypothesized founding source. Possible admixture (gene flow) events between branches of the tree were evaluated using a stepwise likelihood approach, where the software searched the tree for the optimal location of each translocation or dispersal event (Pickrell and Pritchard 2012). To further specifically evaluate gene flow between pre-defined populations, we conducted a three-population test (Reich et al. 2009, Pickrell and Pritchard 2012). Thus, we used these analyses to specifically identify

previously unknown admixture events due to dispersal and confirm genetic contribution of augmentation events.

Finally, we estimated mean kinship between populations to evaluate genetic differences in reintroduced herds from their documented founding source. This information can also inform future augmentation decisions by identifying potential source and recipient populations based on their level of genetic similarity (Ballou and Lacy 1995, Frankham et al. 2017). Kinship, also called coancestry, represents the probability that two randomly sampled alleles from two individuals are identical by descent (Manichaikul et al. 2010a). Mean kinship calculated between populations serves as a measure of population similarity, with higher values interpreted as populations that are more related (Frankham et al. 2017). Thus, genetic differentiation between populations is represented by one minus mean kinship (Frankham et al. 2017). We estimated mean kinship between populations using KING v2.1.4 (Manichaikul et al. 2010a). To assess how characteristics of translocations influenced the current kinship between reintroduced populations and their founding source, we evaluated six herd attributes that may affect divergence of reintroduced populations using boxplots. These attributes included founder population size, number of generations since herd establishment, number of augmentations from the founding source, number of source populations, number of augmentations from other sources, and level of connectivity with neighboring herds.

## Results

### Genomic Dataset and Quality Control

We genotyped 541 bighorn sheep samples using the HD Ovine array, resulting in a dataset composed of 606,006 SNPs. Using a sample call rate of 0.85, we filtered 30 samples from the dataset, and subsequently evaluated 511 samples from 17 different populations. We met our sample size goal of at least 20-25 bighorn sheep for fourteen Rocky Mountain bighorn sheep populations, excluding Highlands and Galton (Table 1). We included Highlands and Galton populations in the MDS and fastStructure analyses, where overall sample size was less likely to affect the results, but excluded these two low sample size populations from the Treemix analysis and interpreted their mean kinship results with caution (Highlands  $n=17$ ; Galton  $n=5$ ). After filtering, 33,289 SNPs were used for kinship estimates; 6,155 SNPs were used for the remaining analyses.

### Population Structure

To evaluate population structure, we estimated the number of genetic populations and ancestry proportions for each individual and population using a fastStructure analysis. Our fastStructure analysis of seventeen bighorn sheep populations suggested there were six genetic clusters ( $K$ ). Three clusters consisted of pairs of native populations that were geographically proximate, including Glacier with Galton, Spanish Peaks with Taylor Hilgard, and Stillwater with Beartooth-Absaroka. Two other genetic groups encompassed source and receiving populations of reintroductions. Wild Horse Island and Paradise formed a distinct cluster because Wild Horse Island was the sole source for the Paradise reintroduction and later augmentation. The Castle Reef cluster

encompassed the greatest number of populations, as Castle Reef bighorn sheep or their descendants founded the other populations in the genetic group, including Fergus, Middle Missouri, Petty Creek, Lost Creek, Highlands, and Tendoys. The sixth cluster included geographically distant Dinosaur and outgroup Sierra Nevada. In the  $K=7$  analysis, Petty Creek formed its own cluster, and in the  $K=9$  analysis, Lost Creek formed its own cluster, which may be due to past augmentation from sources not included in this study.

We detected past augmentations between genetic groups, where the recipient population contained partial ancestry from the cluster of the source population and the populations were geographically distant, such that movement of breeding animals between the areas was unlikely. We detected the following translocation events, indicating that translocated individuals survived and reproduced successfully at the release site: Wild Horse Island to Taylor Hilgard, Lost Creek to Taylor Hilgard, Wild Horse Island to Tendoys, and Castle Reef to Tendoys (Figure 2). The success of an augmentation from Castle Reef to Spanish Peaks was unclear, as we detected genetic ancestry from the cluster including Castle Reef in fewer than five individuals in Spanish Peaks. We also detected augmentations from populations that were not directly sampled but were also augmentation sources to herds in our study. The Whiskey Mountain population (Figure 2; Wyoming hunt unit 10) provided two augmentations that likely made a genetic contribution to Wyoming hunt units 5 and 22 in the Beartooth-Absaroka (Wild Sheep Working Group 2015, Love Stowell et al. 2020). Managers moved individuals from Whiskey Mountain to augment the reintroduced Dinosaur population three times, which was detected by a shared cluster between Dinosaur and augmented

Beartooth-Absaroka hunt units 5 and 22. Our results also suggested past gene flow between the populations found in Galton and Ural-Tweed, which was not included in our study (Figure 2). Managers moved bighorn sheep from Ural-Tweed to Wild Horse Island for an augmentation, and genetic ancestry from the same cluster found in Galton and Wild Horse Island indirectly suggested both genetic contribution of the augmentation to Wild Horse Island and past connectivity between the populations found in Galton and Ural-Tweed. Finally, our results suggested historical gene flow between the Glacier population and geographically proximate Castle Reef population, as Castle Reef bighorn sheep shared genetic ancestry from the same cluster with the southern unit of the Glacier population.

#### Genetic Distinctiveness

We used the MDS analysis to further evaluate clusters of populations in a hierarchical fashion without assumptions required by the fastStructure software. We identified four clusters of populations in the MDS analysis and evaluated the four MDS clusters separately (Figure 3). The cluster shown in Figure 3A encompasses all populations found outside of Montana, including the Sierra Nevada, Dinosaur, and the Beartooth-Absaroka, as well as Stillwater, which is located in Montana but near the Montana-Wyoming border. Most Stillwater individuals separated from the Beartooth-Absaroka, although the two populations were assigned into a single fastStructure cluster. However, four Stillwater individuals did not separate from Beartooth-Absaroka genotypes. Outgroup Sierra Nevada and geographically distant Dinosaur each separated from the other populations.

Figure 3B, which generally depicts the extensive history of translocations from Castle Reef, included nine out of seventeen populations, similar to the fastStructure results. Wild Horse Island, Lost Creek, and Petty Creek populations were all founded by Castle Reef animals but separated more from their founding source than the Fergus, Middle Missouri, and Highlands populations, indicating different influences on the evolution of these populations. The Paradise population separated somewhat from its founding and only augmentation source, Wild Horse Island. Results for individuals from the Tendoys reflected the herd's translocation history from five different sources. Most individuals in the Tendoys clustered with the Castle Reef population, which provided an augmentation, rather than the Lost Creek population, which founded the Tendoys population. Four Tendoys individuals clustered with Wild Horse Island, the source of one augmentation. Three Tendoys individuals plotted partway between Castle Reef/Lost Creek and Wild Horse Island clusters, likely representing hybrids from these two lineages. One Tendo individual had an outlier genotype in the overall analysis (Figure 3C), which may represent an individual descended from neighboring populations in Idaho or two augmentations from areas not in the study.

Figure 3D includes the Taylor Hilgard and Spanish Peaks populations, which separated in the subset MDS analysis, despite being grouped into the same fastStructure cluster, suggesting a lack of current genetic connectivity. When compared to all other populations, Spanish Peaks plotted closer to the nearby native populations of Stillwater and Beartooth-Absaroka, whereas Taylor Hilgard plotted closer to its augmentation sources, Lost Creek and Wild Horse Island (Figure 3C). Figure 3E includes Glacier and

Galton, where Glacier genotypes generally separated within the population based on location, north and south of a large lake drainage, and Galton was distinct from Glacier.

#### Population Tree and Genetic Contributions from Augmentations

We used Treemix to evaluate the lineages of populations defined by geography using a bifurcating tree that accounted for gene flow. The population tree generated by Treemix was consistent with fastStructure, as the nodes on the tree generally grouped together populations found in the same fastStructure clusters (Figure 4). Sierra Nevada was defined as the outgroup. Geographically distant Dinosaur was the least related to all other evaluated Rocky Mountain bighorn sheep populations. Stillwater and Beartooth-Absaroka were grouped together, followed by Spanish Peaks and the Taylor Hilgard. Glacier formed its own branch, as Galton was not evaluated due to low sample size. The remaining populations grouped together were influenced by a history of translocations from Castle Reef and its descendant populations. The population founded by Castle Reef that showed the highest divergence was Wild Horse Island, and the founding of Paradise by Wild Horse Island was accurately represented in the population tree. Reintroduction of the Tendoy herd by founders from Lost Creek was depicted in the population tree, despite the possibility for genetic connectivity with neighboring herds in Idaho and later augmentations directly from Castle Reef. Petty Creek and Lost Creek showed greater drift parameters from Castle Reef than Fergus and Middle Missouri, consistent with MDS and fastStructure results, although this may be because these populations received augmentations from other herds not included in this study, rather than genetic drift.

The Treemix model identified four augmentation events between specific populations (Figure 4, orange lines; Table 2). All four identified gene flow events represented known augmentations where shared ancestry between populations was identified by fastStructure (Table 2). The direction of the plotted augmentation event was the least stable feature of the Treemix analysis, and we reversed the direction of augmentation events identified 3<sup>rd</sup> and 4<sup>th</sup>, as the direction was known and unassisted gene flow was unlikely due to geographic separation of the identified areas (Table 2). Translocation weight estimated the proportion of alleles contributed by the source population, assuming admixture occurred in one generation (Pickrell and Pritchard 2012). The three-population test, which tested for admixture between populations defined by geography, further supported genetic contributions of Wild Horse Island, Castle Reef, and Lost Creek to the Tendoy from translocations. In addition, the three-population test suggested unassisted gene flow (natural movement of breeding animals) between Stillwater and the Beartooth-Absaroka, consistent with Figure 3A.

#### Comparing Translocation History and Genomic Analyses

To identify which reintroduction and augmentation efforts made a genetic contribution to the recipient population, we synthesized genomic evidence from fastStructure, MDS, and Treemix for 24 different translocation events where both populations were included in the study (Table 3). For all eight reintroduced herds with founding source data, genetic contribution of the suspected founding group(s) to the contemporary population was suggested by at least two analyses. Fifteen out of 24 translocations were augmentations, including 11 unique pairs of source and recipient

populations. Four out of 11 augmentation pairs could not be assessed for genetic contribution, as the source herd was the same as or genetically similar to the founding source. Of the remaining seven source and recipient augmentation pairs, we detected five augmentation pairs in multiple analyses, including Wild Horse Island to Taylor Hilgard, Lost Creek to Taylor Hilgard, Wild Horse Island to Tendoys, Castle Reef to Tendoys, and Beartooth-Absaroka (due to augmentation from Whiskey Mountain) to Dinosaur. In addition, our analyses indirectly suggested the genetic contribution of an augmentation from Ural-Tweed to Wild Horse Island. Augmentations to Spanish Peaks and Stillwater from Castle Reef were not detected in multiple analyses, suggesting that after these two augmentation events, the translocated individuals did not survive or reproduce.

#### *Genetic similarity*

We estimated mean kinship between bighorn sheep populations to evaluate genetic similarity and inform future augmentation efforts based on mean kinship (Table 1). Mean kinship values ranged from -0.868 (most unrelated) to -0.001 (most related). All mean kinship values between herds were negative, indicating that allelic correlations were less than expected under an assumption of Hardy-Weinberg equilibrium (Frankham et al. 2017). A lack of Hardy-Weinberg equilibrium between herds was consistent with the geographic isolation of most populations and a limited number of recent translocations (Figure 2). The Sierra Nevada outgroup and geographically distant Dinosaur population had the lowest mean kinship with all other populations. The highest mean kinship value was found between the Middle Missouri and Castle Reef populations (-0.001, standard deviation of 0.032), likely because the Middle Missouri herd was

founded by one translocation of 28 Castle Reef bighorn sheep in 1980 and had no other augmentations.

We estimated mean kinship between reintroduced herds and their founding source to evaluate six attributes that could affect population evolution since reintroduction. These attributes included founder population size, number of generations since herd establishment, number of augmentations from the founding source, number of source populations, number of augmentations from other sources, and level of connectivity with neighboring herds. As the number of augmentations from other areas and the number of source populations increased, mean kinship with the founding source generally decreased. All six examined herd attributes likely influenced evolution of reintroduced herds to differing extents, which complicated our interpretation of which attributes were dominant. However, application of this approach with a greater sample size of populations could serve as a method to further evaluate which population attributes influenced reintroduced population evolution and genetic divergence from the founding source.

### Discussion

We examined the genomic consequences of bighorn sheep restoration to enhance understanding of evolutionary processes such as gene flow from genetic connectivity or genetic drift from isolation that affect population genetics and to inform genetic management of fragmented populations through future translocation strategies. First, our genetic structure results indicated genetic connectivity due to natural dispersal of individuals within large, spatially-structured populations, but not between most

populations. Second, genetic similarity between reintroduced populations and their documented founding source indicated that translocation, rather than recolonization, was the source of all populations initially expected to be reintroduced. Third, genetic similarity between reintroduced populations and their founding sources was mainly influenced by later augmentations, rather than founding population size. Fourth, we detected genetic contributions from augmentations consisting of 18-49 animals including males and females, but not from augmentations of 2 males. This information can guide selection of source populations and translocation strategies to aid in restoration of bighorn sheep.

#### Natural Genetic Connectivity Detected Only in Large Populations

Genetic structure and connectivity across native populations prior to fragmentation due to human activities can serve as a baseline goal for bighorn sheep restoration in other areas. Glacier and Beartooth-Absaroka served as examples of large, continuous native populations, and our results suggested genetic connectivity within these populations and that gene flow within these herds was influenced by geographic distance and at least one natural barrier. Genetic differences between the north and south portions of Glacier suggested partial fragmentation due to a large, central lake that may serve as a barrier to extensive gene flow (Figure 2; Figure 3). In addition, our results suggested isolation-by-distance within Stillwater and Beartooth-Absaroka, as results for Stillwater, Yellowstone, northern hunt units 1-3, and southern hunt units 5 and 22 suggested small genetic differences within a larger population (Figure 2; Figure 3). Love Stowell *et al.* (2020) also detected a north-south pattern of isolation-by-distance in the

Beartooth-Absaroka population. Between populations, distances over 100 km and non-contiguous mountain ranges generally reduced detection of past and present unassisted gene flow. For example, native Galton and Castle Reef were genetically different and about 170 km apart in linear distance (Figure 2). Populations outside of Montana and Wyoming, including Sierra-Nevada and Dinosaur, were distinct from one another and all other populations (Figure 3). In contrast, our results for restored herds did not suggest isolation-by-distance, but instead a strong genetic influence of translocations. This is consistent with patterns observed in restored white-tailed deer (*Odocoileus virginianus*) populations, where little genetic differentiation existed due to translocations to repopulate previously occupied areas (Budd et al. 2018).

Our results identified native populations that had historical gene flow but likely did not have genetic connectivity at the time of sampling. To identify populations that were recently fragmented, we evaluated MDS and three-population test results. Our MDS and three-population test results suggested that most populations were genetically isolated from one another, except for Stillwater and Beartooth-Absaroka. This is likely because Stillwater and Beartooth-Absaroka are in geographic proximity and part of a continuous, spatially-structured population. Although female bighorn sheep often associate in genetically related groups (Rubin et al. 1998, Boyce et al. 1999), rams often disperse during breeding season to genetically connect ewe groups (Geist 1971) and our genotyping approach accounted for male genetic contributions. In addition, for most populations we sampled animals across a broad distribution via helicopter search and capture. Thus, we expect that our results represent genetic isolation among examined

herds, rather than genetic sub-groups within a larger population. However, examined herds could have gene flow with nearby populations not evaluated in this study, and additional genetic sampling of neighboring herds may be useful to more thoroughly evaluate genetic isolation in specific populations.

Gaps between formerly connected populations are areas where managers could prioritize reestablishing connectivity. Filling gaps in distribution can be an important part of species restoration, in addition to establishing new populations (Watson and Watson 2015, Stewart et al. 2017). Broadly distributed and spatially-structured populations can also help prevent extirpation of an entire population after an epizootic by localizing outbreaks in smaller groups and lowering probability of disease spread (Altizer et al. 2003, Lopez et al. 2005). We detected two pairs of native herds that had past genetic connectivity but were likely not connected at the time of sampling, including Spanish Peaks/Taylor Hilgard and Castle Reef/south Glacier. These populations were similar in fastStructure and Treemix analyses, but the three-population test and MDS suggested a lack of contemporary gene flow. The linear distances between recently fragmented populations were comparable to those for herds with recent gene flow. Stillwater and Beartooth-Absaroka are about 25 km apart in linear distance, whereas about 35 km separate south Glacier and the nearest bighorn sheep range connected to Castle Reef and 28 km separate Spanish Peaks and Taylor Hilgard. However, the lack of signal for genetic connectivity between the Glacier and Castle Reef populations may be due to a lack of sampling of animals in the bighorn sheep range closest to Glacier which is likely connected to Castle Reef.

Translocation, Not Recolonization, Started All  
Populations Thought to be Reintroduced

To evaluate evolution of newly founded populations and inform reintroduction planning, we compared herds thought to be reintroduced with their suspected founding source. Other studies suggested that bighorn sheep reintroduction efforts may be more successful when founders are sourced from either matching environmental conditions, due to greater recruitment when ecotypes are matched, or native populations, due to typically higher levels of genetic diversity (Fitzsimmons et al. 1997, Singer et al. 2000, Bleich et al. 2018). We evaluated eight populations that originated from reintroductions where a founding source was in our study. Castle Reef was the source of six out of eight reintroduced herds, and the remaining two were started by reintroduced populations initially founded by Castle Reef, rather than recolonization. The overall success of reintroductions from Castle Reef suggested that sourcing from the native population may have been more important than matching environmental conditions. Sourced from mountain foothills, Castle Reef animals successfully established populations not only in environments similar to the source location (Lost Creek and Highlands), but also in semiarid prairie/river breaks environments (Fergus and Middle Missouri) and an island with weather influenced by the Pacific Ocean maritime effect (Wild Horse Island; Montana Department of Fish, Wildlife and Parks, 2010). Translocated bighorn sheep establishing populations in areas with different environmental conditions than their source has been documented in other studies (Rominger et al. 2004, Wiedmann and Sargeant 2014, Malaney et al. 2015). For example, translocated bighorn sheep can adjust the timing of parturition to match the environmental characteristics of a new area

(Whiting et al. 2011, 2012). Local adaptation in bighorn sheep herds is still possible (Wiedmann and Sargeant 2014), given that native populations found in different ecological regions were genetically differentiated, but genetic distinctiveness could also be explained by genetic drift (Figure 2). Future research to evaluate the possibility for local adaptation would involve assessing patterns of correlation between individual genotypes and environmental characteristics across space, for which there are many analytical approaches (Rellstab et al. 2015, Balkenhol et al. 2017, Selmoni et al. 2019). If ecologically matched native herds have low genetic diversity or are not available for a reintroduction, multiple sources could be used, as maximizing genetic diversity might increase adaptive potential in a new environment (Broadhurst et al. 2008, Olson et al. 2013, White et al. 2018).

#### Evolution of Reintroduced Populations was Mainly Influenced by Augmentations

After selecting source population(s) for reintroduction, geneticists recommend using a large number of founders to replicate the genetic profile of the founding source, but recommendations can range from 20-50 animals and depend on the species (Taylor and Jamieson 2008, Weeks et al. 2011, Jamieson and Lacy 2012). It is useful to evaluate how much reintroduced populations genetically diverged from their founding source, to assess if the released number of founders successfully represented the source's genetic profile and to determine if genetic drift occurred, which is the main process by which small populations lose genetic variation, as in reintroduced populations of Alpine ibex (*Capra ibex ibex*; Biebach & Keller, 2009; Lacy, 1987; Templeton, 2006). Thus, greater genetic divergence from the source population may indicate a loss of genetic variation in

the reintroduced herd due to chance. We expected that reintroduced populations established with a greater number of founders would have greater mean kinship with the founding source, as a large number of animals would be more genetically representative of the source and experience a lower rate of genetic drift. When we compared founder sizes ranging from 6 to 53 bighorn sheep to kinship with the founding source, we did not find a clear relationship. For some populations a limited number of generations have passed since founding, thus genetic drift may not yet be detectable. Kinship with the founding source may also have been driven by other genetic influences in many of the reintroduced herds, as six out of eight reintroduced populations received translocations from at least two sources. Three reintroduced populations that had the greatest divergence from their founding source (Wild Horse Island, Lost Creek, and Petty Creek) each were founded by 25 or fewer animals from the same source, received an augmentation from at least one other source, and had 8-11 generations for genetic drift or selection. Thus, a founder effect, genetic drift or selection over time, and genetic input from a different source likely influenced population genetics of these reintroduced populations. In contrast, the population with the highest mean kinship with its founding source (Middle Missouri) had one reintroduction event of 28 animals, six generations of isolation, and no augmentations. While multiple influences on the evolution of reintroduced populations complicated identifying which affected divergence the most, we observed an inverse relationship between the number of source populations and augmentations from other sources and mean kinship of reintroduced populations with their founding source, suggesting that this influence was important in our study. Future

genomic studies could target reintroduced populations with different founder sizes and no augmentations to gain clearer insights to inform the number of bighorn sheep recommended for reintroductions.

#### Augmentations of Two Males were Less Successful than Larger, Mixed Sex Groups

Augmentations provided an opportunity to assess the impact of specific gene flow events on fragmented population evolution and inform animal selection for future efforts. For translocation to be used for augmenting genetic diversity of small populations, managers need to 1) identify which populations need augmentation based on demographic attributes and levels of population fragmentation, genetic diversity, and inbreeding, 2) identify source populations based on mean kinship, other direct measures of diversity, and other management concerns like disease history, 3) determine how many and which individuals should be moved, and 4) monitor recipient populations for realized genetic contribution, connectivity with nearby populations, genetic diversity, and demographic response, to determine if additional augmentations are needed (Johnson et al. 2010, Adams et al. 2011, Harrisson et al. 2016, Frankham et al. 2017). Future research could evaluate the relationship between levels of inbreeding within herds and population performance to aid in determining which populations may require augmentation.

Identifying which animals will contribute genetically to a recipient population includes considerations such as adaptation, number, sex, age, and disease status. To synthesize results across the fastStructure, MDS, and Treemix analyses, we defined a translocation event as detected only if we found evidence for its genetic contribution in

the intended recipient population in at least two out of three analyses (Table 3). As in reintroductions, we observed successful augmentations (defined as genetically detected) across different ecological regions defined by Montana Fish, Wildlife, and Parks and extensive latitudes from the Whiskey Mountain population in Wyoming to the Dinosaur population in Colorado and Utah, suggesting that matching environmental attributes is not always necessary. Because our definition of augmentation success was based on detected genetic contribution and not individual fitness, additional assessment evaluating hybrid fitness would be valuable (Olson et al. 2012).

We found that five detected translocations consisted of 18 to 49 animals, whereas two undetected augmentations each consisted of two males. During breeding season, adult male bighorn sheep can wander long distances between mountain ranges (Geist 1971) and potentially depart the augmentation destination, which may result in no genetic contribution to the intended recipient population. In addition, because bighorn sheep are a polygynous species, a small number of dominant rams may competitively exclude translocated males due to female mate preference for residents or poor condition after transport/release, suggesting that translocating a greater proportion of females may be more effective for augmentation (Sigg et al. 2005, Mulder et al. 2017). However, we suspect that an augmentation of two males from Ural-Tweed to Wild Horse Island made a genetic contribution, due to divergence of Wild Horse Island from its founding source, Castle Reef, and genetic similarity between Galton and Wild Horse Island (Figure 2). In this case, the small size of the recipient population and boundaries to dispersal from an island increased the possibility for genetic contribution. After an augmentation of

females, translocated ewe groups may socially segregate from the resident population following release, which was observed in Taylor Hilgard after augmentation from Lost Creek in 1989 (Roy and Irby 1994, Robinson et al. 2019). However, observed mixing of rams and ewes with different origins during breeding season or social mixing in later years resulted in hybrids descended from native and translocated animals after 24 years or about 4 generations (Figure 4; Roy & Irby, 1994). Future research could more specifically evaluate if translocated bighorn sheep males or females are more likely to reproduce successfully.

Augmentations are often promoted for genetic rescue (Tallmon et al. 2004, Hogg et al. 2006, Whiteley et al. 2015). To increase effectiveness of a genetic rescue, source and recipient populations should have been previously connected but recently isolated to allow differentiation over multiple generations in the past 500 generations (Falconer et al. 1996, Allendorf and Luikart 2009, Frankham et al. 2017). Many types of information should be evaluated to determine optimal sources for augmentation, but our results can provide guidance on selecting sources for genetic rescue augmentations by combining information on genetic differentiation and mean kinship. One possible approach, if managers want to maintain populations that are currently differentiated, would be to identify possible sources for future augmentations within clusters of populations in MDS or fastStructure analyses (Figure 2; Figure 3). Within identified clusters, managers can select a source population that has low mean kinship with the intended recipient herd (Table 1). Minimizing mean kinship between source and recipient populations within identified clusters would be an approach to retain genetic diversity and minimize

inbreeding at the population level while still considering the possibility for local adaptation (Ballou and Lacy 1995, Frankham et al. 2017). For example, augmentations could be implemented within the cluster associated with Castle Reef (Figure 2), and mean kinship minimized between source and recipient by consulting a mean kinship table associated with that cluster.

### Conclusions and Management Implications

There has been great concern that bighorn sheep augmentation efforts may spread respiratory pathogens novel to the recipient population or disrupt a stable relationship between resident pathogens and host, which could result in devastating epizootic events (Cunningham 1996, Aiello et al. 2014, Cassirer et al. 2016). Disease concerns have resulted in widespread testing of bighorn sheep herds for a variety of pathogen species and strains associated with epizootic events, but the results of these tests can be inconclusive due to imperfect detection probability and lack of understanding as to what individual test results imply for the overall population (Butler et al. 2017, 2018). Thus, when considering translocations between bighorn sheep populations, agencies could recognize the risk of mixing pathogens, consider how to mitigate this risk, and monitor outcomes for adaptive management. Future research to address this issue could evaluate genetic variation at the major histocompatibility complex (MHC) associated with immune response in bighorn sheep populations.

Our results provide insight regarding the population genomics of native and reintroduced herds, genetic contributions of past translocation efforts, and strategies for future bighorn sheep restoration efforts. This research serves as an example of how

genomic analyses can provide information regarding the genetic outcomes of previous management approaches and inform future decisions. Building on other studies that drew conclusions from only a few populations or limited genetic markers, our study design maximized insight from an observational study by employing standardized sampling of fourteen bighorn sheep herds with differing management histories distributed across the northern Rocky Mountain region, a standardized set of SNP markers, and a suite of contemporary analytical tools. Successful genetic contribution of most reintroduction and augmentation efforts evaluated in this study in the context of the continued struggle of Rocky Mountain bighorn sheep conservation suggests there are multiple interacting influences on restoration success. Not only is there uncertainty regarding how genetic attributes affect population performance, but the specific drivers behind population trend and demography are also frequently unclear. Thus, genetic management should generally be integrated into conservation planning with other management considerations (IUCN/SSC 2013, Ralls et al. 2017). Assessment of multiple population attributes during restoration efforts, such as genetics, migratory patterns, mortality causes, and disease, can enable an adaptive management framework and improve the longevity of managed populations (IUCN/SSC 2013). Genomic data alone cannot dictate decisions concerning population and genetic management, but rather can be integrated with management judgements as to what population genetics are valuable to conserve and what should be built for the future. Our results demonstrate that genomic analyses are a tool for evaluating the genetic effects of translocations and planning future genetic management of small, fragmented populations.

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## Tables and Figures

Table 1. Mean kinship between Rocky Mountain and Sierra Nevada bighorn sheep populations evaluated using the HD Ovine array. Standard deviation from the mean is in parentheses. Smaller values indicate lower mean kinship. Sample size for each population is next to each population name on the y-axis; populations with fewer than 20 genotypes are labeled in red.

|                    |    | Beartooth-Absaroka | Castle Reef       | Dinosaur          | Fergus            | Galton            | Glacier           | Highlands         | Lost Creek        | Middle Missouri   | Paradise          | Petty Creek       | Sierra Nevada     | Spanish Peaks     | Stillwater        | Taylor Hilgard    | Tendoy            |
|--------------------|----|--------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Beartooth-Absaroka | 90 |                    |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| Castle Reef        | 25 | -0.168<br>(0.038)  |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| Dinosaur           | 20 | -0.210<br>(0.098)  | -0.278<br>(0.078) |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| Fergus             | 30 | -0.202<br>(0.116)  | -0.019<br>(0.096) | -0.320<br>(0.138) |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| Galton             | 5  | -0.224<br>(0.054)  | -0.191<br>(0.039) | -0.343<br>(0.085) | -0.228<br>(0.116) |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| Glacier            | 95 | -0.208<br>(0.110)  | -0.169<br>(0.120) | -0.341<br>(0.147) | -0.206<br>(0.163) | -0.163<br>(0.121) |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| Highlands          | 17 | -0.194<br>(0.125)  | -0.034<br>(0.133) | -0.325<br>(0.158) | -0.052<br>(0.155) | -0.235<br>(0.137) | -0.205<br>(0.168) |                   |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| Lost Creek         | 25 | -0.276<br>(0.072)  | -0.087<br>(0.061) | -0.383<br>(0.090) | -0.130<br>(0.111) | -0.285<br>(0.072) | -0.264<br>(0.142) | -0.135<br>(0.153) |                   |                   |                   |                   |                   |                   |                   |                   |                   |
| Middle Missouri    | 25 | -0.201<br>(0.052)  | -0.001<br>(0.032) | -0.307<br>(0.078) | -0.035<br>(0.098) | -0.212<br>(0.047) | -0.198<br>(0.129) | -0.058<br>(0.140) | -0.101<br>(0.069) |                   |                   |                   |                   |                   |                   |                   |                   |
| Paradise           | 25 | -0.252<br>(0.043)  | -0.096<br>(0.035) | -0.347<br>(0.069) | -0.128<br>(0.102) | -0.185<br>(0.037) | -0.229<br>(0.133) | -0.151<br>(0.141) | -0.171<br>(0.069) | -0.096<br>(0.037) |                   |                   |                   |                   |                   |                   |                   |
| Petty Creek        | 25 | -0.343<br>(0.058)  | -0.129<br>(0.045) | -0.437<br>(0.068) | -0.156<br>(0.100) | -0.345<br>(0.060) | -0.328<br>(0.145) | -0.183<br>(0.156) | -0.201<br>(0.083) | -0.126<br>(0.047) | -0.209<br>(0.047) |                   |                   |                   |                   |                   |                   |
| Sierra Nevada      | 5  | -0.731<br>(0.142)  | -0.798<br>(0.145) | -0.715<br>(0.135) | -0.827<br>(0.171) | -0.841<br>(0.158) | -0.868<br>(0.227) | -0.846<br>(0.250) | -0.855<br>(0.172) | -0.794<br>(0.147) | -0.844<br>(0.154) | -0.863<br>(0.147) |                   |                   |                   |                   |                   |
| Spanish Peaks      | 20 | -0.104<br>(0.055)  | -0.133<br>(0.045) | -0.257<br>(0.091) | -0.168<br>(0.114) | -0.212<br>(0.059) | -0.188<br>(0.113) | -0.166<br>(0.131) | -0.228<br>(0.079) | -0.163<br>(0.059) | -0.211<br>(0.053) | -0.300<br>(0.065) | -0.773<br>(0.151) |                   |                   |                   |                   |
| Stillwater         | 24 | -0.100<br>(0.120)  | -0.206<br>(0.122) | -0.277<br>(0.150) | -0.244<br>(0.167) | -0.264<br>(0.127) | -0.252<br>(0.159) | -0.235<br>(0.169) | -0.310<br>(0.141) | -0.236<br>(0.128) | -0.279<br>(0.129) | -0.375<br>(0.143) | -0.828<br>(0.227) | -0.147<br>(0.121) |                   |                   |                   |
| Taylor Hilgard     | 30 | -0.147<br>(0.134)  | -0.114<br>(0.148) | -0.288<br>(0.174) | -0.148<br>(0.180) | -0.212<br>(0.150) | -0.199<br>(0.173) | -0.149<br>(0.181) | -0.185<br>(0.168) | -0.142<br>(0.158) | -0.162<br>(0.162) | -0.283<br>(0.178) | -0.817<br>(0.265) | -0.097<br>(0.147) | -0.190<br>(0.175) |                   |                   |
| Tendoy             | 25 | -0.167<br>(0.074)  | -0.024<br>(0.069) | -0.295<br>(0.101) | -0.051<br>(0.119) | -0.185<br>(0.063) | -0.174<br>(0.127) | -0.066<br>(0.142) | -0.117<br>(0.084) | -0.041<br>(0.071) | -0.087<br>(0.073) | -0.165<br>(0.076) | -0.805<br>(0.163) | -0.137<br>(0.079) | -0.209<br>(0.133) | -0.111<br>(0.149) |                   |
| Wild Horse Island  | 25 | -0.272<br>(0.068)  | -0.130<br>(0.072) | -0.378<br>(0.095) | -0.162<br>(0.124) | -0.202<br>(0.078) | -0.248<br>(0.142) | -0.180<br>(0.153) | -0.207<br>(0.098) | -0.132<br>(0.078) | -0.030<br>(0.081) | -0.240<br>(0.088) | -0.876<br>(0.183) | -0.236<br>(0.079) | -0.302<br>(0.140) | -0.177<br>(0.164) | -0.098<br>(0.108) |

Mean Kinship  -0.8  -0.6  -0.4  -0.2

Table 2. Translocation events identified by Treemix based on the population tree (Figure 4). Translocation weight can range from 0 to 0.5 and estimated the proportion of alleles contributed by the source population.

| Translocation Order | Source             | Recipient      | Translocation Weight |
|---------------------|--------------------|----------------|----------------------|
| 1                   | Wild Horse Island  | Taylor Hilgard | 0.45                 |
| 2                   | Wild Horse Island  | Tendoy's       | 0.39                 |
| 3                   | Lost Creek         | Taylor Hilgard | 0.15                 |
| 4                   | Beartooth-Absaroka | Dinosaur       | 0.29                 |

Table 3. Evidence for all known translocations between herds in this study detected using MDS (multidimensional scaling), fastStructure, and Treemix analyses. An asterisk after the total number of bighorn sheep indicates that biologists suspected the translocation failed and did not contribute to the receiving population. For the MDS analysis (Figure 3), a translocation detection was designated as “possible” if both herds were found in the same group that was evaluated in a subset MDS analysis and “not detected” if herds were not evaluated together in a subset MDS analysis. For fastStructure (Figure 2), a translocation was considered “detected” if a common cluster was found for more than five animals in either herd, “possible” if a common cluster was found for less than five animals in either herd or there was an alternative source herd within the same cluster as the herd providing animals, and “not detected” if a common cluster was not found in both herds. For Treemix (Figure 4), a translocation was considered “detected” if identified as a migration event by the software, “possible” if herds were plotted on nearby branches in the tree, and “not detected” if herds were not plotted on nearby branches in the tree.

| Herd Receiving Animals | Herd Providing Animals | Year | Number Animals | Translocation Details             | MDS      | fast-Structure | Treemix       |
|------------------------|------------------------|------|----------------|-----------------------------------|----------|----------------|---------------|
| Dinosaur               | Beartooth-Absaroka     | 1983 | 19             | Augmentation of reintroduced herd |          |                |               |
| Dinosaur               | Beartooth-Absaroka     | 1984 | 17             | Augmentation of reintroduced herd | Possible | Detected       | Detected      |
| Dinosaur               | Beartooth-Absaroka     | 1989 | 21             | Augmentation of reintroduced herd |          |                |               |
| Fergus                 | Castle Reef            | 1961 | 12             | Founder                           |          |                |               |
| Fergus                 | Castle Reef            | 1980 | 28             | Augmentation from founder source  | Possible | Detected       | Possible      |
| Highlands              | Castle Reef            | 1967 | 22             | Founder 1                         |          |                |               |
| Highlands              | Castle Reef            | 1969 | 31             | Founder 2                         |          |                |               |
| Highlands              | Castle Reef            | 2000 | 15             | Augmentation from founder source  | Possible | Possible       | Not evaluated |

|                   |                   |      |    |                                   |              |              |               |
|-------------------|-------------------|------|----|-----------------------------------|--------------|--------------|---------------|
| Highlands         | Castle Reef       | 2001 | 17 | Augmentation from founder source  |              |              |               |
| Highlands         | Castle Reef       | 2008 | 65 | Augmentation from founder source  |              |              |               |
| Highlands         | Fergus            | 2014 | 9  | Augmentation of reintroduced herd | Possible     | Possible     | Not evaluated |
| Lost Creek        | Castle Reef       | 1967 | 25 | Founder                           | Possible     | Detected     | Possible      |
| Middle Missouri   | Castle Reef       | 1980 | 28 | Founder                           | Possible     | Detected     | Possible      |
| Paradise          | Wild Horse Island | 1979 | 14 | Founder                           |              |              |               |
| Paradise          | Wild Horse Island | 2011 | 22 | Augmentation from founder source  | Possible     | Detected     | Possible      |
| Petty Creek       | Castle Reef       | 1968 | 16 | Founder                           | Possible     | Detected     | Possible      |
| Spanish Peaks     | Castle Reef       | 1974 | 2  | Augmentation of native herd       | Not detected | Possible     | Not detected  |
| Stillwater        | Castle Reef       | 1970 | 2* | Augmentation of native herd       | Not detected | Not detected | Not detected  |
| Taylor Hilgard    | Lost Creek        | 1989 | 18 | Augmentation of native herd       | Not detected | Detected     | Detected      |
| Taylor Hilgard    | Wild Horse Island | 1993 | 26 | Augmentation of native herd       | Not detected | Detected     | Detected      |
| Tendoy's          | Lost Creek        | 1984 | 39 | Founder                           | Possible     | Detected     | Possible      |
| Tendoy's          | Castle Reef       | 2002 | 30 | Augmentation of reintroduced herd | Possible     | Detected     | Possible      |
| Tendoy's          | Wild Horse Island | 2012 | 49 | Augmentation of reintroduced herd | Possible     | Detected     | Detected      |
| Wild Horse Island | Castle Reef       | 1947 | 6  | Founder                           | Possible     | Not detected | Possible      |

Figure 1. Rocky Mountain bighorn sheep (*O.c. canadensis*) ram from the Taylor Hilgard population in Montana, U.S.A.



Figure 2. Map of fastStructure (K=6) results for Rocky Mountain (*O.c. canadensis*) and Sierra Nevada (*O.c. sierrae*) bighorn sheep populations genotyped using the HD Ovine array. Approximate distributions of native populations are brown polygons; reintroduced populations are black polygons. A pie chart of herd-level fastStructure group assignments is next to each population. All known translocation events between Rocky Mountain bighorn sheep populations in this study are shown by arrows. Arrows point generally to the recipient population and do not represent exact release location. Arrow thickness is proportional to number of translocations; arrow color corresponds to the predominant fastStructure group assignment of the source population. Approximate bighorn sheep ranges, including populations not in this study, are shown in gray polygons for Idaho, Wyoming, and Montana (Montana Fish, Wildlife and Parks 2008, Wyoming Game and Fish Department 2012, Thomas 2019). Hunt unit boundaries for Beartooth-Absaroka are labelled and truncated to bighorn sheep range. State boundaries are designated by dashed lines outlined in gray; national park boundaries in the study area are designated by dashed lines.

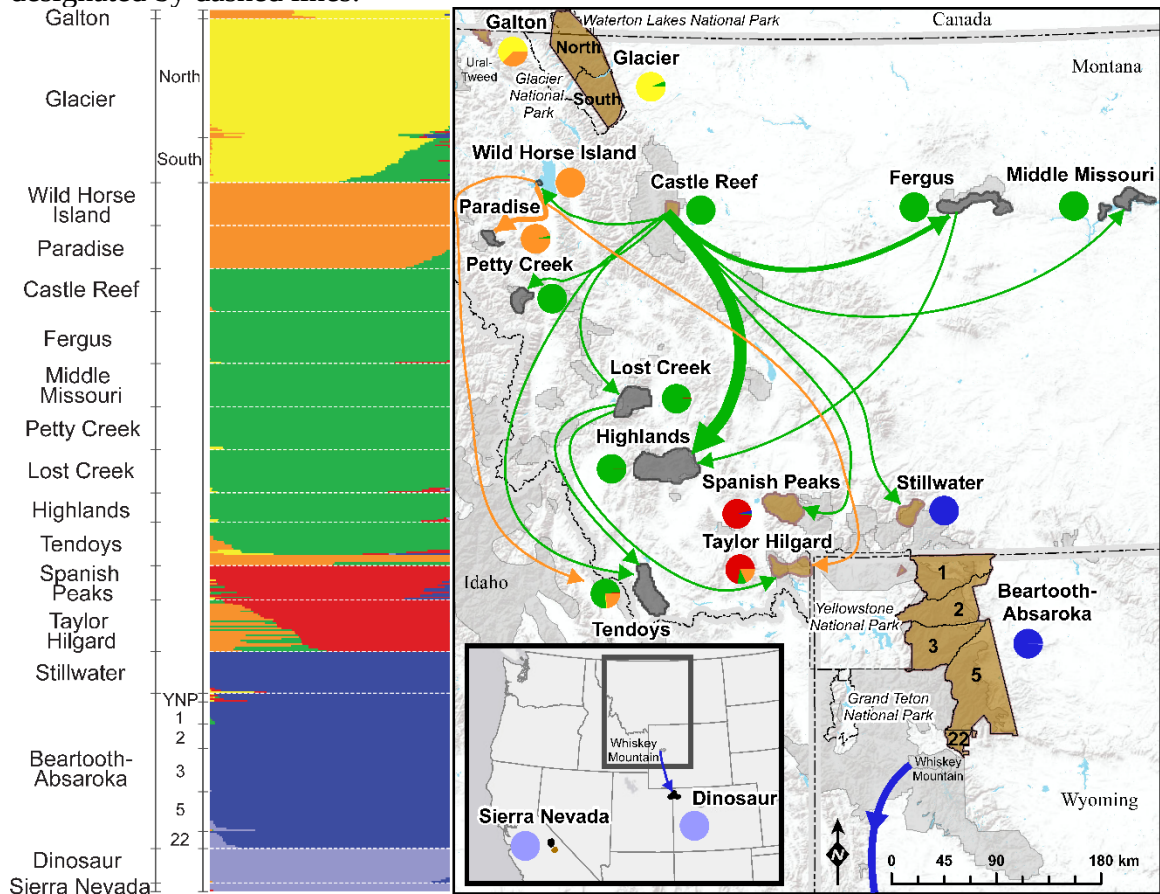


Figure 3. Multi-dimensional scaling (MDS) results for individual HD Ovine array genotypes from seventeen bighorn sheep populations, including an analysis of all herds (C) and subset analyses (A, B, D, and E) based on clusters of populations in panel C. The legend defines symbols for each population and pie charts of herd-level fastStructure group assignments (K=6).

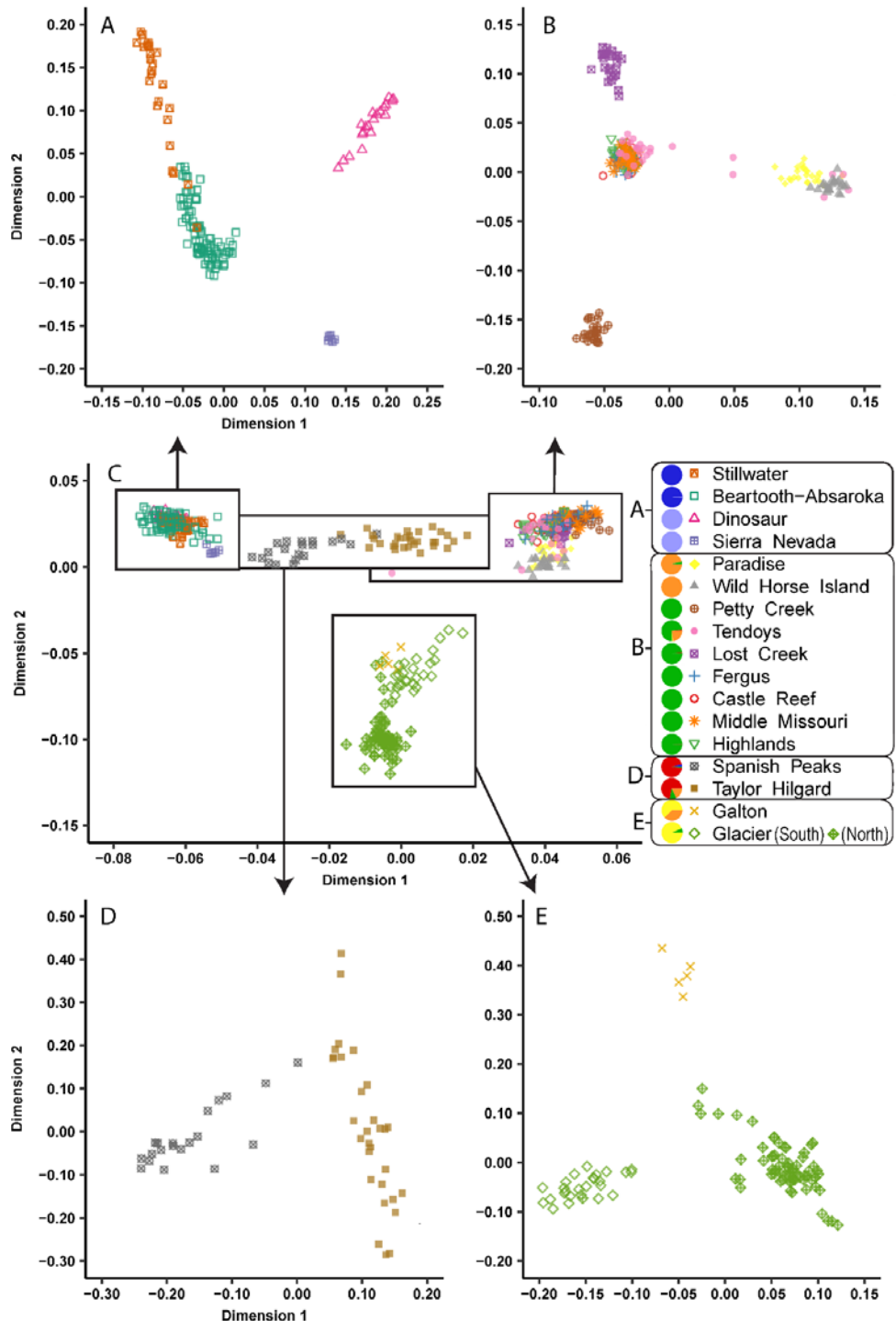
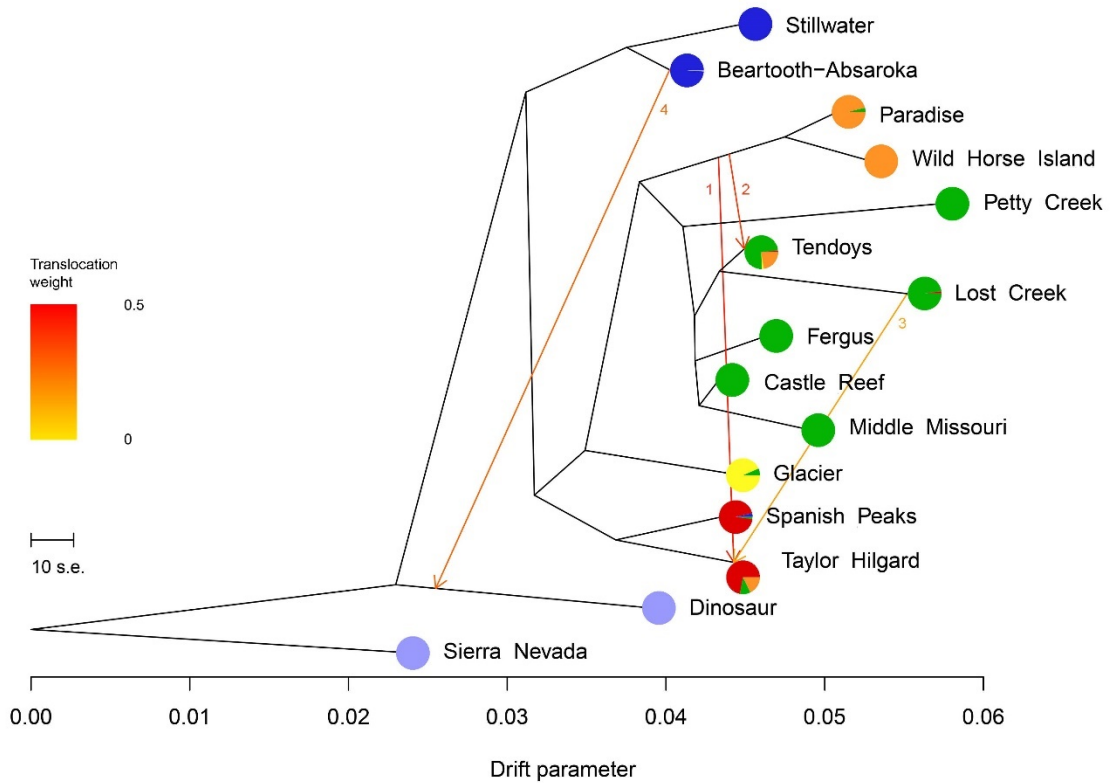


Figure 4. Treemix population tree with four detected translocations (orange lines) plotted for fourteen Rocky Mountain bighorn sheep populations with 20 or more genotypes. Sierra Nevada was defined as the outgroup. Horizontal axis scale bar defines ten times the mean standard error of the sample covariance matrix; horizontal branch length is proportional to genetic drift amount. Pie charts of herd-level fastStructure group assignments (K=6) are shown for each population.



CHAPTER FOUR

GENETIC DIVERSITY OF BIGHORN SHEEP POPULATION IS ASSOCIATED  
WITH DISPERSAL, AUGMENTATION, AND BOTTLENECKS

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Abstract

Understanding the interaction between genetic diversity and demography of populations is important to advancement of biological conservation. We compared the genetic diversity of bighorn sheep (*Ovis canadensis*) herds with population origin (native or reintroduced), minimum population size, unassisted gene flow via dispersal, and assisted gene flow via augmentations, to determine which demographic predictors were the most important. In addition, we compared population genetic diversity with median juvenile survival to evaluate potential for inbreeding depression. We evaluated 488 bighorn sheep from 19 wild populations, including native and reintroduced populations that received 0-165 animals in augmentations. Using the Illumina High Density Ovine array, we generated a dataset of 30,168 single nucleotide polymorphisms and calculated average pairwise kinship for each population, which is inversely related to genetic diversity and represents the expected level of inbreeding in the next generation. Multiple linear regression analysis determined that greater gene flow via dispersal, number of animals contributed in augmentations, and minimum population size were correlated with higher genetic diversity at the population level, and origin was less important. We observed no clear relationship between average pairwise kinship and median juvenile survival, suggesting that inbreeding depression did not suppress population growth in the examined populations. However, this study highlights that genetic isolation of populations could result in inbreeding at concerning levels. By determining the relative importance of different influences on genetic diversity, the results of this study can aid in

identifying populations of genetic concern and defining management priorities for conservation of genetic diversity in wild populations.

Key Words: *Ovis canadensis*, kinship, genetic diversity, genetic rescue, population bottleneck, gene flow

### Introduction

Genetic diversity describes variation found within DNA, provides the raw material for evolution, and correlates with greater fitness (Charlesworth and Charlesworth 1987, Reed and Frankham 2003). As wild plant and animal populations become increasingly fragmented due to human activities, small and isolated populations gradually lose genetic diversity due to chance and inbreeding (mating between relatives) (Frankham et al. 2017). Inbred individuals and populations can exhibit reduced reproductive fitness, termed inbreeding depression (Wright 1984, Charlesworth and Charlesworth 1987, Keller and Waller 2002). In addition, populations with low genetic diversity have a reduced ability to adapt to environmental change, novel diseases, and evolving resident pathogens (Hoffmann et al. 2003, Hughes and Boomsma 2004, Spielman et al. 2004a, Kellermann et al. 2006, Whiteman et al. 2006, MacPherson et al. 2018). As a result, level of inbreeding in a population can influence population persistence (Madsen et al. 1996, Saccheri et al. 1998, Soulé and Mills 1998, Penn et al. 2002).

Thus, understanding the interaction between genetic diversity and demography of populations is important to advancement of biological conservation. Despite the

importance of maintaining genetic variation in wild populations, there is not yet a comprehensive theory that fully explains the causes of genetic diversity (Frankham 1996, Lanfear et al. 2014, Ellegren and Galtier 2016). Research questions that evaluate the determinants of genetic diversity should now be revived, as significant technical advances in genomics greatly increase our ability to assess genetic diversity through genotyping thousands of genetic markers (Leffler et al. 2012).

Among different species, genetic diversity can depend on breeding system and abundance. Specifically, species that are longer lived or with lower fecundity generally have lower genetic variation than those that are shorter lived or have higher fecundity (Romiguier et al. 2014). In addition, abundant species generally have higher genetic diversity than rare species due to larger population sizes (Hague and Routman 2016). For populations of a single species, there are multiple competing hypotheses regarding the relationship between population size and genetic diversity. While greater population size may enhance genetic diversity, selection can be more effective in large, randomly-mating populations, which may alternatively lead to reduction in genetic variation (Tajima 1990, Gillespie 1991, Charlesworth and Charlesworth 2010, Cutter and Payseur 2013). Other studies have found no clear relationship between population size and genetic diversity (Nabholz et al. 2008, Leffler et al. 2012, Perry et al. 2012).

Change in population size over time may also be important (Alcala and Vuilleumier 2014). There are multiple influences on population size over time that may affect genetic diversity, including bottlenecks, founder events, and gene flow. A bottleneck is a sudden reduction in population size, such as a die-off event, which can

result in loss of genetic variation (Leberg 1992, England et al. 2003, Spielman et al. 2004b). Theory and data suggest that historic population minimum is the most important factor in determining a population's genetic diversity (Wright 1938, Avise et al. 1988). Similar to a bottleneck, a founder event occurs when a small number of individuals immigrate or are reintroduced to an unoccupied area to start a new population. A founder effect refers to loss of genetic variation by chance when the new population is started by a limited number of animals (Templeton 1980). Thus, it can be important to consider a population's origin, such as distinguishing between native (indigenous) and reintroduced populations (Mayr 1963, Jamieson 2011).

Gene flow via unassisted dispersal or augmentation (translocations implemented by managers) can enhance genetic diversity (Spielman and Frankham 1992, Falconer and Mackay 1996). However, natural gene flow between populations can be disrupted by human development, such as roads, which can lead to inbreeding depression (Epps et al. 2005, Holderegger and Di Giulio 2010). To address this concern, managers can add unrelated individuals to an inbred population to enhance its viability and genetic diversity, termed genetic rescue (Frankham, 2015; IUCN/SSC, 2013; Tallmon et al., 2004; Whiteley et al., 2015). Mean kinship is an optimal metric to determine if genetic rescue is recommended for a population and can be used as a guide to maximize genetic diversity and minimize inbreeding (Finger et al. 2011, Frankham et al. 2017). Specifically, Frankham et al. (2017) recommended that populations have a mean kinship value less than 0.1 to avoid inbreeding depression (Van Dyke 2008, Manichaikul et al. 2010a, Frankham et al. 2017).

In addition, determining if genetic factors are limiting population growth can help determine if management action, such as augmentation, might improve population viability (Keller and Waller 2002, Tallmon et al. 2004). Inbreeding can affect fitness at various life stages, and different vital rates can affect population growth in differing proportions (Morris and Doak 2002, Mills 2007). Thus, to evaluate if inbreeding is negatively affecting population growth, mean kinship could be compared with the vital rate that has the greatest effect on population trend, such as juvenile survival in large herbivores (Paterson et al. In prep, Mills and Smouse 1994, Gaillard et al. 1998). When considering augmentation efforts, managers must not only decide which populations should receive additional animals, but also the identity of source populations and the number of animals to translocate. Thus, it would be useful to improve understanding regarding what aspects of augmentation decisions and other influences on population size over time most affect genetic diversity in populations of conservation concern.

Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) management has experienced many challenges that are common in conservation, including fragmented populations, disease, and areas of historic range that remain unoccupied despite restoration efforts. The species historically had high gene flow across its geographic range, but many populations are now genetically differentiated, suggesting recent fragmentation due to human activities (Luikart and Allendorf 1996, Epps et al. 2005). Some populations still represent historic conditions and are large and distributed continuously, whereas others are small and isolated with a history of drastic bottlenecks due to epizootic events that often result in mortality of over half of the population

(Cassirer et al. 2018). Managers moved over 21,500 bighorn sheep in 1,460 translocations with the intention of establishing new populations in historic range and improving genetic diversity and size of existing populations (Hogg et al. 2006, Wild Sheep Working Group 2015). However, recent concerns regarding resident pathogens and disease spillover from domestic sheep have resulted in managers keeping many populations small and genetically isolated to avoid the spread of disease (Cassirer et al. 2016, Butler et al. 2018). This complex history has resulted in concern that genetic factors could limit population growth and viability of the species (Singer et al. 2000, Hogg et al. 2006, Johnson et al. 2011). Since bighorn sheep populations vary greatly in their origin, population size, connectivity, and augmentation history, they provide a unique opportunity to observe the response of genetic diversity to a spectrum of natural and management influences.

This study addressed the following objectives: 1) evaluate genetic diversity of bighorn sheep populations with varied management histories, 2) compare genetic diversity with population origin, size, unassisted gene flow via dispersal, and assisted gene flow via augmentations to determine if there is a correlation and which are most important, and 3) determine if populations are experiencing inbreeding depression by comparing genetic diversity with juvenile survival. We expected that native origin, large population size, and greater gene flow via augmentations or natural dispersal would result in higher genetic diversity than reintroduced origin, small population size, and genetic isolation. We also predicted that augmentation history and origin would be the most important influences, due to observed effects of these variables on genomic similarity

between populations (Flesch et al. In press). Finally, we expected that populations with low genetic diversity would have lower juvenile survival, which would suggest the presence of inbreeding depression. Comparing vital rates and influences on population size over time with genetic diversity in multiple wild populations can serve to: determine relative importance of these influences to enhance genetic management strategies, identify priorities for conservation of genetic diversity in different types of populations (e.g. native vs. reintroduced), and improve identification of populations of concern if genetic data are not available.

## Methods

### Using Kinship to Evaluate Genetic Diversity

While there are many metrics to assess genetic diversity and inbreeding status of populations, the kinship coefficient is optimal for genetic management decisions (Frankham et al. 2017). Kinship, also termed coancestry, represents the probability that two randomly sampled alleles from two individuals are identical by descent (Manichaikul et al. 2010b). Kinship can be estimated from pedigrees and genetic marker information (Ballou and Lacy 1995, Manichaikul et al. 2010a). An individual's inbreeding coefficient is equal to the level of kinship between its mother and father (Falconer and Mackay 1996). Thus, the mean of all pairwise kinship values from a sample of individuals in a population represents the expected level of inbreeding in the next generation (Frankham et al. 2017). In addition, mean kinship of a population is inversely related to genetic diversity, meaning that greater mean kinship indicates lower genetic diversity (Frankham et al. 2010).

There are multiple advantages of using kinship over other inbreeding and relatedness metrics. Kinship metrics estimate identity by descent between individuals relative to that found within individuals, so the estimate is not biased by being measured relative to a reference population generated from all samples included in the calculation (Manichaikul et al. 2010a). The metric is also invariant to which samples are included in the calculation and is not impacted by linkage disequilibrium among genetic markers (Manichaikul et al. 2010a). The metric's maximum value is 1, with higher values meaning that individuals are more related; zero or negative values mean that the individuals are unrelated (Manichaikul et al. 2010a).

Minimizing mean kinship is an optimal approach to retain genetic and allelic diversity and minimize inbreeding in wild populations (Ballou and Lacy 1995, Fernández et al. 2004, Frankham et al. 2017). Its utility has been verified in *Drosophila* experiments (Montgomery et al. 1997), and biologists use mean kinship to make breeding decisions for threatened species in captivity and rare domestic livestock breeds (Falconer and Mackay 1996, Ballou et al. 2010, Frankham et al. 2010). Based on the fact that kinship is expected to be 0.125 for a second-degree relationship (i.e. half-siblings) and 0.0625 for a third-degree relationship (i.e. first cousins), Frankham et al. (2017) recommended that populations have a mean kinship value less than 0.1 to avoid inbreeding depression (Van Dyke 2008, Manichaikul et al. 2010a, Frankham et al. 2017).

### Study Populations

We evaluated 19 wild populations of Rocky Mountain bighorn sheep located in the United States and Canada, including the U.S. states of Montana, Wyoming, Colorado,

and Utah, and the Canadian provinces of Alberta and British Columbia (Figure 1). In the following descriptions of these populations, we use the terms “population” and “herd” synonymously. We aimed to sample at least 20-25 individuals per herd, based on sample size simulations that indicated lower sample sizes introduced an unacceptable level of uncertainty to mean kinship estimates (Flesch et al. 2018).

We sampled ten native herds, including seven small to moderately sized populations (70-227 animals) and three large populations (406-726 animals). Seven native herds represented natural population structure prior to widespread fragmentation of the species range, as they are part of large, spatially-structured populations, defined as a collection of subpopulations that occupy distinct geographic areas but are linked by animal movement. These populations included three units in the Beartooth-Absaroka mountain ranges of Wyoming, three units in Glacier and Waterton Lakes National Parks, and Castle Reef. The Wyoming units are in the eastern front of the Greater Yellowstone Area and include Clark’s Fork/Trout Peak, Wapiti Ridge, and Franc’s Peak, with their boundaries defined by administrative units. The Glacier area was split into north, central, and south for our analyses, and the divisions were informed by previous genetic and movement analyses (T. Graves unpublished data?) (Flesch et al. In press). South of Glacier, Castle Reef is expected to have connectivity across four administrative units in the Rocky Mountain Front of Montana (Montana Department of Fish, Wildlife and Parks, 2010).

We evaluated nine reintroduced herds that were established by translocations within the past 75 years. Reintroduced herds included eight small to moderately sized

populations (70-277 animals) and one large population (416 animals). Founder size for the reintroduced herds ranged from 6 to 53 animals. We considered a translocation to be part of the original founding group if it occurred within three years of the first reintroduction event to an unoccupied area. Five to eleven generations occurred since establishment of evaluated reintroduced herds, based on a generation time of 6 years (Hogg et al. 2006, Johnson et al. 2011).

#### Sample Collection and DNA Extraction

We collected all genetic samples from 2002 to 2018, and all herds were sampled over a generation time of less than two generations. All but two populations were sampled over a time span of 1 day to 4 years (less than one generation). North Glacier and Central Glacier were sampled over a period of 7 and 9 years (about 1.2 and 1.5 generations), respectively. Animal capture and handling protocols were approved by Institutional Care and Use Committees at Montana State University (Permit # 2011-17, 2014-32), Montana Department of Fish, Wildlife, and Parks (Permit # 2016-005), Parks Canada (Permit # WL-2005-638), Wyoming Game and Fish Department (Permit # 854), U.S. Geological Survey (Permit # 2004-01, DINO-2008-SCI-0010), and University of Montana (Permit # 024-07MHWB-071807 and 012-16MMMCWRU-022916). Genetic samples included gene cards, biopsy punches from ear cartilage, whole blood samples, and tissue from hunter-harvested or road-killed animals. We extracted DNA using Maxwell 16 LEV Blood DNA Kit for whole blood and the Maxwell 16 SEV Tissue Kit for biopsy punch and tissue samples per kit instructions. For gene cards, we modified the Promega LEV Blood DNA kit procedure; we incubated two to three 5mm gene card

punches with proteinase K and lysis buffer in a DNA IQ spin basket (Promega), spun at 3000 XG for five minutes, and loaded the flow-through into the Maxwell 16 LEV Blood DNA cartridge. For genotyping, we used samples with a minimum DNA concentration of 20 ng/ $\mu$ L, a minimum of 300 ng of DNA, and a 260 nm/280 nm ratio of 1.0 to 1.5.

#### Genomic Dataset and Quality Control

We contracted GeneSeek (Lincoln, NE, USA) to genotype genetic samples using the Illumina High Density (HD) Ovine array, also termed a SNP (single nucleotide polymorphism) chip. The SNP chip included 606,006 SNPs at a density of one SNP per 4.279kb. The Ovine array was originally developed for evaluation of domestic sheep, but its development included five bighorn sheep and four Dall's Sheep (*Ovis dalli*) (Kijas et al. 2009, 2014). Species divergence between domestic and bighorn sheep took place about three million years ago, but the two species can interbreed and produce viable offspring (Young and Manville 1960, Bunch et al. 2006). In addition, the two species have the same number of chromosomes and are expected to have high genomic synteny (Poissant et al. 2010). Thus, about 24,000-33,000 SNPs in the Ovine array are informative for Rocky Mountain bighorn sheep (Flesch et al. In press, Miller et al. 2015), and the domestic sheep reference genome enables mapping SNPs to chromosomes (Kohn et al. 2006). Since only a select number of individuals were used to create the SNP chip, this genotyping approach can have an ascertainment bias (Albrechtsen et al. 2010). However, differences in mean kinship within populations can be differentiated at a sample size of 20-25 individuals per population (Flesch et al. 2018).

We loaded all Illumina genotype data and concatenated multiple datasets in Golden Helix SNP and Variation Suite v8.6.0 (*SNP & Variation Suite* 2016). Using Golden Helix software, we mapped genotype data to the domestic sheep reference genome Oar v.4.0, removed samples with a call rate less than 0.85, removed markers on sex chromosomes and with unknown mappings, and exported the genotypes into PLINK v1.90 for filtering (Purcell et al. 2007, *SNP & Variation Suite* 2016). We completed the remaining analyses using R version 3.4.4 and bash code version 4.3.48 in the RStudio environment (*GNU bash, version 4.3.48(1)-release (x86\_64-pc-linux-gnu)* 2013, RStudio Team 2015, R Core Team 2017). We used minimal filtering prior to kinship analysis based on KING software guidelines (Manichaikul et al. 2010a). We removed monomorphic and extremely rare markers using a minor allele frequency of less than 0.0001 (de Cara et al. 2013) and removed markers with poor performance by requiring a SNP call rate greater than 0.99 (Huisman et al. 2016).

### Estimating Genomic Kinship

We calculated genomic kinship between all possible pairs of individuals within each population using KING v2.1.4 (Manichaikul et al. 2010a). Since the mean of all pairwise kinship values from a population represents the level of inbreeding in the next generation with random mating, we calculated the average of all pairwise kinship values for samples within each population. We termed this approach average pairwise kinship, which is similar to the method employed by Finger et al., (2011). The formal definition of mean kinship includes self-kinship values (Van Dyke 2008, Frankham et al. 2017), but we excluded self-kinships in calculating a mean for each herd, as uneven sample sizes

among populations biased relative comparisons of mean kinship among herds.

Specifically, with the inclusion of self-kinship, mean kinship of herds with smaller sample sizes would be biased higher than that of herds with larger sample sizes.

After calculating average pairwise kinship, we could not calculate an uncertainty interval around these values, because uncertainty could be influenced by many factors, including the quality of samples, genotyping approach, number of genetic markers, and the specific kinship estimator. There is no known approach to account for all of these influences in a single measure of uncertainty, so we present our average pairwise kinship estimates without uncertainty intervals.

#### Evaluating Influences on Genetic Diversity using Linear Models

We completed multiple linear regression to evaluate the relationship between average pairwise kinship and four predictor variables, including origin, minimum population size, connectivity, and augmentation history (Table 1). Population origin was defined as native (indigenous) or reintroduced. The predictor variable of origin represented the potential for a recent founder effect within the past 75 years (12.5 bighorn sheep generations).

We considered two forms of population size for the analysis: recent population size at the time of sampling and historic minimum count, which would represent a past bottleneck. Area biologists and agency reports provided count data (Keating 1985, George et al. 2009, Montana Department of Fish, Wildlife and Parks 2010). We expected counts among populations would have different detection probabilities and observer platforms (i.e. aerial vs. ground surveys), so we considered the raw counts to be an index

for relative abundance across the evaluated populations. We calculated recent population size using the mean of all available credible counts over a 5-year period prior to the last genetic sampling year. Historic minimum count represented the lowest credible count in the population's count history after 1980. This minimum count could be a result of epizootic events, population growth following reintroduction, or other unknown factors. Fourteen out of 19 evaluated herds (74%) experienced a documented all-age die-off suspected to be an epizootic event after 1970, so disease events were a predominant cause of documented bottlenecks in our dataset. Recent population size and minimum count had a Pearson correlation of 0.893, so including both variables in the same linear model would violate the independence assumption of linear regression (Sokal and Rohlf 1995). Based on genetic theory that historic bottlenecks would be the most important influence on genetic diversity, we selected minimum count data to include in the analysis (Wright 1938).

Connectivity represented level of gene flow due to unassisted dispersal of breeders between the examined population and neighboring populations. Classification of herds into gene flow categories was based on input from area biologists and previous research that evaluated genetic similarity and connectivity among populations (Flesch et al. In press). Herds were classified as "isolated" if there was no known recent gene flow, "some" if there was limited indication or some possibility for gene flow, and "high" if the population was a part of a continuous, spatially-structured population.

Finally, we evaluated augmentation history of herds to account for assisted gene flow via translocations. Thirteen out of 19 populations received at least one

augmentation. Two documented augmentation events were removed from the analysis because the translocations did not make a detectable genetic contribution in the recipient populations (Flesch et al. In press)., We considered multiple aspects of augmentation events that managers can control for the analysis, including the number of source populations, animals contributed, and augmentation events. Pearson correlation values for these variables ranged from 0.944-0.964. Given the very high correlations, we selected the number of animals contributed in augmentations to include in the linear models, as augmentation recommendations are often based on the number of migrants per generation (Wright 1931, Mills and Allendorf 1996).

In general, we expected that all four covariates were reasonable to include in a single linear model, due to expected independence of each predictor's influence on average pairwise kinship. In addition, we did not expect any two-way interactions. We summarized the data graphically to visualize the relationships among the predictors and with average pairwise kinship, and we verified that all populations had observations for each predictor (Table 1). We evaluated all possible subsets of a global model that included all four predictors, because our relatively small sample size of herds ( $n=19$ ) could cause precision to vary among models, and an all combination analysis is expected to perform better than ad hoc strategies in terms of variable selection (Doherty et al. 2012).

For all evaluated linear models, we ensured that statistical assumptions were met. The first assumption was that the average pairwise kinship response was independent among populations. We considered the possibility for a cluster effect, as some native herds were geographically proximate (Figure 1), but the Durbin Watson Test for all

models indicated that the residuals were not autocorrelated (Fox 2015). The second assumption was that the relationship between average pairwise kinship and the explanatory variables of origin, connectivity, minimum count, and animals contributed in augmentations was linear. We evaluated this assumption using scatterplots and boxplots of average pairwise kinship vs. the predictor variables and residual plots. The relationships appeared to be approximately linear, and the residual plot had random scatter, indicating this assumption was met. The third assumption was that for each value of the covariates, the variance of average pairwise kinship was constant. To examine this assumption, we evaluated the residual plots and determined there was even spread around the lowess curve to satisfy this assumption. The fourth assumption was that for each value of the explanatory variables, the average pairwise kinship values were normally distributed. We considered this assumption by examining the QQ plot of residuals, and this assumption was reasonably satisfied.

We evaluated additional linear regression diagnostics, including correlation among predictors, outliers, and high leverage points. All models had variance inflation factor (VIF) values less than 3, indicating low collinearity (Fox and Monette 1992, Fox 2015, Fox and Weisberg 2018). Bonferroni outlier tests for all candidate models indicated there were no studentized residuals with a p-value less than 0.05 (Fox 2015). There were no high leverage points, as all Cook's distance values were less than 0.3 (Cook 1977).

To evaluate relative model plausibility, we used an information-theoretic approach (Burnham and Anderson 1998). We used Akaike's Information Criterion with

small-sample bias adjustment ( $AIC_c$ ) to compare models, where the best-fitting model has the lowest  $AIC_c$  value (Akaike 1973, Hurvich and Tsai 1989). In addition, relative plausibility of each candidate model was represented by Akaike weights, which range from 0 (lowest plausibility) to 1 (highest plausibility) (Burnham and Anderson 1998). Because each predictor was included in an equal number of models, we estimated the importance of individual predictor variables using the sum of Akaike weights for the candidate models that included the predictor (Burnham and Anderson 1998).

#### Comparing Average Pairwise Kinship with Juvenile Survival to Detect Inbreeding Depression

Because juvenile survival can be affected by inbreeding depression more than adult survival (Ralls et al. 1979, Cohas et al. 2009) and generally has the greatest impact on population trend over other vital rates in large ungulates, including bighorn sheep (Paterson et al. In prep, Gaillard et al. 1998), we compared average pairwise kinship with juvenile survival to evaluate the possibility for inbreeding depression and its effects on population growth. Juvenile survival was defined using a biological year, meaning the probability of survival was calculated from May in chronological year  $t$  to April in chronological year  $t + 1$  (Paterson et al. In prep). Juvenile survival estimates were available for ten herds, and median juvenile survival with a 90% credible interval was calculated from the full time series that demographic data were available following the most recent augmentation (Paterson et al. In prep). Poor recruitment due to lamb pneumonia can last from 3-5 years to decades following an epizootic event (Cassirer et al. 2013, Manlove et al. 2016), so we also compared median juvenile survival with

population disease history. Population disease history was defined by 1) if the population had a known epizootic event since 1980, and 2) if *Mycoplasma ovipneumonia* was detected by PCR, as this pathogen has been associated with bighorn sheep epizootic events (Butler et al. 2018, Garrott et al. 2020). *Mycoplasma ovipneumonia* was only recorded as absent from populations if the probability the pathogen was present was less than 0.06 (assuming 10% prevalence), given the number of swabs collected per animal, sample size, and detection probability (Butler et al. 2017, 2018, Garrott et al. 2020).

## Results

### Genomic Kinship and Herd Attributes

We genotyped 488 bighorn sheep samples with a SNP call rate greater than 0.85. We met our sample size goal of at least 20-25 individuals for eighteen Rocky Mountain bighorn sheep populations, excluding Highlands (Table 1). Filtering markers on sex chromosomes and with unknown mappings removed 29,411 SNPs. Filtering based on a minor allele frequency of less than 0.0001 removed 469,939 SNPs; requiring a SNP call rate greater than 0.99 removed 76,488 SNPs. After filtering, we used 30,168 SNPs to estimate genomic kinship between all individuals in each population. Average pairwise kinship per population ranged from 0.014 to 0.080, and positive values suggested that all populations were in Hardy-Weinberg equilibrium. For quantitative predictor values, minimum count per population ranged from 16 to 562 individuals; number of animals contributed in augmentations ranged from 0 to 165 individuals (Table 1).

### Connectivity and Other Predictors were Correlated with Average Pairwise Kinship

We evaluated 15 linear models, and eight top-performing linear models with a delta AIC<sub>c</sub> less than 9 had  $r^2$  values that averaged 0.65 and ranged from 0.55 to 0.75, indicating precise fits (Table 2). All top-performing models contained connectivity as a predictor. The most plausible model of average pairwise kinship contained connectivity and number of animals contributed in augmentations. This model was 1.7 times more likely than the next best approximating model, which contained the same predictors and minimum count. All estimated coefficients in the top three models had 95% confidence intervals that did not overlap with 0. Coefficients were relatively stable across models, so we further discuss the results from the full model:

$$\begin{aligned} \hat{\mu} \{Mean\ kinship \mid & Connectivity, Minimum\ count, Animals\ contributed\ in \\ & augmentations, Origin\} \\ &= 0.06347 - 0.01800(Connectivity\ [Some]) \\ &\quad - 0.02372(Connectivity\ [High]) \\ &\quad - 0.00003(Minimum\ count) \\ &\quad - 0.00015(Animals\ contributed\ in\ augmentations) \\ &\quad - 0.00453(Origin\ [Native]) \end{aligned}$$

The relationship between average pairwise kinship and all predictors were in the expected direction. Connectivity had the highest importance weight (0.99), followed by number of animals contributed in augmentations (0.83), minimum count (0.36), and origin (0.13). Populations with high connectivity generally had lower average pairwise kinship, implying greater genetic diversity, than those that were genetically isolated

(Figure 2A). The modeled distribution of average pairwise kinship for some connectivity overlapped with distributions of isolated and high connectivity. Similar to connectivity, increasing the number of animals contributed in augmentations was associated with a decrease in average pairwise kinship (Figure 2B). This result suggests that management intervention to supplement existing populations via augmentation was effective in increasing genetic diversity. Greater minimum count was associated with decreasing average pairwise kinship in an inverse relationship (Figure 2C), suggesting that significant bottlenecks lowered genetic diversity. Finally, origin was the least important predictor, and the model suggested that native herds may have lower average pairwise kinship and thus greater genetic diversity than reintroduced herds, but the confidence interval of this predictor overlapped zero (Figure 2D).

#### Median Juvenile Survival and Average Pairwise Kinship

Median juvenile survival was calculated post-augmentation for ten populations for a time series that ranged from 6 to 33 biological years (Paterson et al. In prep). We observed no clear relationship between average pairwise kinship and median juvenile survival (Figure 3). Herds that experienced epizootic events within the past 40 years generally had lower median juvenile survival than those that did not (Figure 3). The lack of relationship between average pairwise kinship and median juvenile survival suggested that inbreeding depression was not detected.

## Discussion

We determined that influences on population size over time were correlated with genetic diversity for 19 different bighorn sheep populations. Greater gene flow, including unassisted connectivity and animals contributed in augmentations, were more important predictors than historic minimum population size and origin. This result suggests that influences on population genetics that occurred in more recent generations (i.e. gene flow), could compensate for past loss of genetic diversity in earlier generations, such as a founder effect in reintroduced populations. In addition, lack of an observed relationship between average pairwise kinship and median juvenile survival suggested that inbreeding depression did not suppress population growth in the examined populations.

### Native and Reintroduced Populations had Similar Levels of Genetic Diversity

We did not expect that origin of populations (native vs. reintroduced) would be the least important predictor of genetic diversity, due to previous research that suggested reintroduced bighorn sheep herds generally have lower genetic diversity due to a founder effect (Fitzsimmons et al. 1997, Hedrick et al. 2001, Olson et al. 2013). Seven out of nine reintroduced populations were established or augmented by managers sourcing from multiple populations, which may have mitigated a founder effect. For example, one island population (Wild Horse Island) was founded by six individuals in 1947 and only augmented by a translocation of 2 males in 1987 from a different herd than the founder source, and this population had only the fifth highest average pairwise kinship in the

study. Thus, our results suggest that gene flow and admixture (interbreeding between differentiated populations) after reintroduction can serve to minimize average pairwise kinship. Specifically, sourcing reintroductions and augmentations from different populations can be an effective management tool to maximize genetic diversity in reintroduced populations. Similarly, reintroduced bighorn sheep herds in Nevada, most with past augmentations, generally did not have lower genetic diversity than native populations (Jahner et al. 2018). Admixture was also more important in maximizing genetic diversity within reintroduced populations of Alpine ibex (*Capra ibex*) than releasing a large number of founders from the same source (Biebach and Keller 2012). This admixture approach to reintroduction has also been effective in multiple Australian mammal species (White et al. 2018, McLennan et al. 2020).

Previous bighorn sheep research has suggested sourcing only from native bighorn sheep populations for translocations due to higher success rates and expected greater genetic diversity (Singer et al. 2000). In contrast, our research suggests that genetic diversity of reintroduced herds may not always be a concern to influence this decision, if reintroduced herds had gene flow that enhanced genetic diversity. Considering reintroduced populations as potential sources for translocations would be helpful to translocation planning by providing a larger pool of candidate source populations. However, lack of a clear relationship between origin and average pairwise kinship in our analysis also suggests that this predictor could be explored further with additional observations and predictors. Additional variables beyond admixture, such as rate

population growth after population establishment, can also be important to maintaining genetic diversity after reintroduction (Lacy 1994).

In our analysis we were not able to directly evaluate number of generations since reintroduction and founder size due to a limited sample size of reintroduced herds. However, our results suggest a slight inverse relationship between the number of founders and average pairwise kinship, as expected (Templeton 1980, Aguilar et al. 2008). Biologists typically seek to avoid strong founder effect by sampling a sufficiently large number of individuals to serve as founders, such that the group will be representative of the source's population genetics (Jamieson and Lacy 2012). At least 30 breeding individuals are generally recommended for reintroduction in an unoccupied area (Frankham et al. 2017). Future research could evaluate the optimal number of founders for bighorn sheep reintroductions, as optimal founder size can vary by reintroduction area and species, based on factors such as carrying capacity and expected survival rate after translocation (Tracy et al. 2011).

#### Bottlenecks of Greater Magnitude were Associated with Lower Genetic Diversity

Lower historic minimum counts of population size were correlated with higher average pairwise kinship, suggesting that past bottlenecks negatively affected genetic diversity as expected. Most examined herds experienced bottlenecks due to epizootic events, and these disease events can reduce genetic diversity of bighorn sheep populations (Ramey et al. 2000). Similarly, studies on marine and freshwater fish found a positive relationship between abundance and genetic diversity, including influences from overfishing (McCusker and Bentzen 2010, Pinsky and Palumbi 2014). However, it

was interesting to observe that minimum population size was not one of the most important predictors of average pairwise kinship, because population size is important to persistence of bighorn sheep herds (Berger 1990). However, small population size or bottlenecks can interact with other influences on population growth that are genetic or not, such as predation (Rominger et al. 2004). Lower importance of minimum population size in predicting average pairwise kinship also suggests that evaluating genetic diversity alone may not be an effective approach to estimate census population size for this species, similar to desert lizards (Grundler et al. 2019). Due to high correlation between current population size and historic minimum count, we were not able to distinguish between these variables for this analysis, and future research could further evaluate the influences of current population size and fluctuations in population growth over time on genetic diversity.

#### Augmentation was Associated with Greater Genetic Diversity

An increasing number of animals contributed in augmentation efforts was correlated with lower average pairwise kinship, as expected. Thus, translocations to supplement existing populations were generally effective in minimizing inbreeding. A similar correlation with genetic diversity was observed in other bighorn sheep populations for the number of translocated animals and population sources (Jahner et al. 2018, Love Stowell et al. 2020). Gene flow via translocations can also enhance bighorn sheep juvenile survival in the generation following augmentation (Olson et al. 2012). Increase in genetic diversity following augmentation has been documented for other species, such as the Florida panther (*Puma concolor coryi*) and Swedish adder (*Vipera*

*berus*) (Madsen et al. 1999, Johnson et al. 2010). This result is consistent with genetic management guidelines that suggest a certain number of migrants (i.e. 1-10 animals per generation) to effectively maintain genetic variation (Wright 1931, Mills and Allendorf 1996). Future research could seek to differentiate between the influences of the number of animals moved, number of augmentation events, and number of population sources. When planning an augmentation, managers can identify optimal source populations by evaluating the level of mean kinship between populations to minimize inbreeding and other translocation considerations, such as disease concerns (Flesch et al. In press, Finger et al. 2011, Ewen et al. 2012, Garbe et al. 2016, Frankham et al. 2017).

#### Unassisted Gene Flow was the Most Important Predictor of Average Pairwise Kinship

Populations with high connectivity had greater genetic diversity than isolated populations, and natural dispersal of breeders between populations was the most important influence in maximizing genetic diversity. This result is consistent with the observation that reduction of natural gene flow between bighorn sheep populations due to highways can result in decreased genetic diversity (Epps et al. 2005). Gene flow was also more important than decline in population size for Atlantic salmon (*Salmo salar*) (Consuegra et al. 2005). Conversely, Wang et al., (2017) found that island size, population size, and time since isolation were more important drivers of genetic diversity than the distance to larger islands and mainland that represented potential for connectivity, emphasizing the importance of evaluating this research question in different systems. When managing bighorn sheep populations, enabling natural gene flow may be highest priority to avoid negative impacts of inbreeding, when this approach does not

conflict with other management considerations. Our study considered categorical variables to describe connectivity based on expert knowledge and previous genomic analyses (Flesch et al. In press), but future research could more specifically evaluate the relationship between the number of animals dispersing and average pairwise kinship to establish a more specific target level of natural gene flow between populations.

#### Inbreeding Depression was not Detected

Populations with greater levels of average pairwise kinship were not associated with lower juvenile survival, suggesting that inbreeding depression was not present in the examined populations. Similarly, Johnson et al., (2011) suggested that inbreeding depression would not negatively impact population growth of Sierra Nevada bighorn sheep populations for eight bighorn sheep generations (48 years) after genetic analysis, despite a history of reintroductions and bottlenecks. Research using experimental translocations of wild fish to evaluate the consequences of small population size was not able to detect an effect of low genomic diversity on individual growth and survival, which would indicate inbreeding depression (Yates et al. 2019). All examined populations had average pairwise kinships lower than the concerning mean kinship threshold of 0.1 (Frankham et al. 2017), suggesting that inbreeding in the observed populations was not high enough to negatively affect median juvenile survival. Average pairwise kinships evaluated in this study were a slight underestimate of mean kinship, due to our exclusion of self-kinship values. However, we expect that only one evaluated population (Middle Missouri) would exceed a mean kinship value of 0.1 if self-kinships were included in the calculation, and this population had the highest median juvenile

survival, supporting the idea that inbreeding did not negatively affect juvenile survival in our study populations.

Alternatively, other influences on juvenile survival may have masked its relationship with average pairwise kinship. There are many other variables, such as maternal age, body weight at weaning, and disease, that are correlated with juvenile survival in mammals (Hadley et al. 2007, Proffitt et al. 2008, Cassirer et al. 2013, Manlove et al. 2016). Herds that had documented all-age die-offs within the past 40 years generally had lower median lamb survival than those that did not (Figure 3). Thus, epizootic events were likely a more important influence on juvenile survival than average pairwise kinship, which is consistent with previous research that found poor recruitment after an epizootic event can last from 3-5 years to decades (Cassirer et al. 2013, Manlove et al. 2016). Thus, the effect of epizootics on juvenile survival may have masked any potential impact of inbreeding depression on juvenile survival. Future research could evaluate the relationship between individual genomics and juvenile survival after an epizootic. For example, inbred California sea lion juveniles (*Zalophus californianus*) exhibited less effective immune responses to parasites (Acevedo-Whitehouse et al. 2006). While inbreeding depression may be correlated with lower disease resistance (Balloux et al. 2004), immune response can also be associated with specific loci (Acevedo-Whitehouse et al. 2003, 2006, Alves et al. 2019). Thus, after an augmentation intended to increase genetic diversity, managers should not expect an immediate, obvious response in terms of improved survival in an epizootic event (Boyce et al. 2011, Ewen et al. 2012). However, high genetic diversity in a population is still important as it enables evolution

of more resilient populations to occur through natural selection of individuals that survive epizootic events (Alves et al. 2019).

### Conclusions

Our study found that greater connectivity, number of animals contributed in augmentations, and minimum population size were correlated with higher genetic diversity at the population level, and origin was less important. These results also demonstrated that use of the HD Ovine SNP chip and kinship coefficients can be used to effectively evaluate genetic diversity in bighorn sheep populations. There are multiple metrics to calculate kinship and the optimal method may depend on the research question and dataset (Goudet et al. 2018). Mean kinship is used to make breeding decisions to minimize inbreeding in most, if not all, wildlife captive-breeding programs to support reintroductions, such as the black-footed ferret (*Mustela nigripes*) and California condor (*Gymnogyps californianus*) (Russell et al. 1994, Ballou and Foose 1996, Ralls and Ballou 2004). In addition, other studies have also found that kinship is effective for informing genetic rescue decisions in plants and animals to maximize genetic diversity and minimize inbreeding (Finger et al. 2011, Garbe et al. 2016).

Despite lack of evidence for inbreeding depression in the examined populations, this study also highlights which herd attributes may result in inbreeding at concerning levels. For example, our results suggested that populations that are isolated with no natural connectivity or augmentations are of highest concern, regardless of origin. Thus, managers can prioritize herds of highest genetic concern based on average pairwise kinship, mean kinship, or population attributes that were ranked in importance by our

analysis if genetic data are not available. This approach would provide managers with additional information to weigh the costs and benefits of augmentation efforts.

Kinship and other genomic analyses alone cannot dictate augmentation and management decisions, as species life history, population demography, and habitat concerns are also important (Van Dyke 2008). However, increasing the role of genomic analyses and considerations in wildlife management would improve understanding regarding what circumstances can result in genetics limiting the distribution, population growth, or size of a species of conservation concern (Van Dyke 2008). Thus, our study can help inform genetic management and conservation of other fragmented wild populations to retain genetic diversity for population persistence and evolution into the future.

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use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Tables and Figures

Table 1: Data for 19 bighorn sheep herds integrated into linear models that compared the relationship between average pairwise kinship and origin, connectivity, animals contributed in augmentations, and minimum count. Sample size describes the number of individual bighorn sheep genotypes used to estimate average pairwise kinship.

| <b>Herd</b>                  | <b>Sample Size</b> | <b>Connectivity</b> | <b>Animals Contributed in Augmentations</b> | <b>Minimum Count</b> | <b>Average Pairwise Kinship</b> |
|------------------------------|--------------------|---------------------|---------------------------------------------|----------------------|---------------------------------|
| <b>Native</b>                |                    |                     |                                             |                      |                                 |
| Glacier (South)              | 26                 | High                | 0                                           | 136                  | 0.014                           |
| Clark's Fork / Trout Peak    | 27                 | High                | 0                                           | 562                  | 0.015                           |
| Wapiti Ridge                 | 27                 | High                | 0                                           | 552                  | 0.021                           |
| Glacier (North) and Waterton | 25                 | High                | 0                                           | 106                  | 0.021                           |
| Franc's Peak                 | 23                 | High                | 23                                          | 270                  | 0.030                           |
| Castle Reef                  | 25                 | High                | 23                                          | 99                   | 0.035                           |
| Stillwater                   | 24                 | Some                | 3                                           | 23                   | 0.038                           |
| Glacier (Central)            | 44                 | High                | 0                                           | 103                  | 0.042                           |
| Taylor Hilgard               | 30                 | Isolated            | 68                                          | 25                   | 0.055                           |
| Spanish Peaks                | 20                 | Isolated            | 2                                           | 80                   | 0.057                           |
| <b>Reintroduced</b>          |                    |                     |                                             |                      |                                 |
| Dinosaur                     | 20                 | Some                | 165                                         | 100                  | 0.018                           |
| Tendoy                       | 25                 | Some                | 99                                          | 16                   | 0.029                           |
| Highlands                    | 17                 | Isolated            | 140                                         | 80                   | 0.034                           |
| Fergus                       | 30                 | High                | 28                                          | 108                  | 0.038                           |
| Paradise                     | 25                 | Isolated            | 22                                          | 93                   | 0.043                           |
| Lost Creek                   | 25                 | Some                | 2                                           | 58                   | 0.045                           |
| Wild Horse Island            | 25                 | Isolated            | 2                                           | 38                   | 0.052                           |
| Petty Creek                  | 25                 | Isolated            | 4                                           | 63                   | 0.064                           |
| Middle Missouri              | 25                 | Isolated            | 0                                           | 79                   | 0.080                           |

Table 2: Predictor variables, number of parameters ( $K$ ), log-likelihood ( $\text{Log}L$ ), Akaike's Information Criterion with small-sample bias adjustment ( $\text{AIC}_c$ ),  $\Delta\text{AIC}_c$ , and Akaike weights ( $w$ ) for candidate models ( $i$ ) that predicted bighorn sheep population average pairwise kinship. Eight models with  $\Delta\text{AIC}_c$  less than 9 are shown out of fifteen evaluated models.

| <b>Candidate Model</b>                                                    | <b><math>K</math></b> | <b><math>\text{Log}L</math></b> | <b><math>\text{AIC}_c</math></b> | <b><math>\Delta\text{AIC}_c</math></b> | <b><math>w_i</math></b> |
|---------------------------------------------------------------------------|-----------------------|---------------------------------|----------------------------------|----------------------------------------|-------------------------|
| Connectivity, Animals contributed in augmentations                        | 5                     | 61.29                           | -107.96                          | 0.00                                   | 0.444                   |
| Connectivity, Minimum count, Animals contributed in augmentations         | 6                     | 62.97                           | -106.95                          | 1.02                                   | 0.267                   |
| Connectivity                                                              | 4                     | 57.93                           | -105.00                          | 2.96                                   | 0.101                   |
| Connectivity, Animals contributed in augmentations, Origin                | 6                     | 61.74                           | -104.49                          | 3.47                                   | 0.078                   |
| Connectivity, Minimum count                                               | 5                     | 59.02                           | -103.43                          | 4.54                                   | 0.046                   |
| Connectivity, Minimum count, Animals contributed in augmentations, Origin | 7                     | 63.36                           | -102.54                          | 5.43                                   | 0.029                   |
| Connectivity, Origin                                                      | 5                     | 57.95                           | -101.28                          | 6.68                                   | 0.016                   |
| Connectivity, Minimum count, Origin                                       | 6                     | 59.02                           | -99.05                           | 8.92                                   | 0.005                   |

Figure 1: Approximate distributions of bighorn sheep (*Ovis canadensis*) populations that were evaluated. Native populations that did not receive animals from other areas through augmentation are dark brown. Native populations that received animals from other areas through augmentation are beige. Reintroduced populations are shown in black. Approximate bighorn sheep ranges, including populations not in this study, are shown in gray polygons for Idaho, Wyoming, and Montana (Montana Fish, Wildlife and Parks 2008, Wyoming Game and Fish Department 2012, Thomas 2019). State boundaries are dashed lines outlined in gray; national park boundaries in the study area are dashed lines.



Figure 2: Empirical linear model estimates of the relationship between average pairwise kinship for 19 bighorn sheep populations and connectivity (A), total number of animals contributed in augmentations by connectivity (B), minimum count by connectivity (C), and origin (D). High connectivity represented by green, some connectivity is blue, and isolated is red. Whiskers and bands around estimates represent the 95% confidence interval. Observed data are shown as points. Relationships are based on the linear model:  $\hat{\mu}\{\text{Mean kinship} \mid \text{Connectivity}, \text{Minimum count}, \text{Animals contributed in augmentations}, \text{Origin}\} = 0.06347 - 0.01800(\text{Connectivity} [\text{Some}]) - 0.02372(\text{Connectivity} [\text{High}]) - 0.00003(\text{Minimum count}) - 0.00015(\text{Animals contributed in augmentations}) - 0.00453(\text{Origin} [\text{Native}])$ .  $R^2$  for the model is 0.747.

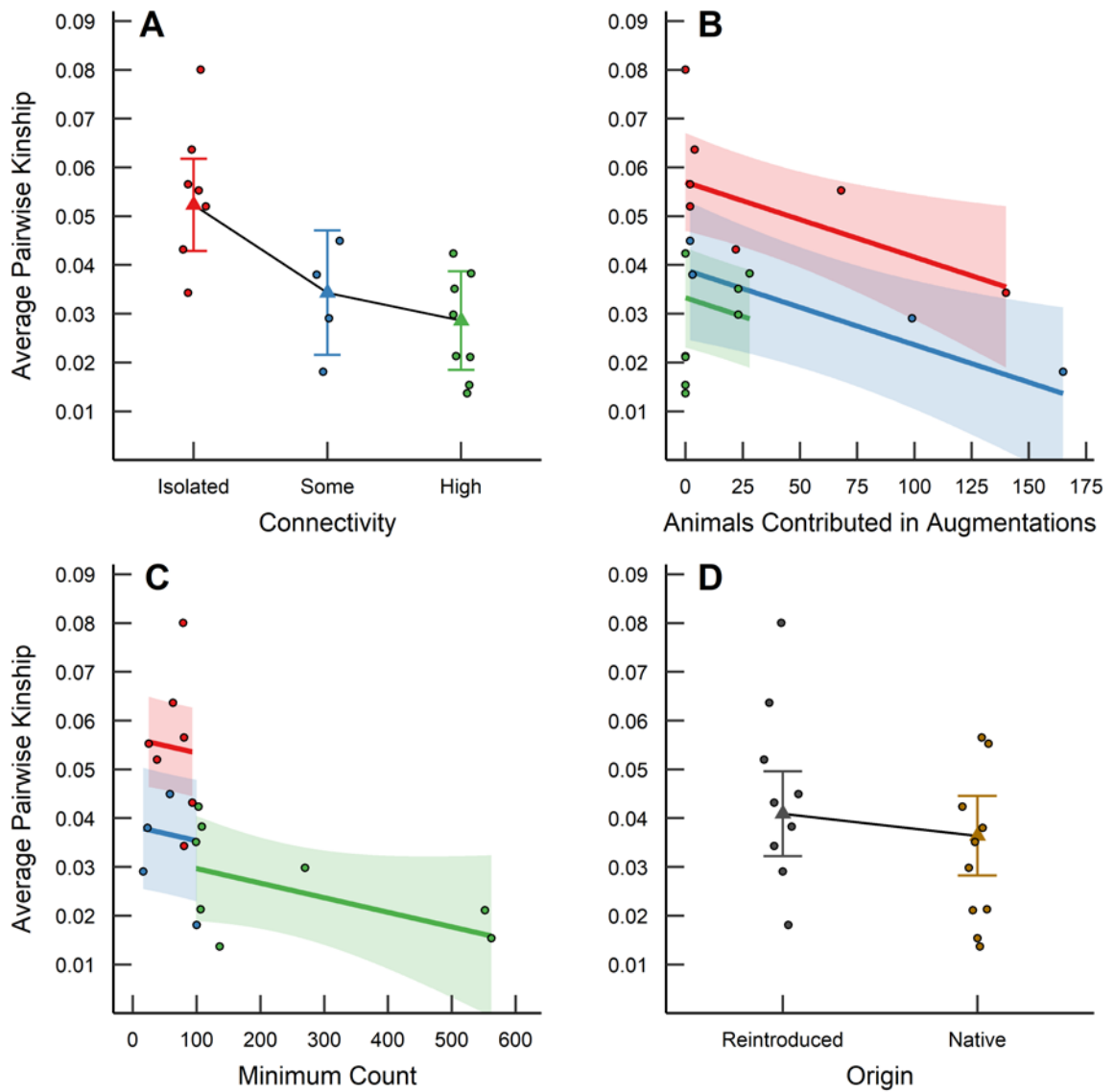
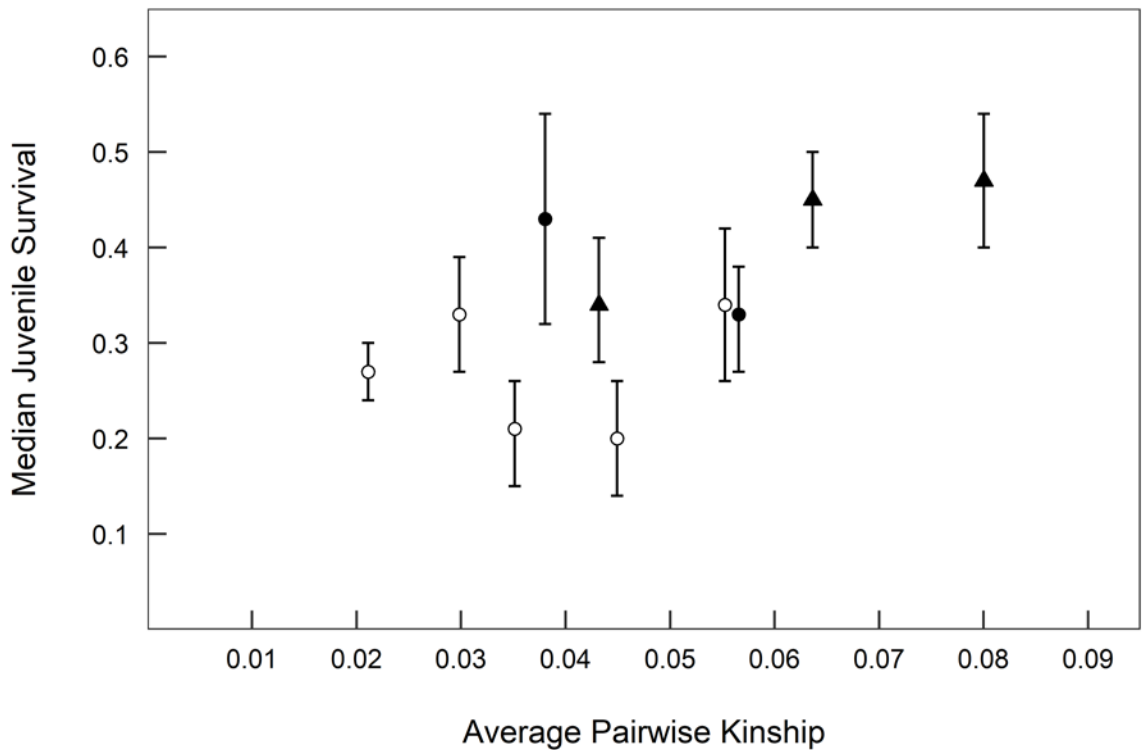


Figure 3: Median juvenile survival and average pairwise kinship for 10 bighorn sheep populations (Paterson et al. In prep). Estimates for populations with *Mycoplasma ovipneumoniae* detected are circles; estimates for populations that were tested for *Mycoplasma ovipneumoniae* with negative results are shown as triangles (Butler et al. 2018, Garrott et al. 2020). Whiskers indicate the 90% credible interval for median juvenile survival. All median juvenile survival values were estimated using a time series of count data one year after the most recent augmentation of animals from another area. Estimates for populations that had at least one recorded all-age die-off in the past 40 years are white; estimates for populations without a known all-age die-off in the past 40 years are black.



## CHAPTER FIVE

## GENERAL CONCLUSIONS

This dissertation evaluated population structure, gene flow, and genetic diversity of bighorn sheep populations with diverse management histories. To genetically evaluate bighorn sheep, we generated genome-wide genotypes using the Illumina High Density Ovine array that included thousands of single nucleotide polymorphisms. A genomic approach and evaluation of multiple bighorn sheep populations allowed us to effectively address our research questions.

In Chapter Two, my co-authors and I determined the optimal sample size per population necessary to evaluate intra- and interpopulation relationships. Using genomic data for four populations, we employed empirical simulations to evaluate kinship and identity by state metrics, as well as  $F_{ST}$  between populations. We concluded that a sample size of 20 to 25 was adequate for assessing these metrics when using the Ovine array to genotype Rocky Mountain bighorn sheep herds. This result contrasted with sample size recommendations of other empirical simulation studies of wild populations (Gaughran et al., 2017; Nazareno et al., 2017). Thus, we concluded that a universal sample size rule may not be able to sufficiently address the complexities that impact genomic kinship and inbreeding estimates.

In Chapter Three, my co-authors and I conducted a population genomic analysis of 511 bighorn sheep from 17 areas, including native and reintroduced populations that received 0-10 translocations. Our analyses determined that natural gene flow did not occur between most populations. We synthesized genomic evidence across analyses to

evaluate 24 different translocation events. We detected eight successful reintroductions and five successful augmentations, based on genetic similarity with the source populations. One native population founded most of the examined reintroduced herds, suggesting that environmental conditions did not need to match for populations to persist following reintroduction. Other studies have also documented translocated bighorn sheep establishing populations in areas with different environmental conditions than their source (Malaney et al., 2015; Rominger et al., 2004; Wiedmann and Sargeant, 2014). However, local adaptation in bighorn sheep herds is still possible (Wiedmann and Sargeant, 2014), given that native populations found in different environments were genetically different.

In Chapter Four, we determined that influences on population size over time were correlated with genetic diversity (assessed using average pairwise kinship) for 19 bighorn sheep populations. Greater gene flow, including unassisted connectivity and animals contributed in augmentations, were more important predictors than historic minimum population size and origin (i.e. native vs. reintroduced). This result suggested that influences on population genetics that occurred in more recent generations, such as gene flow, could compensate for past loss of genetic diversity in earlier generations, such as a founder effect in reintroduced herds. We did not observe a relationship between average pairwise kinship and median juvenile survival, suggesting that inbreeding depression did not suppress population growth in the examined populations. All examined populations had average pairwise kinship values lower than the concerning mean kinship threshold of

0.1 (Frankham et al. 2017), so inbreeding in the observed populations may not have been sufficiently high to negatively affect juvenile survival.

Insights gained from this project can help inform bighorn sheep management and restoration efforts. Specifically, this research may help determine what populations have low genetic diversity and might benefit from augmentation, as well as aid in selection of potential source populations for augmentation or reestablishment projects. In general, a low-cost genomic analysis technique new to wildlife research had demonstrated potential to help address bighorn sheep genetic research questions. By conducting genomic research of bighorn sheep, we also sought to provide information that could benefit genetic management of other wildlife species, such as genetic viability planning for isolated populations. This effort could lead to an innovative framework for genomic research techniques to research challenging and complex ecological questions that impact wildlife populations worldwide.

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