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The role of disease, habitat, individual condition, and herd attributes on bighorn sheep recruitment and population dynamics in Montana

Dr. Robert Garrott
Prof., Montana State University
310 Lewis Hall, Bozeman, MT 59717
406-994-2270
rgarrott@montana.edu

Dr. Kelly Proffitt
Research Biologist, MFWP
1400 S. 19th Ave., Bozeman, MT 59715
406-994-6365
kproffitt@mt.gov

Dr. Jay Rotella
Prof., Montana State University
310 Lewis Hall, Bozeman, MT 59717
406-994-5676
rotella@montana.edu

Dr. Jim Berardinelli
Prof., Montana State University
103 Anim. Biosci. Bldg, Bozeman, MT 59717
406-994-5574
jgb@montana.edu

Dr. Jennifer Thomson
Prof., Montana State University
103 Anim. Biosci. Bldg, Bozeman, MT 59717
406-994-7434
jennifer.thomson@montana.edu

Elizabeth Flesch
Ph.D. Candidate, Montana State University
310 Lewis Hall, Bozeman, MT 59717
406-994-4573
elizabeth.flesch@gmail.com

Carson Butler
M.Sc. Graduate, Montana State University
310 Lewis Hall, Bozeman, MT 59717
307-739-3487
carson_butler@nps.gov

Ethan Lula
M.Sc. Candidate, Montana State University
310 Lewis Hall, Bozeman, MT 59717
406-994-4573
ethanlula@montana.edu

Rashelle Lambert
M.Sc. Candidate, Montana State University
103 Anim. Biosci. Bldg, Bozeman, MT 59717
406-994-5574
Melissa.herrygers@msu.montana.edu

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Project Background

The history of bighorn sheep (*Ovis canadensis)* conservation shares many similarities with the conservation history of other North American ungulates, but is also quite distinctive. Similar to other ungulates, bighorn sheep existed in continuous and broadly distributed populations and likely numbered in the millions prior to colonization of western North America. Following settlement of western North America bighorn sheep and other ungulate species experienced drastic reductions in numbers and extirpation from much of their former range which prompted a dedicated restoration effort by wildlife management agencies throughout the 20th century. This effort was successful in recovering most ungulate species back from perilously low populations (Picton and Lonner 2008). Restoration efforts of most ungulates entailed regulating harvest, protecting habitat, and translocating animals to facilitate colonization of previously occupied habitat; a prescription that has been successful to the point that wildlife managers are now challenged by conflicts between broadly distributed and abundant wildlife populations and humans. However, such issues are rarely described as challenges for bighorn sheep management.

There are currently estimated to be approximately 80,000 wild bighorn sheep in North America, representing a four-fold increase compared to the beginning of restoration efforts, but still likely at least a ten-fold decrease from historic numbers (Buechner 1960, Toweill and Geist 1999). The total population of bighorn sheep in North America is the sum of hundreds of patchily distributed individual populations. In Montana, most populations are isolated and number less than 150 animals (Butler, Garrott and Rotella 2013) and this pattern has been described across their range (Berger 1990). This stands in contrast to the comparatively continuous distribution of other ungulates such as deer, elk and antelope. The most obvious factor hindering further bighorn sheep restoration is continued, widespread expression of respiratory disease. However, high predation rates, habitat loss and, poor genetic diversity and "unique factors" are also cited as factors limiting bighorn sheep populations (Festa-Bianchet *et al.* 2006, Hogg *et al.* 2006, Johnson *et al* 2010). Given multiple potential limiting factors, managers often face difficult decisions regarding bighorn sheep conservation with insufficient information on the drivers of demographic processes. The small size of many populations makes management decisions even more challenging by heightening the consequences of these decisions. However, there still exist numerous populations that, for unknown but presumably tangible reasons, are well distributed, robust and require minimal management intervention. Thus, additional information regarding general bighorn sheep ecology would be useful for management agencies to have more confidence in predicting outcomes of different management actions.

As an initial start to establishing a statewide bighorn sheep research project, Montana Fish, Wildlife and Parks (MFWP) supported a six-month contract to Montana State University (MSU) during fiscal year 2012/2013 to consolidate all herd-specific bighorn sheep classification data into a single standardized database and analyze these data to learn as much as possible from existing data routinely collected by area biologists (Butler, Garrott, and Rotella 2013). This effort revealed a substantial amount of variation in population size and annual recruitment rates (as indexed by lamb:ewe ratios) among herds as well as within each herd through time, even after accounting for numerous weather metrics and respiratory disease epizootics. Further, the report's findings suggested population-specific responses of bighorn recruitment to annual weather variability. Collectively, the report indicated there is much to be learned about the factors that drive bighorn sheep demographic rates and accordingly, much to be learned about potential management strategies that can be used to influence demographic rates in desirable ways.

In 2013, MFWP and MSU initiated a collaborative six-year research program designed to assess factors driving bighorn sheep population dynamics across Montana. The integrated study design entails using standardized methods to investigate demographic rates, body condition and nutrition, respiratory pathogens, movements, habitat use, and herd attributes across a diverse set of populations occupying a diverse set of landscapes (Figure 1). Similar designs have proven efficient at producing reliable and generalizable findings useful for management agencies. In recognition of the improved inference associated with incorporation of additional study populations, this research program has strived to incorporate data from a companion MSU bighorn sheep study (Greater Yellowstone Area Mountain Ungulate Project), has worked with the MFWP wildlife health lab to incorporate data from additional populations captured for health monitoring purposes, and has collaborated with Wyoming Game & Fish Department (WGF) to develop sampling methods that are comparable across states. This study has and will continue to greatly benefit from inclusion of these parties in the research project. This annual report is the third produced by this research project. All findings reported herein should be considered preliminary, as data collection and analysis are ongoing.

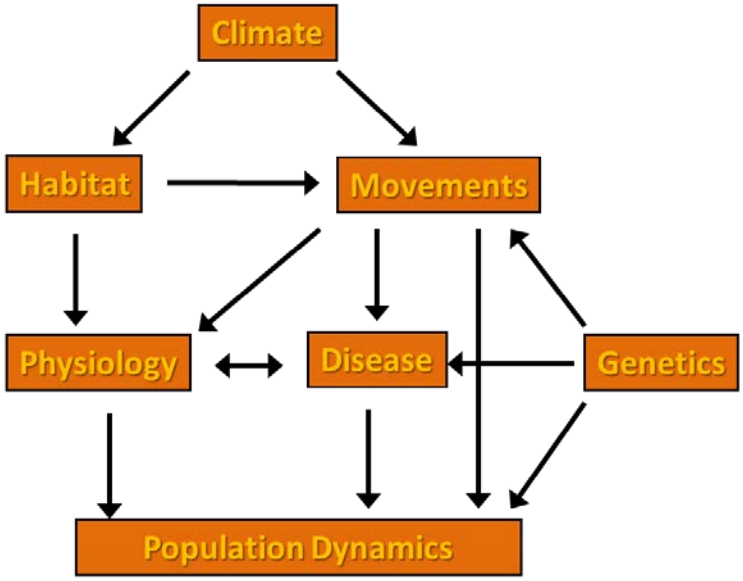


Figure 1. *Conceptual diagram of integrated study design used by this research program*

Locations

Research conducted under this grant is focused within the range of eight distinct bighorn sheep populations across varying ecological settings in Montana. Bighorn sheep populations incorporated into this study occupy portions of Deer Lodge, Fergus, Lewis & Clark, Madison, Missoula, Phillips, Sanders, Stillwater and Teton Counties, as well as the Flathead Indian Reservation. Populations and associated hunting districts (HD) included in the research program include Perma-Paradise (HD 124), Petty Creek/Grave Creek Range (HD 203), Lost Creek (HD 213), Hilgard (HD 302), Castle Reef (HD 422), Fergus (HD 482), Stillwater (HD 500), and Middle Missouri Breaks/Larb Hills (HD 622).

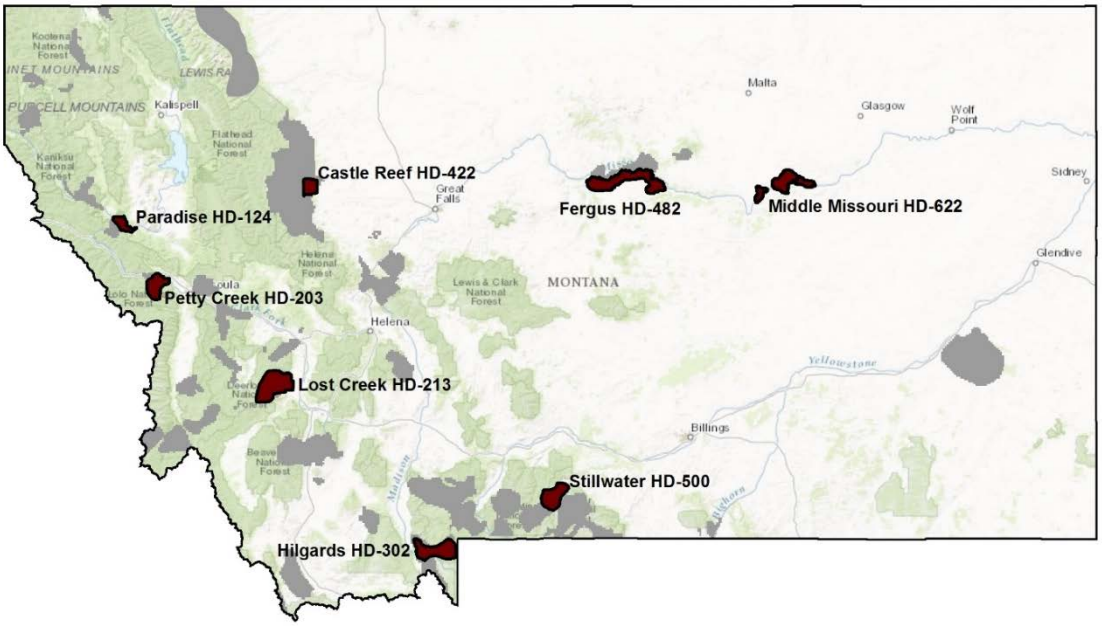


Figure 2. *Ranges of the eight study populations included in the Montana Bighorn Sheep Study. Polygons shaded in gray show ranges of the other bighorn sheep populations in Montana that are not part of this research effort.*

Study Objectives (Year 3 of 6-year study)

During the third year of this bighorn sheep research program, the primary objectives were:

1) Continue to capture, sample, and instrument animals in each study population in order to reach original capture and monitoring goals
2) Assess respiratory pathogen communities and associations with demographic performance
3) Assess variation in body condition and physiological status among sampled populations
4) Collect first deployment of GPS collars and begin spatial analysis of data.
5) Monitor demographic rates in instrumented populations
6) Collect and provide samples for a bighorn sheep genetics study and complete preliminary genomic analyses

Objective # 1: *Capture, sample, and instrument animals in each study population*

1.1 Animal Capture and Sampling

1.1.1. Capture Methods

All captures were planned for winter months. Animals have been captured using three different capture methods including helicopter net-gunning (performed by Quicksilver Air Inc.), drop-netting, and chemical immobilization using B.A.M. (30 mg Butorphanol/adult, 10 mg Azaparone/adult, 12 mg

Metatomidine/adult). Chemically immobilized animals were administered oxygen at 2 liters/minute and were also subcutaneously administered 5-7 mL Liquamycin (oxytetracycline antibiotic). Reversal entailed intramuscularly administering 200 mg Tolazaline followed by 31.25 mg Atipamezole. All capture and handling procedures followed protocols approved by the Montana State University Institutional Animal Care and Use Committee (Permit # 2014-32).

1.1.2 Sample Collection

A series of measurements and samples were taken from each animal captured. Sex was determined based on genitalia and age was estimated using incisor eruption patterns (Hemming 1969). Thirty-five mL of blood was drawn from the jugular vein. Nasal swabs, tonsil swabs and fecal samples were also collected. Lactation of adult females was assessed by palpating the teats. Ultrasonography was used to measure subcutaneous rump fat thickness of adult females and body condition was also assessed using skeletal palpation methods. Additionally, weight and hind foot length (Zannése *et al.* 2006, Garel *et al.* 2010) were measured for all adult females.

Figure 3. *From left to right: Ewe from Hilgard population captured via drop net being weighed by MSU and FWP personnel, principle investigator Bob Garrott, MS student John Thornburgh and PhD candidate Blake Lowrey sampling a chemically immobilized ewe from the Stillwater population, and Quicksilver Air Inc. capturing bighorn sheep in the Perma-Paradise population via net gun .*

1.2 Study populations & Sampling Accomplishments

In this third year of the study, sampling goals consisted of resampling an additional 30 animals from six of the seven original study populations and instrumenting 10 adult females with paired GPS/VHF (Models: TGW4400 [GPS] and MOD400 [VHF], Telonics Inc, Mesa, Arizona) or Iridium satellite linked GPS collars (Model: TGW-4570, Telonics, Mesa, Arizona). In addition, the Middle Missouri/Larb hills herd was added to the study in 2016, with a sampling goal of 20 adult females sampled and fitted with paired GPS/VHF radio collars (Table 1).

An important principle underlying this research program is that inferences obtained from research are most broadly applicable to wildlife management needs by addressing the same questions in multiple wildlife populations occupying different ecological conditions. Accordingly, populations included in this research program were carefully selected by MFWP regional wildlife managers to capture varying respiratory disease histories, habitat types, management histories, as well as demographic performance. Descriptions of the eight study populations, as relevant to the above characteristics, are outlined below along with sampling accomplishments in each to date.

Paradise:
This population, also known as Perma-Paradise, is located in northwestern Montana in the Northwest Montane ecoregion. The population was established with a reintroduction in 1979 and was never augmented. Currently the population numbers approximately 300 animals, experiences moderate recruitment in most years, and is believed to be isolated from other bighorn populations. There is no known history of respiratory disease in this population.

Original capture and sampling objectives were fully met at Paradise in December 2014 and helicopter operations successfully met resampling goals in December 2016.

Petty Creek:
Also known as the Grave Creek Range population, this population is located in western Montana in the Northwest Montane ecoregion. The population was established with an initial reintroduction in 1968 and received a small augmentation in 1985. The population is currently estimated at approximately 160 animals and is thought to be isolated from other populations. The population typically experiences strong annual recruitment rates and it is not known to have a respiratory disease history.

Attempts to attract animals at Petty Creek to drop-net sites in Winter 2014/2015 were unsuccessful. Accordingly, a helicopter contract was solicited and seventeen adult females were captured and sampled, with all 15 pairs of GPS/VHF collars deployed via helicopter net-gunning February, 2016. The population is scheduled for resampling winter 2017/2018.

Lost Creek:
This population is located in southwestern Montana within the Mountain Foothills ecoregion. The population was established with a reintroduction in 1967 and was augmented in 1985. It is believed to be relatively isolated and traditionally has had high recruitment rates and historically been of moderate population size. The population has experienced two significant respiratory disease outbreaks, the most recent occurring in 2010. The population currently numbers ~60 animals due to the recent disease event and recruitment remains low.

In Winter 2014/2015 seven animals (6 adult females and 1 adult male) were captured and sampled using a drop-net on January 3rd, and six adult females were captured and sampled using ground-based chemical immobilization throughout March. All 12 adult females were fit with paired GPS/VHF radio-collars, however 2 of these animals died before winter 2015/2016, leaving five sets of radio-collars to be deployed over winter 2015/2016. In December 2015, five adult females were captured via ground darting and sampled, all of which were instrumented with paired GPS/VHF radio-collars. An additional adult female was captured and collared via chemical immobilization March 20th 2016, resulting in a total

of 19 animals sampled and all 15 collars deployed. In December 2016, 24 additional animals were sampled and 9 adult females instrumented with paired GPS/VHF collars.

Hilgard:
Also known as the Taylor-Hilgard population, this native population is located in southwestern Montana within the Mountain Foothills ecoregion. The population has been augmented on three occasions during the late 1980s and early 1990s due to concerns over low numbers after a respiratory disease even in 1987. A second major mortality event due to disease occurred in 1997, but the population experienced a robust recovery without management intervention. The population is believed to be isolated from other bighorn populations and currently numbers at least 200 animals with strong annual recruitment in recent years.

Sampling and radio-collaring of the Hilgard population continues to be enhanced beyond the original research objectives. Just prior to the initiation of this study in winter 2011/12 the MFWP biologist responsible for the Hilgard population instrumented 5 adult females and 5 mature rams with VHF collars that have been incorporated into the demographic studies. In addition to our research capture and sampling of 29 animals in this herd during the winter of 2013/14, 52 animals were captured and translocated from the Hilgard population in winter 2014/2015 and data and samples that will contribute to the research program were collected from 50 of these animals. Ten of the translocated animals were also instrumented with Lotek LifeCycle™ GPS collars purchased with funds provided by the Montana Auction License Fund, allowing us to include this newly established population in our routine research monitoring. Additionally, as part of a supplementary translocation, 35 animals were sampled February 20, 2016. The continued increased data and sample collection that has resulted from this collaboration will undoubtedly improve insights that will be obtained from the research program. Resampling goals were successfully met in 2016 and 10 adult females were fitted with Iridium satellite-linked GPS collars. These collars transmit for approximately 5 years and provide location data to researchers and managers every two days, in addition to real time mortality alerts, via satellite transmission. This provides researchers and managers with a useful tool for improving population estimates, identifying causes of mortality and understanding herd movement.

Castle Reef:
This native population is located along the Rocky Mountain Front in the Prairie Mountain Foothills ecoregion of central Montana. The population received a single small augmentation in 1944 and has experienced three respiratory disease outbreaks between 1924 and 1936, a fourth outbreak in 1984, and the most recent outbreak in 2010. The population is currently estimated at approximately 160, but is part of a metapopulation complex along the Rocky Mountain Front representing an aggregate total of 650-700 animals. Historically recruitment has been moderate to high, but since the most recent respiratory disease even, recruitment has been very low, but appears to be returning to “normal” levels over the past two years.

Twenty animals were captured and sampled using a drop net in December 2014 and January 2015 and three additional animals were captured and sampled using ground-based chemical immobilization in March 2015. Fifteen adult females were instrumented with paired GPS/VHF radio-collars and 1 was instrumented with a VHF radio-collar. An additional three animals were captured and sampled in December 2015 and four adult females were captured in March 2016, to redeploy two radio-collars from animals that had died. In December 2016, 27 animals were captured and sampled using a combination of

helicopter net gunning (10 animals) and a drop net (17 animals) and seven Iridium linked GPS collars were deployed on adult females. Ground darting was used to capture and sample an additional animal January 6, 2017 and there are plans for an additional helicopter capture effort February, 2017.

Fergus:

This restored population is located in east-central Montana on the south side of the Missouri River in the Prairie Breaks ecoregion. The population was established with a reintroduction in 1947, with three augmentations between 1959 and 1961, and the most recent augmentation occurring in 1980. This population consistently experiences very high recruitment rates and is the second largest bighorn population in the state, numbering approximately 500 animals. There is free exchange of animals with the population on the north side of the Missouri River, creating a metapopulation of nearly 1000 animals with no known respiratory disease outbreaks since 1980.

Capture and sampling objectives were fully met and exceeded in December 2014. Collaboration and coordination between Montana State University, MFWP, and the Hells Canyon Initiative (another collaborative bighorn sheep research program) has allowed the Montana Bighorn Sheep Study to increase sampling effort in the Fergus population beyond project goals with minimal additional costs or effort. As a result of collaboration with the Hells Canyon Initiative, 15 additional VHF radio-collars were deployed on adult females in the Fergus population. In addition, concurrent with the research capture, 30 additional bighorn sheep were captured and translocated out of this population. Much of the same data and samples were collected from the 30 animals captured for translocation as were collected from the animals captured for the research project. Helicopter net gunning efforts in December 2016 successfully met recapture sampling and instrumentation objectives. In concert with this effort, 20 of the sampled animals were translocated to the Beartooth Wildlife Management Area.

Middle Missouri/Larb Hills:

This herd is located in the plains/Missouri River Breaks area of northeastern Montana and was established with the reintroduction of 28 bighorn sheep in 1980. The herd is composed of two distinct subpopulations thought to be linked by ram movement during the rutting season. The smaller portion of the herd occupies typical Missouri River breaks habitat in the Mickey-Brandon Buttes area with the larger subpopulation occupying the Iron Stake Ridge/Larb Hills region distant from the breaks in prairie hills habitat. After establishment the population grew to >90 animals, but experienced an approximately 50% decline between 1997-2001. Cause of the decline was never determined, but disease and possibly poor nutrition were suspected. Since the die-off the population has recovered and currently numbers >225 and experiences strong annual recruitment

This population was included into the study using surplus funds in order to enhance our understanding of bighorn sheep populations that utilize a prairie-breaks habitat type. Previously only one herd of this type (Fergus) was included in the study despite the fact that many of the state's most robust bighorn sheep populations occupy this type of habitat. The addition of the Middle Missouri/Larb hills herd, along with Fergus herd, will provide the study with a dataset for the prairie-breaks habitat type more comparable to the mountainous terrain associated with the other study herds.

Capture and sampling objectives for this population were fully met in December 2016. Twenty adult females were captured via helicopter net-gunning, sampled and instrumented with paired GPS/VHF radio collars.

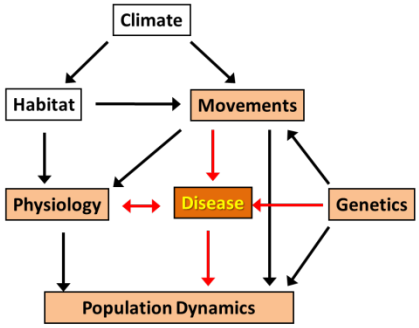
Stillwater:
This native population is located in south-central Montana within the Southern Mountains ecoregion. The population is believed to be relatively isolated, is small (~60 animals) and has moderate recruitment. There are no known respiratory events in the population in recent times, but the population has been augmented twice (1970, 1984).

Ground-based chemical immobilization was used throughout winter 2014/2015 to capture and sample 16 adult females, 15 of which were fit with paired GPS/VHF radio-collars. In order to more closely reach the capture and sampling objective and redeploy a pair of GPS/VHF radio-collars, which were originally deployed on an animal that died, three additional adult females were captured and sampled using chemical immobilization in December 2015 for a total of 19 animals sampled. Due to limited animal availability and logistical constraints associated with ground based chemical immobilization, resampling goals were modified for the Stillwater herd to capture and sample an additional 15 animals with 5 adult ewes fitted with paired GPS/VHF collars. As of January 2016, 7 animals have been sampled with 3 ewes fitted with collars. There are plans to continue sampling through March, 2017.

Table 1. *Sampling accomplishments to date in each of the eight study populations. Increased sampling in the Hilgard and Fergus populations resulted from coordination with MFWP during translocation captures. The increased number of radio-collars deployed in the Hilgard population also resulted from coordination with MFWP and the increased number of deployed radio-collars in the Fergus population resulted from collaboration with the Hell's Canyon Initiative.*

	ANIMALS SAMPLED					RADIO-COLLARED EWES	
	2013/ 2014	2014/ 2015	2015/ 2016	2016/ 2017	TOTAL	TOTAL COLLARED	CURRENTLY ON AIR
Paradise		30	0	30	**60**	25	21
Petty Creek		0	17	0	**17**	15	15
Lost Creek		7	11	25	**43**	27	23
Hilgard	29	50	0	65	**144**	37	32
Castle Reef		23	3	32	**58**	25	17
Fergus		60	0	30	**90**	40	37
Stillwater		16	3	7	**26**	19	17
Middle Missouri				20	**20**	20	20
TOTAL	**29**	**186**	**34**	**209**	**458**	**208**	**182**

Objective # 2: *Assess variation in respiratory pathogen communities and associations with population performance*



Respiratory disease has been a persistent problem for recovery of bighorn sheep in North America. The severity of respiratory disease epizootics has been variable, ranging from 30% to 90% mortality in affected populations (Besser et al., 2013). The epizootics often involve an extended phase where a high percentage of juveniles die from respiratory disease within four months of birth, however the duration of this phase is also extremely variable, lasting from a single year of poor recruitment to decades of poor recruitment (Plowright et al., 2013). In numerous cases local populations have gone extinct or have been depopulated after many years of chronically poor performance following respiratory disease epizootics (Carlsen and Erickson, 2010).

Anecdotal and experimental evidence suggests that domestic sheep (*Ovis aries*) and, perhaps, domestic goats (*Capra aegagrus hircus*) are likely the original source of the pathogen(s) responsible for respiratory disease in bighorn sheep as 98% of bighorn sheep commingled with healthy domestic sheep in captive studies have developed respiratory disease (Besser et al., 2013). Bacterial organisms belonging to the family *Pasteurellaceae* have long been implicated as important agents for respiratory disease in bighorn sheep, and recent experimental inoculation studies have shown that it is likely leukotoxigenic (lktA) *Pasteureallaceae* organisms, including strains of *Mannheimia haemolytica* and *Bibersteinia trehalosi*, which cause respiratory disease in captive bighorn sheep, but not in domestic sheep (Bavananthasivam et al., 2012; Dassanayake et al., 2013; Dassanayake et al., 2010; Dassanayake et al., 2009; Lawrence et al., 2010). Epidemiologically, *Pasteurella multocida* has also been associated with bighorn respiratory disease epizootics, though to a lesser degree (Besser et al., 2012b). Additionally, experimental and field evidence has emerged, providing strong evidence that the bacteria *Mycoplasma ovipneumoniae* plays an important role in causing respiratory disease epizootics in wild bighorn sheep populations (Besser et al., 2012a, 2012b, 2008) and that transmission of *Mycoplasma ovipneumoniae* from asymptomatic domestic sheep to bighorn sheep is associated with development of respiratory disease in bighorns (Besser et al., 2014).

The high mortality rate observed in bighorn sheep experimentally commingled with domestic sheep and goats represents, perhaps, the most consistent and repeatable finding related to respiratory disease in bighorn sheep. Accordingly, maintaining separation of wild bighorn sheep from domestic sheep and goats to avoid disease transmission is currently recognized as the primary tool management agencies use to reduce the probability of respiratory disease outbreaks (Brewer et al., 2014). Although some number of epizootics have certainly been caused by introduction of novel pathogens (novel pathogen hypothesis) there are numerous examples of respiratory disease outbreaks in bighorn populations where domestic sheep were not known to be in the vicinity (Edwards et al., 2010; Festa-Bianchet, 1988; Ryder et al., 1992) and each of the pathogens which have been tied to bighorn respiratory disease have also been detected in populations with little or no evidence of respiratory disease epizootics (Besser et al., 2013;

D.S. Miller et al., 2012, 2011, H. Edwards *unpublished data,* R. Garrott *unpublished data*). These observations lead to an alternative hypothesis which posits that epizootics have also been triggered by pathogens already resident in a population (resident pathogen hypothesis), which turn virulent and/or increase in transmissibility under certain conditions and that carriage of these respiratory pathogens does not necessarily imply a diseased state for an individual or a population (D. S. Miller et al., 2012). Given the body of evidence that domestic sheep carry the pathogens responsible for bighorn respiratory disease and transmit those pathogens to bighorns in captive studies, these "resident pathogens" in bighorn populations likely originated from sympatric domestic sheep at some point since domestic sheep were introduced to western North America. Distinguishing to what extent these alternative hypotheses (novel vs resident) explain respiratory disease expression would be a useful assessment because the management strategies to reduce disease expression caused by the two hypothesized mechanisms are very different.

The respiratory pathogen aspect of this research effort aims to develop a framework to address these hypotheses and consists of two main initiatives. One initiative is focused on assessing detection probability for the different respiratory pathogens of interest in order to provide recommendations to management agencies for sampling intensity needed to reliably characterize pathogen communities given different sampling protocols. Reliable characterization of pathogen communities establishes a level of baseline information so that when asymptomatic populations that have been previously sampled become affected by respiratory disease, the pathogen communities before and during/after an epizootic can be compared to assess whether novel pathogens were introduced between healthy and diseased states. The second initiative is an assessment of respiratory pathogen communities in numerous populations displaying a range of demographic performance to determine whether there are any associations between certain pathogen communities hosted by the population and poor demographic performance. Lack of associations would suggest that respiratory disease can be managed without the onerous task of eradicating pathogens and provide indirect evidence that disease expression can be caused by pathogens already present in a population.

2.1 Pathogen Sampling Methods

The Montana Bighorn Sheep Study adopted sampling methodologies that improve knowledge of both *Pasteurellaceae* and *M. ovipneumoniae* in study populations. Tonsil swabs were collected to assess presence of *Pasteurellaceae* organisms and the toxic agent they produce (leukotoxin) while nasal swabs were collected to assess presence of *M. ovipneumoniae* (Fig. 4). In order to assess detection probability of the different pathogens, multiple tonsil and nasal swabs were collected from a subsample of captured animals. Further, multiple handling and testing protocols have been employed for both nasal and tonsil swabs to assess detection probability of the different protocols. Samples were collected using the same method as swabs collected from animals as part of the Greater Yellowstone Area Mountain Ungulate Project (MUP), allowing data collected by the two research programs to be pooled. Additionally, MtFWP collected samples using the same method and shared data from those samples in order to augment research sampling.

Figure 4. *Field sampling techniques. A. Collecting nasal swab for M. ovipneumoniae detection. B. Collection of blood for detection of M. ovipneumoniae antibodies. C. Collecting tonsil swab for detecting Pasteurellaceae species. D. Plating tonsil swab onto Columbia Blood Agar plate at the animal.*

All *Pasteurellaceae* pathogens were detected using one set of five diagnostic protocols and *M. ovipneumoniae* was detected using a different set of three diagnostic protocols (Butler 2017). Diagnostic tests offered by a fee-for-service (FFS) laboratory (Washington Animal Disease Diagnostic Laboratory-WADDL) were used to detect and identify respiratory pathogens for four protocols (FFS protocols). Diagnostic tests conducted at a non-FFS diagnostic laboratory (Wyoming Game and Department Wildlife Health Laboratory-WGFD) were used to detect and identify respiratory pathogens for three protocols (non-FFS protocols). A non-FFS diagnostic test also was conducted at WADDL as part of protocol development. For *Pasteurellaceae*, all FFS protocols detected pathogens by culture. The FFS *M. ovipneumoniae* protocol detected this pathogen by PCR. Non-FFS protocols used PCR (sometimes in conjunction with culture) to detect each pathogen, with the exception of *P. multocida*, which was only detected by culture. Exposure of study populations to *M. ovipneumoniae* was also assessed by sending serum from each animal to Washington Animal Disease Diagnostic Laboratory (WADDL) to detect antibodies against *M. ovipneumoniae* using an ELISA.

2.2 Assessing Pathogen Detection Probability-Results

We used occupancy modeling (MacKenzie et al. 2002) to quantify detection probability of the different pathogens under different sampling protocols. The framework requires collecting repeated samples from individual animals at the time of capture and, stated most simply, assessing how frequently lab results from repeated sampling of the same animal agree. One to four tonsil and nasal swabs were

collected by trained personnel from bighorn sheep sampled in nine free-ranging populations in Montana, ten free-ranging populations in Wyoming, and one captive population in Wyoming. A total of 2093 *Pasteurellaceae* diagnostic tests were conducted for 476 bighorn sheep and a total of 768 *M. ovipneumoniae* diagnostic tests were conducted for 469 bighorn sheep. Results from this effort were used in a simulation study to develop recommendations for sampling intensities needed to reliably determine whether a given pathogen is present in a population.

Analysis of replicate samples from individual bighorn sheep revealed that detection probability for regularly-used diagnostic protocols was generally low (<50%) for *Pasteurellaceae* and was high (>70%) for *Mycoplasma ovipneumoniae* (Figure 5). These results indicate that live-sampling of bighorn sheep for respiratory pathogens using diagnostic protocols that are readily available through a FFS laboratory can lead to biased assessments of respiratory pathogen communities. While the diagnostic test to detect *M. ovipneumoniae* offered by the FFS laboratory used in our study (WADDL) uses PCR with a high detection probability, only culture tests are offered by FFS laboratories to detect and identify *Pasteurellaceae* pathogens in bighorn sheep. Diagnostic protocols that relied solely on a FFS culture tests for detection had low estimated detection probabilities (<0.50) for all *Pasteurellaceae* pathogens that were assessed. Low detection probability of these protocols may be due in large part to diminished viability of targeted organisms during the process of delivery to the laboratory rather than sensitivity of the diagnostic test itself (Safaee et al. 2006, Wild and Miller 1994). Nevertheless, this is a limitation whenever samples must be shipped to a laboratory for culture tests.

Low detection probability of *Pasteurellaceae* pathogens using FFS protocols makes simple assessment of species presence at the population-level unreliable when species are at low prevalence and populations are not intensively sampled. Although these specific findings apply to live-sampling bighorn sheep by swabbing the nasal cavity or tonsillar crypts, incongruent findings among studies investigating pathogen communities present in pneumonic and healthy lungs from the same respiratory disease epizootics (Besser et al. 2012, Shanthalingam et al. 2014) suggest that detection error affects these assessments as well. Thus, an assessment of detection probability applied to the sampling of lung tissues is warranted.

Naïve prevalence estimates of *Pasteurellaceae* pathogens are strongly biased when FFS diagnostic protocols are used, unless protocols are conducted multiple times per animal. Given poor detection power and biased prevalence estimates, any true associations between the presence of *Pasteurellaceae* organisms and historic or current respiratory disease in bighorn sheep would likely be unobservable using these protocols. In contrast to the *Pasteurellaceae* pathogens, high detection probability for *M. ovipneumoniae* likely leads to more consistent detection and less biased naïve prevalence estimates in bighorn sheep populations where it is hosted. These findings suggest that prevalence of any pathogen is estimated with poor precision unless intensive sampling is employed (i.e., many animals are sampled and protocols are conducted multiple times per animal). Although *M. ovipneumoniae* could be reliably detected in a population by conducting a single protocol on a modest number of animals, its prevalence was estimated with low precision unless more sampling effort was invested. Therefore, variability in observed pathogen prevalence among different populations or different years within a population could be explained by either sampling variation or true variation in prevalence. Without accounting for differences in detection probability and sampling effort, differences in true prevalence remain unknown.

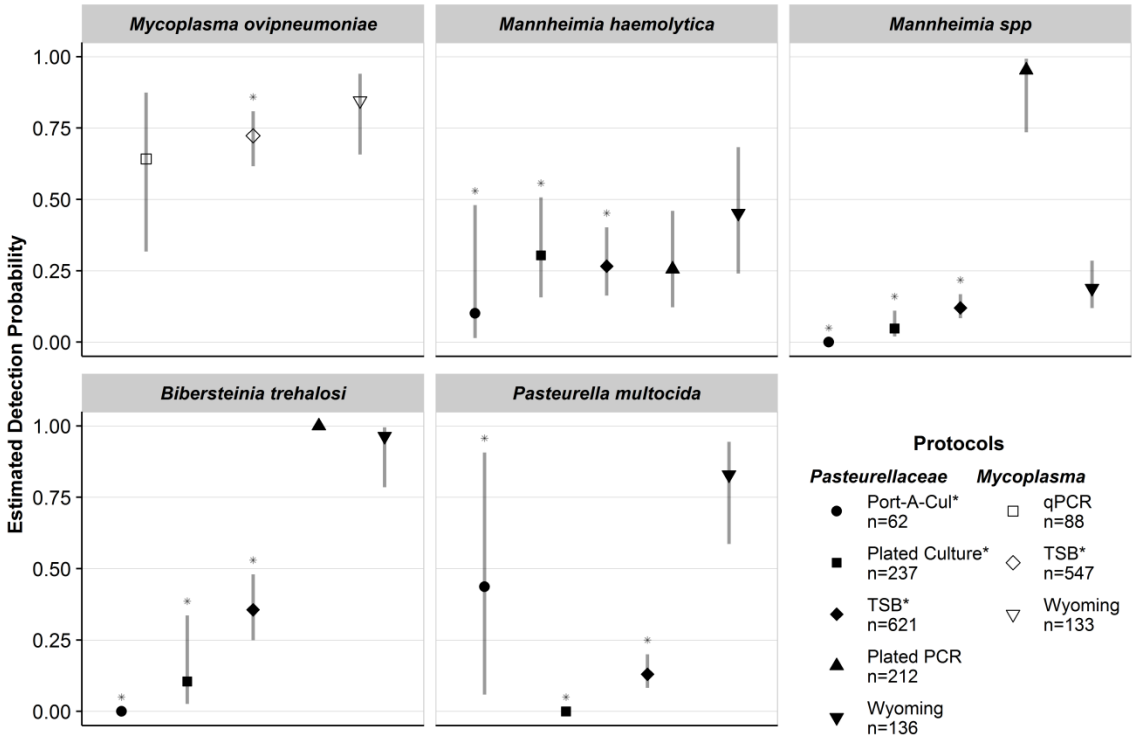


Figure 5. *Estimated detection probabilities and 95% confidence intervals for five bighorn sheep respiratory pathogens. One set of protocols was used to detect the four Pasteurellaceae organisms (shaded) and a separate set of protocols was used to detect Mycoplasma ovipneumoniae (not shaded). Detection probabilities for Mannheimia haemolytica, Mannheimia spp., and Bibersteinia trehalosi are for beta hemolytic or leukotoxigenic strains. Protocols that used diagnostic tests through a fee-for-service laboratory are indicated with an asterisk (*) in the legend and above the upper confidence limit. The total number of samples assessed using each protocol is indicated in the legend.*

A simple and relatively inexpensive measure that wildlife management agencies can take to improve their ability to accurately characterize respiratory pathogen communities is to collect and assess two or three tonsil swabs from each live-sampled animal for *Pasteurellaceae* pathogens using FFS diagnostic protocols. Conducting protocols multiple times per animal would also provide agencies the ability to assess detection probability of their specific diagnostic protocols. Our simulations suggest that 30 to 35 animals need to be sampled from a bighorn sheep population to reliably assess (>80% power) presence of *Pasteurellaceae* pathogens and *M. ovipneumoniae*. Reliable detection of pathogens at the population-level, as defined in this study, still results in a 20% chance of a false negative for each pathogen. The power to simultaneously detect multiple pathogen species or strains in a population is further reduced to the product of the power to detect each individually. Thus, the full set of respiratory pathogens hosted by free-ranging bighorn sheep populations will likely be difficult to characterize with certainty given currently available diagnostic protocols available through FFS labs. However, quantification of this uncertainty is possible and would lead to more accurate inference and informed management decisions.

Poor detection power associated with FFS diagnostic protocols combined with hundreds of bighorn sheep translocations across North America suggest it is probable that *Pasteurellaceae* have been

unknowingly introduced to new regions and populations. Suspected poor detection probability for *M. ovipneumoniae* prior to development of FFS PCR and serology tests (Besser et al. 2008), likely also resulted in the unknown introduction of this pathogen to new regions or host populations. Typically, bighorn sheep populations chosen to be source populations for translocations are those experiencing population growth, and thus, not exhibiting noticeable symptoms of respiratory disease. However, such populations may still host respiratory pathogens capable of causing disease (Miller et al. 1991). Bighorn sheep source populations should be thoroughly sampled for respiratory pathogens using appropriate diagnostic protocols and sampling intensities to determine the extent to which these respiratory pathogens are present. Relative to the total cost of sampling bighorn sheep, additional expenses to improve characterization of respiratory pathogen communities are rather modest.

2.3 Characterizing Respiratory Pathogen Communities and Demographic Attributes of Diverse Bighorn Sheep Populations

Coordinated efforts were used across Montana and Wyoming to rigorously assess respiratory pathogen communities in a diverse set of bighorn sheep populations and then relate estimates of average recruitment and population growth to presence of *Pasteurellaceae* and *M. ovipneumoniae*. Our primary objectives were to assess the pervasiveness of respiratory pathogens in the study populations, assess whether presence of any specific pathogen or combination of pathogens is associated with differences in recruitment or population growth, and determine the extent to which populations hosting different respiratory pathogens maintained satisfactory recruitment or population growth rates. Little or no association between demographic performance and presence of suspected respiratory pathogens were hypothesized and, given the long history of domestic sheep grazing in the two states, it was hypothesized that the respiratory pathogens are resident the majority of study populations.

2.4 Methods Used for Assessment of Respiratory Pathogen Communities and Demographic Attributes

Our survey included 17 bighorn sheep populations that occupy a wide range of habitat types across Montana and Wyoming and have varying disease and management histories. The areas inhabited by these populations represent much of the variation in habitat types that are realized across the species' range. Study populations represent native populations (n=10), restored populations (n=6), as well as native populations that have been augmented in efforts to increase population size (n=1). Connectivity levels varies among study populations, which include well-connected metapopulations (n=10), populations thought to have limited connectivity (n=2), and populations thought to be mostly isolated (n=5). Population structure, in terms of number of subpopulations, ranged from one sub-population to over ten sub-populations. Respiratory disease histories in the populations include no documented history of disease (n=8), a single all-age epizootic (n=4) and multiple all-age epizootics (n=5).

A total of 637 individual bighorn sheep from 17 populations in Montana and Wyoming were captured and sampled between December and March of each year from 2012-2016. Captured animals were live-sampled for presence of *Mycoplasma ovipneumoniae,* leukotoxigenic *M. haemolytica or Mannhemia glucosida* (combined as *M. haemolytica* as these two species are not reliably differentiated by available diagnostic tests), leukotoxigenic *Mannheimia ruminalis* or *Mannheimia spp.* (combined as *Mannheimia spp.* because the ability to identify *Mannheimia ruminalis* from other species was not available until the final year of data collection), leukotoxigenic *B. trehalosi,* and *P. multocida.* Demographic data used in this analysis were primarily collected by Montana Department of Fish Wildlife and Parks (FWP) or Wyoming Game and Fish Department (WGFD) personnel as part of regular bighorn sheep population

surveys from 2006 to 2016. Demographic performance of study populations was characterized by their mean recruitment rates and geometric population growth (lambda or $\bar{\lambda}$). Recruitment rates were indexed as the ratio of lambs to adult females (lamb: ewe ratio) that were counted in the classification surveys. Mean geometric population growth was indexed using counts from annual surveys after accounting for purposeful management additions or removals. Populations that experienced all-age epizootics within the range of years that recruitment data were collected were split into separate populations (before epizootic and after epizootic) for analyses if pathogen data were collected before and after epizootics; if pathogen data were not collected before epizootics, demographic data preceding the die-off were excluded from analysis.

2.5 Respiratory Pathogen Communities Resident in Sampled Bighorn Populations-Results

Collectively, leukotoxigenic *Pasteurellaceae* were detected in every study population except Middle Missouri Breaks. *M. ovipneumoniae* was not detected in any populations where leukotoxigenic *Pasteurellaceae* were not detected, while leukotoxigenic *Pasteurellaceae* were detected in two populations (Perma-Paradise, Petty Creek) where *M. ovipneumoniae* was not detected (Figure 6). Three of the five respiratory pathogens were detected in over 70% of the study populations. *M. ovipneumoniae* was detected in 14 of 17 (82%) study populations and was not detected in the Perma-Paradise, Petty Creek, or Middle Missouri Breaks populations. Leukotoxigenic *M. haemolytica* was detected in 13 of 17 (71%) study populations and leukotoxigenic *Mannheimia spp.* was detected in 14 of 17 (82%) study populations. *P. multocida* and leukotoxigenic *B. trehalosi* were both detected in 9 of 17 (56%) study populations including all Wyoming study populations and two Montana study populations (Stillwater, Hilgard) that are adjacent to Wyoming. Presence of *M. haemolytica, Mannh*eimia *spp.*, or *P. multocida* was not assessed with 80% confidence in any population where they were not detected, preventing association of population characteristics with presence of these pathogens. Presence of *M. ovipneumoniae* was reliably assessed (>80% confidence) in all three populations where it was not detected and presence of *B. trehalosi* was reliably assessed in three (Perma-Paradise, Castle Reef, Fergus) of the eight populations where it was not detected.

These results demonstrate the pervasiveness of the respiratory pathogens among the 17 bighorn sheep populations that were investigated. Intensive sampling found the two most cited agents responsible for respiratory disease, *M. ovipneumoniae* and leukotoxigenic *Pasteurellaceae*, were both present in at least 76% of the study populations within which nearly 6,000 bighorn sheep live. These pathogens are hosted in bighorn sheep populations that inhabit matrices of public and private land as well as those that inhabit the most remote areas of the continental United States that offer the most stringent protections against humans and livestock. These findings demonstrate that the combination of bighorn sheep ecology and anthropogenic use of the landscape results in a propensity for bighorn sheep across a variety of landscapes, including national parks and wilderness areas, to be exposed to these respiratory pathogens. It is not known how long the study populations have hosted these respiratory pathogens. Accordingly, it is not known the extent to which the current pervasiveness of these pathogens in the populations is the result of continued “spillover” events from domestic livestock despite concerted efforts to prevent contact between the species,’ or the result of past eras when domestic sheep were ubiquitous across bighorn sheep range. Regardless, the fact that 76% percent of the study populations, including the most abundant populations, host both *M. ovipneumoniae* and leukotoxigenic *Pasteurellaceae,* highlights the substantial, landscape-level, challenges that wildlife agencies have faced and continue to face in preventing the spread of pathogens to bighorn sheep populations.

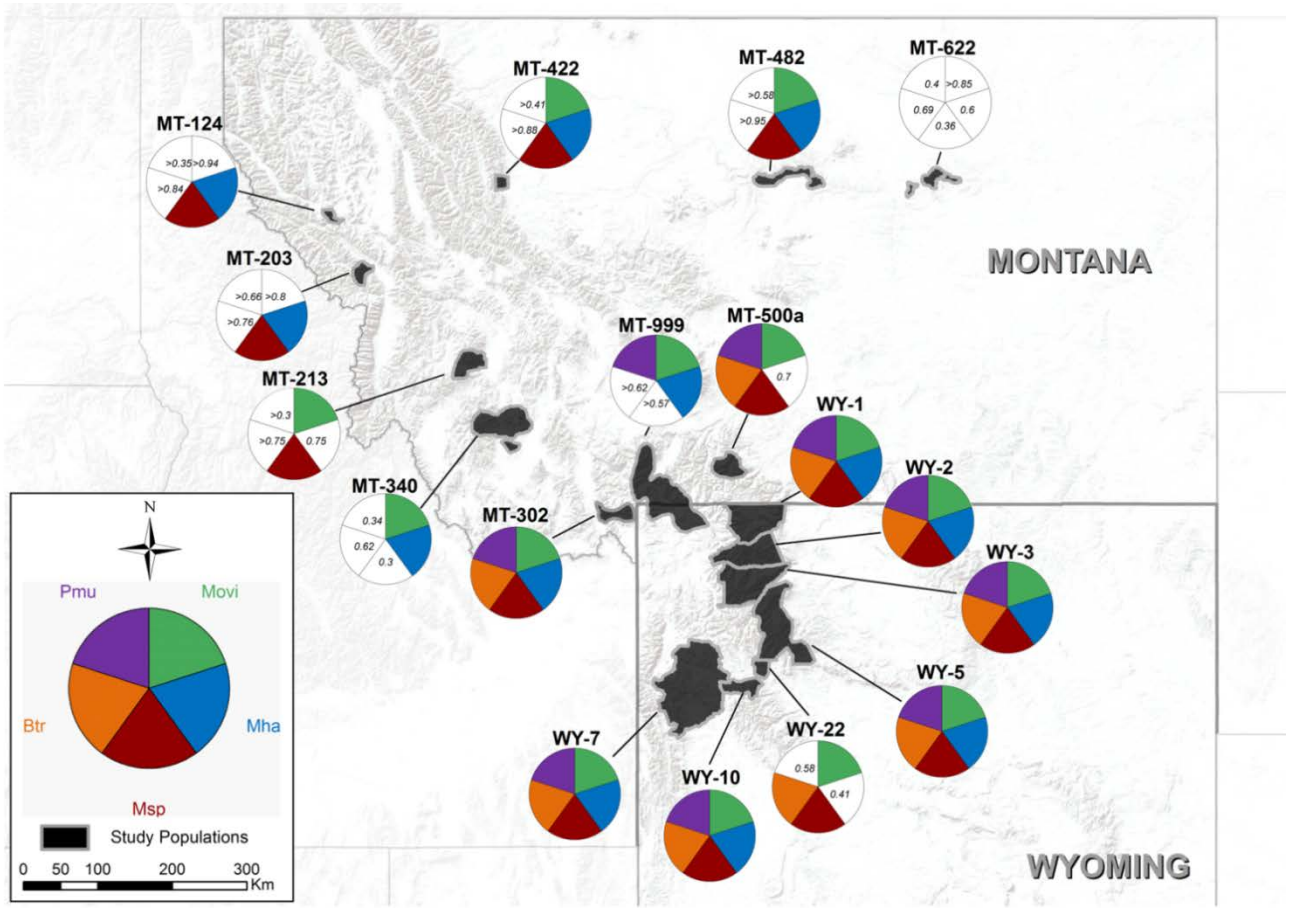


Figure 6. *Map of bighorn sheep study populations and detected respiratory pathogen communities. All sections of the pie-charts are fixed to equal size and represent whether the respective pathogens were detected in the study population. The key for pathogen abbreviations are as follows: Movi= Mycoplasma ovipneumoniae, Mha = leukotoxigenic Mannheimia haemolytica/glucosida, Msp = leukotoxigenic Mannheimia spp., Btr = leukotoxigenic Bibersteinia trehalosi, Pmu = Pasteurella multocida. Where pathogens were not detected, the numbers in the unfilled section indicate the power of the employed sampling methodologies to detect the pathogen at 10% prevalence in the population.*

2.6 Respiratory Pathogen Communities and Recruitment-Results

Mean lamb:ewe ratios of individual populations where any specific pathogen was detected ranged from < 0.20 to > 0.40 and there were at least five populations where any specific pathogen was detected that had mean lamb:ewe ratios > 0.30. Mean lamb:ewe ratios of populations where *M. ovipneumoniae* was not detected were 0.34 (Perma-Paradise, 0.44 (Petty Creek), and 0.47 (Middle Missouri Breaks). Mean lamb:ewe ratios for populations where *B. trehalosi* was not detected (and adequate detection power was achieved) were 0.09 (Castle Reef), 0.29 (Fergus), and 0.34 (Paradise). We found evidence for an association between respiratory pathogen detection and lamb:ewe ratios for *M. ovipneumoniae*, where the estimated mean lamb:ewe ratio in populations it was detected in was 0.25 (95% CI: 0.20-0.31) and the estimated mean lamb:ewe ratio in populations it was not detected in was 0.42 (95% CI: 0.26-0.67). There was no statistical evidence for an association between detection of *B. trehalosi* and the estimated mean lamb:ewe ratio in populations it was not detected in was 0.20 (95% CI: 0.12-0.32) and the mean lamb:ewe ratio in populations it was detected in was 0.29 (95% CI: 0.22-0.37). Associations between presence of any leukotoxigenic *Pasteurellaceae* and lamb:ewe ratios were not explored because leukotoxigenic *Pasteurellaceae* were detected in all but one study population. Interactive effects of *M. ovipneumoniae* and leukotoxigenic *Pasteurellaceae* could not be explored because *M. ovipneumoniae* was never detected in the absence of leukotoxigenic *Pasteurellaceae*, however recruitment was highly variable for 14 populations where both *M. ovipneumoniae* and leukotoxigenic *Pasteurellaceae* were and were not detected are shown in Figure 7.

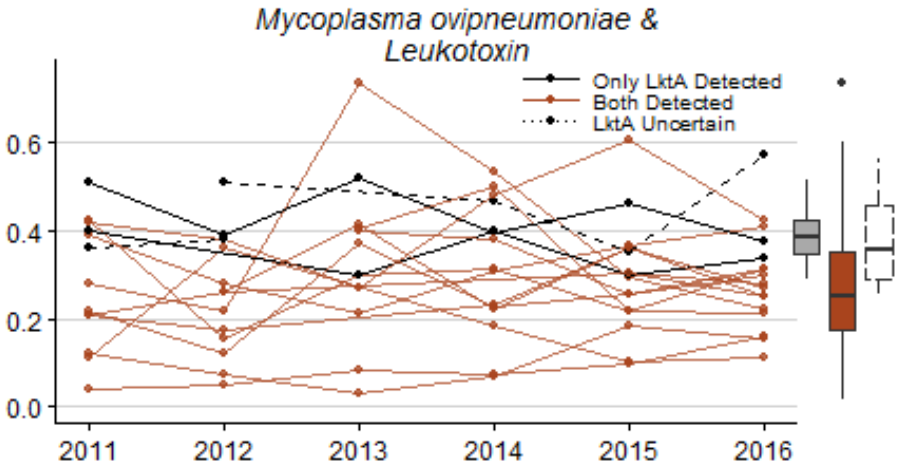


Figure 7. *Lamb:ewe ratios of 14 bighorn sheep populations in Montana and Wyoming where both M. ovipneumoniae and leukotoxigenic Pasteurellaceae were and were not detected.*

2.7 Respiratory Pathogen Communities and Population Growth (λ)-Results

There were adequate data for 13 sampled bighorn populations for analysis of population growth. Eight of the 13 (62%) populations in the population growth analysis were estimated to have positive population growth ($\bar{\hat{\lambda}}$ >1) and five (38%) were estimated to have negative population

growth rates ($\bar{\hat{\lambda}} < 1$). However, the 95% confidence interval for $\bar{\lambda}$ of every population overlapped 1.0. Average estimated geometric population growth ($\bar{\hat{\lambda}}$) of individual populations where any specific pathogen was detected was highly variable ranging from < 0.95 to > 1.10 (Figure 8). Average lambda ($\bar{\hat{\lambda}}$) estimates for populations where *M. ovipneumoniae* was not detected were 1.01 (95% CI: 0.88-1.16) for Perma-Paradise, 1.06 (95% CI: 0.90-1.24) for Petty Creek, and 1.08 (95% CI: 0.90-1.30) for Middle Missouri Breaks. Average lambda estimates for populations where *B. trehalosi* was not detected (and detection power was at least 0.80) were 0.87 (95% CI: 0.66-1.15) for Castle Reef, 1.01 (95% CI: 0.90-1.24) for Perma-Paradise, and 1.10 (95% CI: 0.97-1.24) for Fergus.

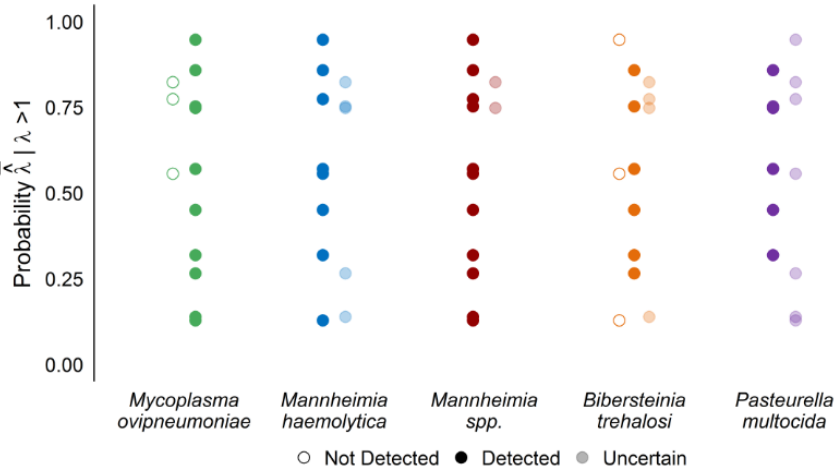


Figure 8. *Estimates of annual population growth rates for 13 bighorn sheep populations in Montana and Wyoming, with respect to detection status of five respiratory pathogens.*

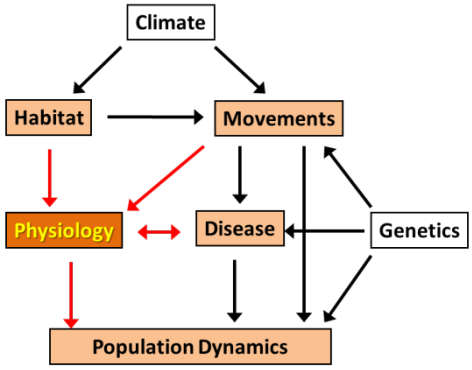
2.8 No Convincing Association Between Respiratory Pathogen Communities Hosted in Bighorn Sheep Populations and Demographic Performance

Although both *M. ovipneumoniae* and leukotoxigenic *Pasteurellaceae* were detected in most (n=14) study populations, these study populations often showed no demographic signs of respiratory disease. Half of the populations where these pathogens were detected met population objectives and had positive estimated growth rates (though never statistically significant), ten (77%) had average lamb:ewe ratios > 0.20 (threshold for "healthy" recruitment defined by the Western Association of Fish and Wildlife Agencies), and six had average lamb:ewe ratios > 0.30. These populations included the herds with the lowest and the highest population size, estimated population growth rate, and average recruitment rates. The number of populations found to host *M. ovipneumoniae* and leukotoxigenic *Pasteurellaceae* and the variation in demographic performance among these populations resulted in the paradoxical finding that, although average demographic performance of populations in these herds was lower than among the few populations where *M. ovipneumoniae* was not detect, most populations that were estimated to have positive growth rates and average recruitment rates greater than 0.30 carried both *M. ovipneumoniae* and leukotoxigenic *Pasteurellaceae.* This pattern suggests that bighorn sheep populations can be successfully managed while hosting all pathogens that have been tied to respiratory disease. However, the significance of this pattern hinges on whether the collection

of study populations here is representative of bighorn sheep populations as a whole and also what explains the variation in demographic performance of populations hosting apparently similar pathogen communities.

There are numerous plausible hypotheses to explain the observed variation in demographic performance. The strong demographic performance of some populations hosting *M. ovipneumoniae* and leukotoxigenic *Pasteurellaceae* could be explained by the presence of less virulent pathogen strains which the available diagnostic tests are unable to distinguish. Differences in virulence could be inherent in the various pathogen strains or attenuated after years of persistence in bighorn sheep populations. Variation in demographic performance could also be explained by differences in prevalence of *M. ovipneumoniae* or leukotoxigenic *Pasteurellaceae*, however, given currently available protocols, this parameter is likely estimated with poor precision in the face of imperfect detection probability, particularly for *Pasteurellacea.* Given variable population-management histories and over a century of exposure to domestic sheep experienced by some populations, natural selection may also have produced increased disease resilience in some populations. High adult and juvenile mortality rates associated with respiratory disease suggest potential for strong selective pressure for physiological or behavioral adaptations against respiratory disease so long as surviving individuals continue to be exposed to the causative agent, traits associated with survival are heritable, and sufficient genetic variability exists. And finally the variation of demographic rates, and presumably disease expression, may be dictated by interactions between the pathogen, the host, and the environment (the classic epidemiologic triad), which is the tradition model of infectious disease causation. This is likely the most challenging hypothesis to evaluate, but our integrated bighorn sheep research program is well positioned to begin addressing this hypothesis.

Objective # 3:
Assess variation in body condition and physiological status among sampled populations



Quantity and quality of forage and associated animal nutritional condition influence the survival and reproduction of ungulates (Keech *et al.* 2000, Cook *et al.* 2004, Bender *et al.* 2008, Parker *et al.* 2009, Cook *et al.* 2013). Recent work in the Pacific Northwest suggests widespread occurrence of inadequate summer nutrition that limits adult fat accretion, pregnancy rates, and calf and yearling growth rates in elk (Cook *et al.* 2013). These results highlight the need to evaluate potential bottom-up (i.e. habitat) drivers of ungulate population dynamics. The evaluation of nutritional status across populations with varying demographic characteristics may provide insights as to the extent nutrition explains variation in demographic rates and may also be associated with expression of respiratory disease. This research project is assessing body

condition and nutrition using two distinct, but integrated, methods. Body condition of adult females was assessed using field-based measurements including ultrasonography and traditional body condition scoring, while physiological and nutritional condition were assessed using both traditional and state-of-the-art serum-based assays.

3.1 Field-Based Body Condition Assessments

3.1.1 Methods

We used ultrasonography to measure rump fat thickness (Figure 9). In addition, the lumbar vertebrate, sacrum, base of tail and caudal vertebrate were palpated manually and a body condition score was assigned. The rump fat thickness measurements and body condition scores were used to estimate the percent ingesta-free body-fat of each adult female (%IFBF; personal communication with Tom Stephenson, Sierra Nevada Bighorn Sheep Recovery Coordinator). Body weight and skeletal size (hind foot length) were also measured on all animals. Although these measurements are not direct measures of body condition, differences in skeletal size and body weight across populations may be reflective of nutritional status or other factors related to fitness.

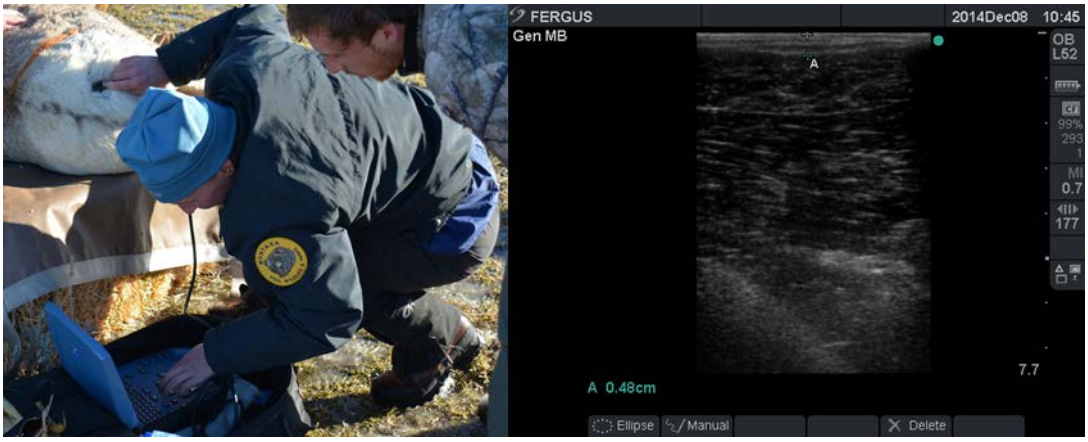


Figure 9. *Measuring rump fat thickness of a bighorn sheep ewe. The ultrasound screen observed by collectors is shown on the right.*

3.1.2 Results

Rump fat thickness measurements have been taken from 269 adult females, body weight has been measured on 288 adult females, and serum has been collected from all captured animals.

Rump fat measurements of adult females (≥3.5 years) varied from 0.10 – 1.62 cm, corresponding to %IFBF estimates ranging from 9.108% to 29.29%. Median %IFBF of lactating adult females (captured in December or January) across populations varied from 13.2 %IFBF (Stillwater) to 17.8 %IFBF (Hilgard 2014), though sample sizes for several populations were relatively small (Figure 10). Median %IFBF for adult females at Petty Creek, which were sampled in February 2016, was 12.30. Due to the late timing of sampling at Petty Creek, lactation (or lack thereof) may not be indicative of lamb production the previous year as only two of 14 adult females were determined to be lactating. Given that the estimated threshold level of winter %IFBF for bighorn

sheep to maintain pregnancy is around 10% (Stephenson *et al.* 2012), preliminary evidence suggests that body condition entering winter does not routinely limit pregnancy rates in the study populations.

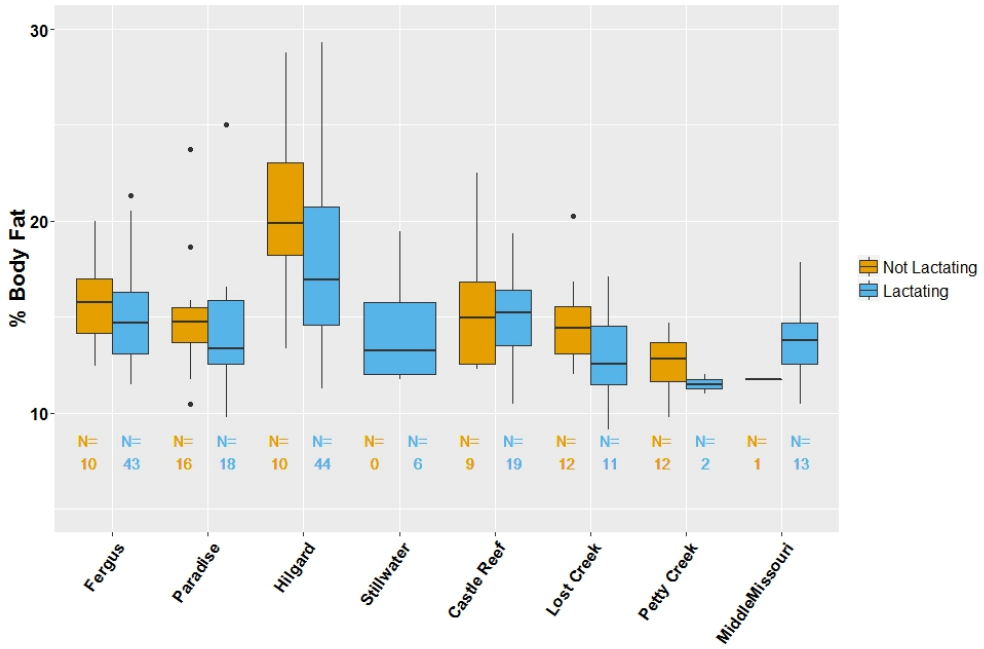


Figure 10. *Boxplot illustrating distribution of percent ingesta-free body-fat (measured using ultrasonography) of lactating and non-lactating adult female bighorn sheep (≥3.5 years) across the sampled study populations and capture years. Horizontal lines through boxes represent median values, length of box represents the middle 50% of observations (IQR), vertical lines (whiskers) represent observations outside the IQR that are within 1.5x the range of the IQR, and points outside the whiskers represent observations greater than 1.5x the range of the IQR. The numbers of animals sampled in each category are shown below the boxes. **Note that Petty Creek was sampled in February, which likely resulted in decreased fat measurements compared to measurements taken earlier in the winter. Additionally, lactation, or lack thereof, in February may not be indicative of lamb production the previous year***

Body weight of adult females (≥3.5 years) varied from 116-196 lbs. Overall, median body weights of lactating and non-lactating adult females were similar (lactating = 158 lbs, non-lactating = 154 lbs). Median body weight of adult females across populations varied from 134 lbs (Stillwater 2016) to 166 lbs. (Castle Reef 2016), though some sample sizes were relatively small (Figure 11). Median body weight of adult females at Petty Creek (measured in February 2016) was 147 lbs.

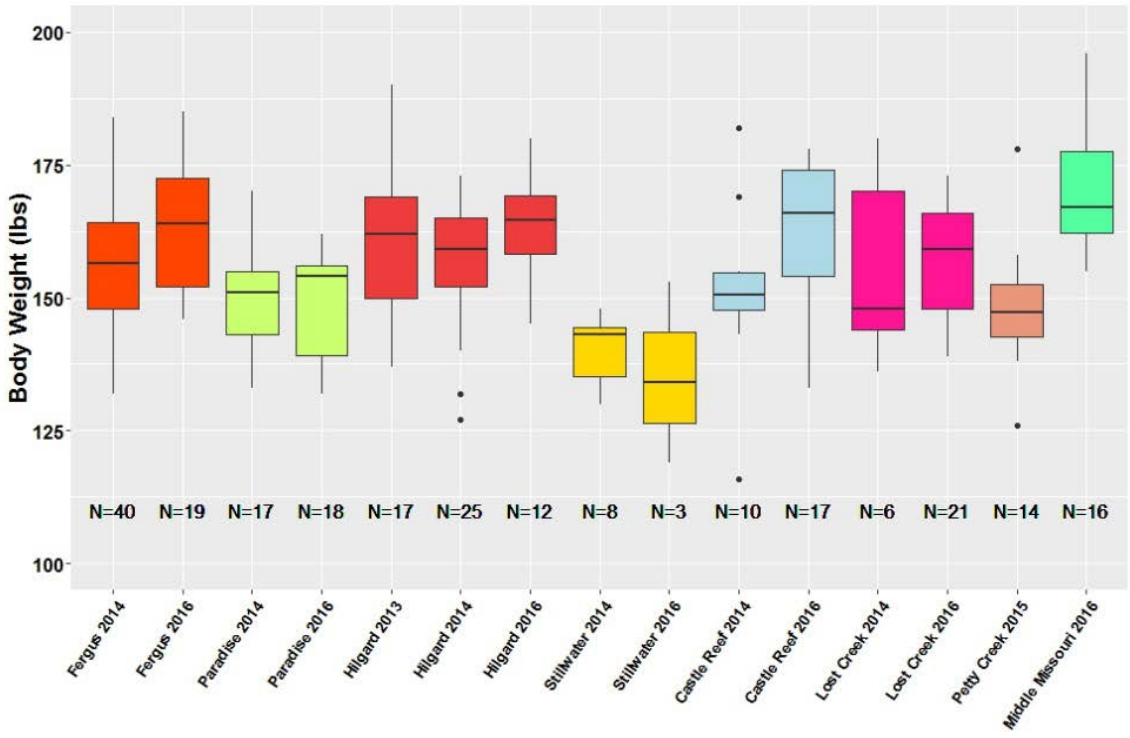


Figure 11. *Boxplot illustrating distribution of body weight measurements of adult female bighorn sheep (3.5 years old or greater) across the sampled study populations and capture years. Horizontal lines through boxes represent median values, length of box represents the middle 50% of observations (IQR), vertical lines (whiskers) represent observations outside the IQR that are less than 1.5x the range of the IQR, and points outside the whiskers represent observations greater than 1.5x the range of the IQR. The numbers of animals sampled in each category are shown below the boxes.*

3.2 Lab-Based Serum Assays

3.2.1 Established Methods

We are collaborating with Dr. Jim Berardinelli and Rashelle Lambert, animal physiologists at Montana State University (MSU) to develop a suite of metabolites and hormones that can be used to assess nutritional health, disease health, and body condition of bighorn sheep in a similar way that is successfully done in the livestock industry and human medicine.

This lab is utilizing nuclear magnetic resonance spectroscopy (NMR), an analytical tool, to study the global metabolism of each herd. The Department of Chemistry and Biochemistry at MSU owns and operates the Nuclear Magnetic Resonance Center under the supervision of Dr. Valerie Copie. Studying global metabolism is known as metabolomics or the study of metabolic intermediates and products of cellular metabolism. Metabolomics is an increasingly studied research field because it explains the functional nutritional and health states that an animal is currently in. NMR and metabolomics is currently being applied in human and domestic livestock research to study a multitude of areas including disease and feed efficiency trials. Metabolites

that are identified in NMR are associated with known metabolic pathways that can be linked to an animal's physiological condition. The benefits of NMR when compared to traditional serum assays is NMR is cost effective, it can quantify many more biological molecules, and it requires less than 1 mL of serum or plasma. One of the disadvantages of NMR is the expert time required to understand and use the programs, however, this obstacle may be overcome with intensive training and interpretation of the biological processes associated with metabolism.

Traditional metabolite assays will be used to quantify non-esterified fatty acids (NEFA) and total protein (TP) because they cannot be identified using NMR. NEFA and TP concentrations have been reported for the bighorn sheep collected in 2014-2015, samples from herds collected during 2015-2017 will be assayed this spring. NEFA is a metabolite correlated to animals' available energy reserves, where high NEFA concentrations reflect the mobilization of fat. TP concentrations reflect dehydration status and presence of acute infections.

3.2.2 Results

From herds captured in the 2014-2015 season, 240 bighorn sheep samples from 13 herds were processed for NMR spectra, profiled into metabolic profiles using a program called Chenomx®, and then analyzed using MetaboAnalyst (http://www.metaboanalyst.ca/). Currently, this lab has built a NMR compound database containing 56 compounds identified in serum of ungulates. Analysis procedures of these compounds were described by Sun et al. (2016) and used to identify potential "biomarkers" among herds. Additionally, such an analyses may be employed to identify significantly important metabolic pathways which coupled with these biomarkers, may be used to differentiate environmental, nutritional and health states of individual herds.

The first step in analyzing the compounds obtained from NMR was to determine whether two different herds could be separated metabolically using partial least squares discriminant analysis (PLS-DA). The two herds chosen were the Fergus herd, located on the plains and sampled in December, and the Absaroka herd, located at a high elevation and sampled in March. The rationale for using these 2 herds was that these herds are located in different geographical and environmental conditions with access to very different nutritional resources. Observations indicated that the Fergus herd had access to a higher plane of nutrition and were in better body condition in December than the Absaroka herd in March. Under these two diverse circumstances, we hypothesized an expected and distinct "shift" in metabolism reflected in patterns of NMR metabolites in serum. The PLS-DA analysis of the NMR profiles from these herds is shown in Figure 12A. It is readily apparent that this analysis indicates a clear separation of NMR-generated metabolic profiles. Furthermore, the analysis revealed that 18 of these compounds were classified as variables of importance (VIP; Figure 12B), that is, these compounds contributed the most to the separation seen in Figure 12A. One interpretation of the results from this type of analysis is that there is a metabolic shift between the two herds, completely separating each other metabolically. This makes physiological sense as the Fergus herd in December should be in better body condition and have access to a higher plane of nutrition than the Absaroka herd in March, which has been on a sub-maintenance diet for a minimum of 3 months. These results support the hypothesis that analysis of NMR metabolic profiles can differentiate herds of bighorn sheep under diverse environmental conditions and nutritional stress.

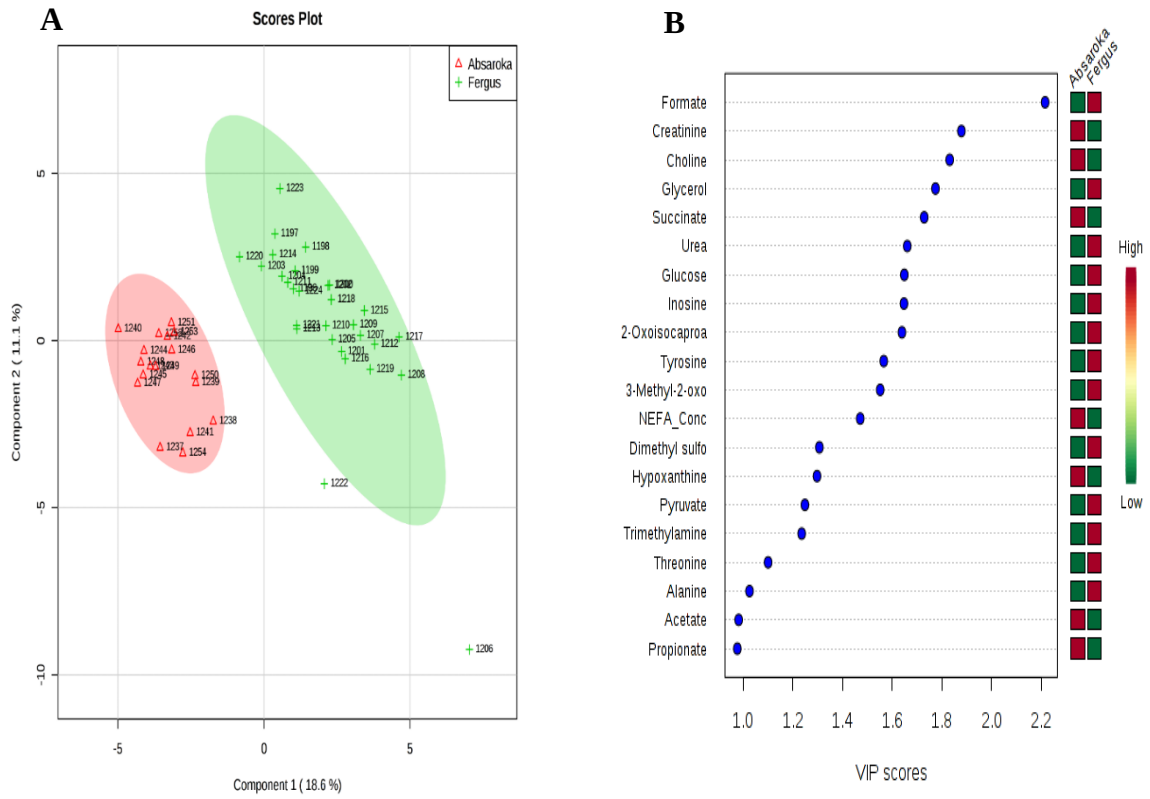


Figure 12. *Partial least squares discriminant analysis (PLS-DA) score map (A) and variable of importance (VIP) scores (B) between herds of bighorn sheep located in the Fergus area collected in December (n = 29,) and in the Absaroka area collected in March (n = 18;). In panel A, Fergus is represented in green and Absaroka is represented in red. In panel B, compounds with a VIP score greater than 1.0 are considered important contributors to the separation in panel A.*

The question becomes which of these 18 VIP compounds, are potential "biomarkers?" In other words which and how many of these contribute to the separation of the Fergus and Absaroka herds. Using the biomarker analysis of MetaboAnalyst, 14 compounds were identified as potential biomarkers (Figure 13).

Using the pathway analysis of MetaboAnalyst, 7 metabolic pathways were identified as having been significantly impacted by the 56 compounds identified through NMR profiling (Table 2). The compounds that are potential biomarkers between the Fergus and Absaroka herds, and are also identified to be found in significantly important metabolic pathways, are creatinine found in arginine/proline metabolism, choline found in glycine, serine, and threonine metabolism, trimethylamine n-oxide found in methane metabolism, urea found in arginine/proline metabolism, threonine found in methane metabolism and glycine, serine and threonine metabolism. Most of these pathways are related to protein anabolism/catabolism and energy balance.

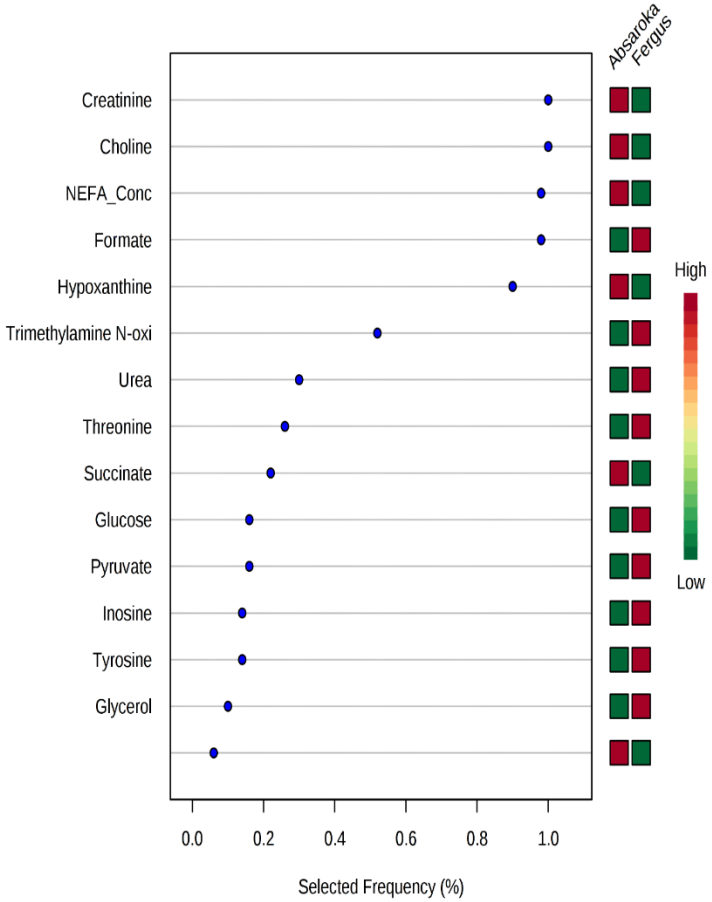


Figure 13. *Biomarker analysis between herds of bighorn sheep located in the Fergus area collected in December (n = 29) and located in the Absaroka area collected in March (n = 18). Compounds with selected frequencies greater than 0.1 are considered potential biomarkers for identifying herds.*

The next step for evaluating the use of NMR metabolic profiling for its' potential for use as a management tool was to determine whether all the herds collected in December could be distinguished between all the herds collected in March. The same analyses were performed as described previously. Figure 14A illustrates that even though the separation in the PLS-DA analysis is not complete as the Fergus and Absaroka herds, it is clear the herds collected in December and the herds collected in March tend to cluster together with only minor overlap between them. Again, using all herds collected within the same season there was a clear metabolic shift between seasons. VIP scores were used to choose the compounds for biomarker analysis (Figure 14B). The 15 compounds identified as potential biomarkers (Figure 14B) and were also found in pathways identified as significantly important (Table 2) are 2-oxoisocaproate found in valine, leucine, and isoleucine metabolism, choline found in glycine, serine, and threonine metabolism, tyrosine found in methane metabolism, creatinine found in arginine/proline metabolism and trimethylamine n-oxide found in methane metabolism. Again, these pathways significantly impact energy metabolism. Changes in these particular pathways may be related to environmental and nutritional stress.

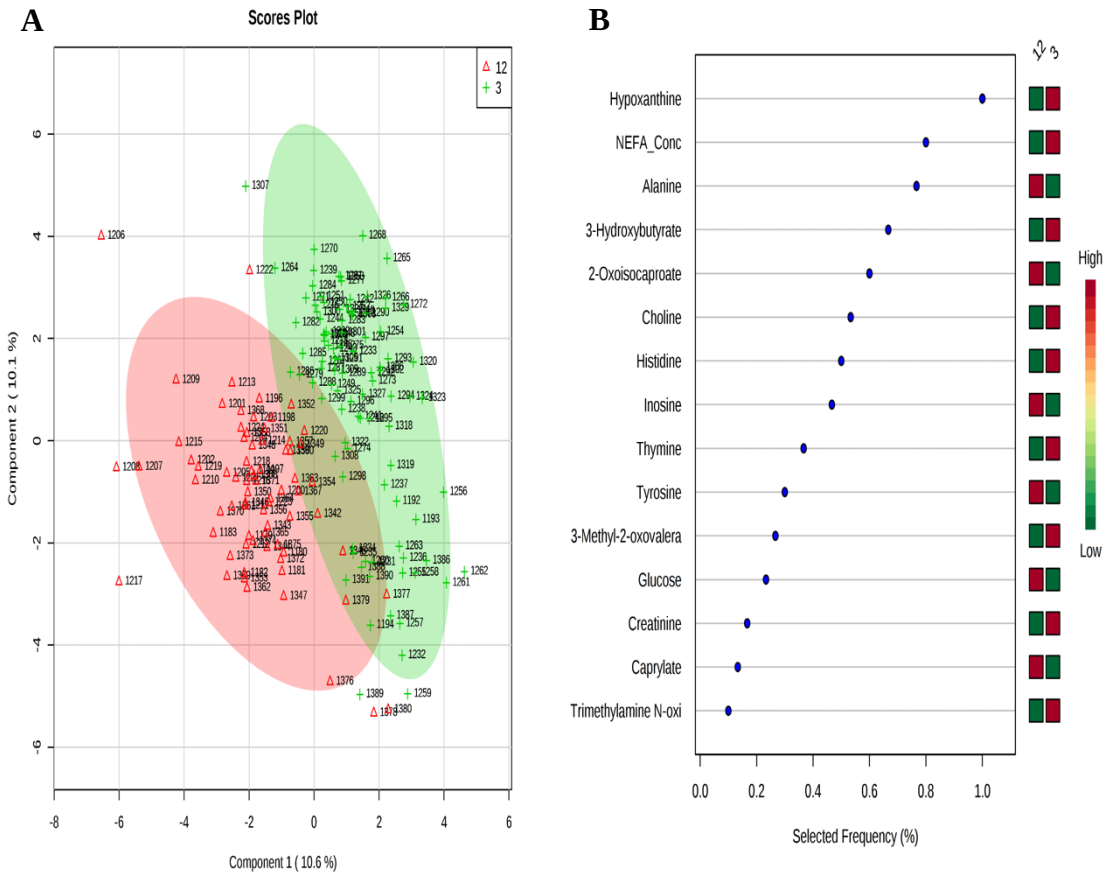


Figure 14. *Partial least squares discriminant analysis (PLS-DA) score map (A) and the biomarker analysis (B) between herds of bighorn sheep sampled in December (n = 73) and herds of bighorn sheep sampled in March (n = 99). In panel A, December is represented in red and March is represented in green. In panel B, compounds with a selected frequency greater than 0.1 are considered potential biomarkers distinguishing herds sampled in December and March.*

The last step in our assessment of NMR profiling was to determine if individual herds can be distinguished from each other within a season. One problem associated with distinguishing individual herds, was to standardize a given herd for comparative purposes. There were no herds where the environmental conditions and the nutritional status were known. To overcome this issue, the lab group used a control group of Rambouillet ewes that were fed a maintenance diet and housed in a controlled environment from December through April of 2014-2015. NMR metabolic profiles of these sheep were assessed at 4 week intervals during this period.

Each herd was compared to the Rambouillet ewes control group in December, January, and March. PLS-DA analysis of NMR profiles from each bighorn sheep herd, within each time frame (December, January, and March) showed complete separation from the control Rambouillet ewes. For the purposes of this report, we show data only for the comparison between the bighorn sheep located in the Fergus area in December and the Rambouillet ewes collected in December (Figure 15A).

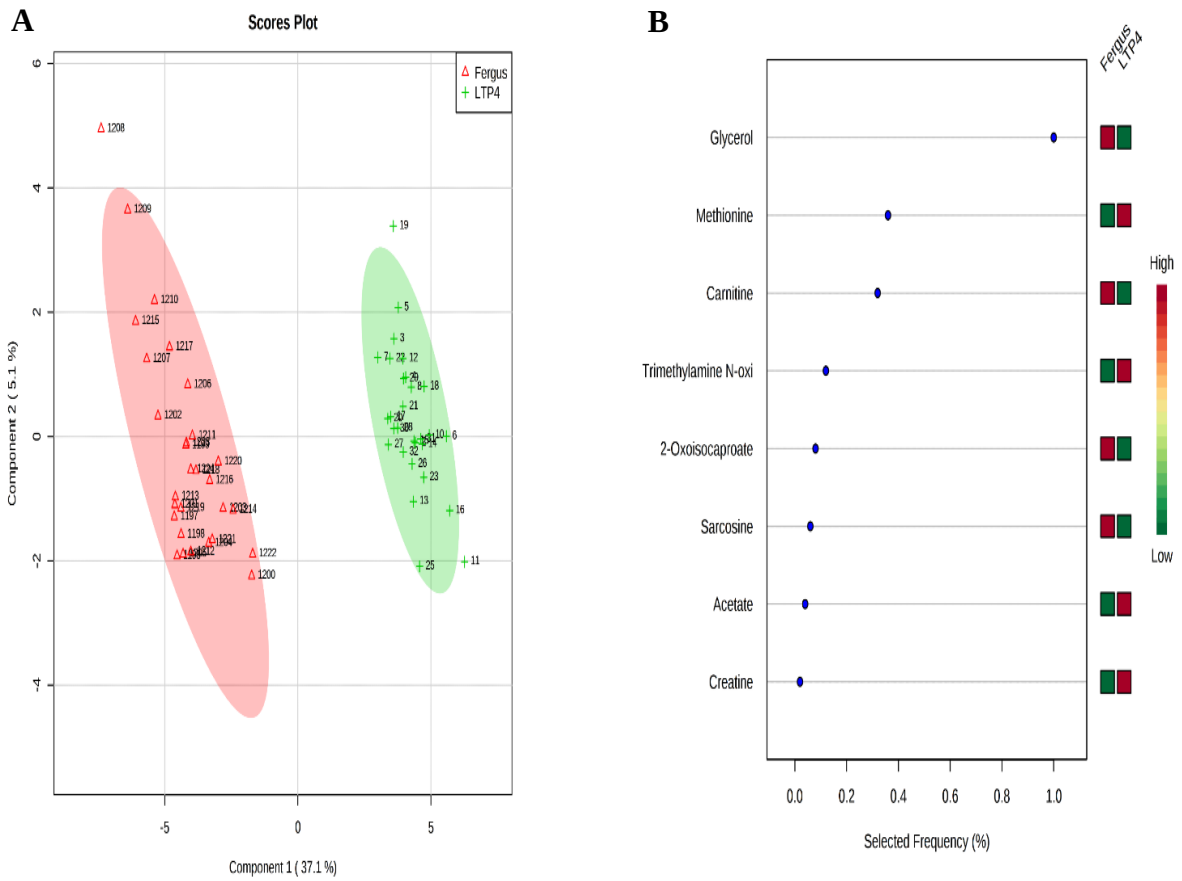


Figure 15. *Partial least squares discriminant analysis (PLS-DA) score map (A) and the biomarker analysis (B) between bighorn sheep located in the Fergus area sampled in December (n = 29) and control Rambouillet ewes sampled in December (n = 29). In panel A, Fergus is represented in red and the control Rambouillet ewes is represented in green. In panel B, compounds with a selected frequency greater than 0.1 are considered potential biomarkers distinguishing the Fergus bighorn sheep herd and the control group of Rambouillet ewes.*

The PLS-DA shows that the Fergus herd and the control group are completely separated; illustrating clear metabolic shifts between the two groups. One interpretation of these results is that this metabolic shift of the Fergus bighorn sheep herd from the Rambouillet control ewes is most likely related to the availability and quality of forage. In this case, the Rambouillet ewes were fed an ad lib, good quality, maintenance diet, while the Fergus diet most likely was less available and of lower quality.

PLS-DA analyses for each individual bighorn sheep herd compared to the Rambouillet control ewes for December, January, and March resemble the pattern shown in Figure 15A. The VIP scores for each individual herd were analyzed through the biomarker analysis pathway. Perhaps the most important finding of these analyses was that within each month, the same potential biomarkers were identified from each herd within a month. Thus, these particular biomarkers have the potential to identify and distinguish herds within a certain time frame.

In December, sarcosine, methionine, and trimethylamine n-oxide were identified as potential biomarkers in every herd. Again, not all figures are shown for each herd, however Figure 4B shows the potential biomarkers found in the Fergus herd. The potential biomarkers in December that were also found in metabolic pathways identified as significantly important (Table 2) were trimethylamine n-oxide found in methane metabolism and sarcosine found in glycine, serine, threonine, arginine and proline metabolism. In January, creatinine, dimethyl sulfone, asparagine and carnitine were the same potential biomarkers found within each herd. These potential

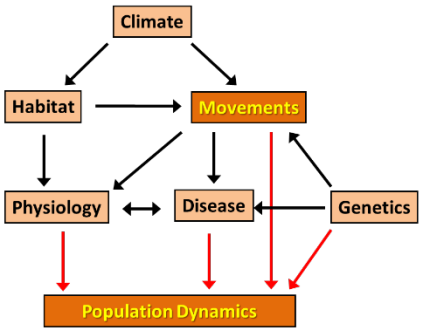
Pathway Impact	Impact Value	p – value	Holm-Bonferroni Correction	Significant Pathway
Aminoacyl- tRNA biosynthesis	**0.14**	**0.0001**	**0.001**	**Significant**
Glycine, serine, threonine metabolism	**0.60**	**0.0001**	**0.001**	**Significant**
Valine, isoleucine, leucine biosynthesis	**0.99**	**0.0001**	**0.001**	**Significant**
Alanine, aspartate, glutamate metabolism	**0.28**	**0.0001**	**0.001**	**Significant**
Arginine & proline metabolism	**0.17**	**0.001**	**0.006**	**Significant**
Methane metabolism	**0.40**	**0.004**	**0.020**	**Significant**
Phenylalanine, tyrosine, tryptophan biosynthesis	**1.00**	**0.008**	**0.032**	**Significant**
Cysteine & methionine metabolism	**0.27**	**0.020**	**0.060**	**Non-significant**
Phenylalanine metabolism	**0.41**	**0.040**	**0.080**	**Non-significant**
Pyruvate metabolism	**0.24**	**0.050**	**0.080**	**Non-significant**

Table 2. *Results from the serum metabolomic pathway analysis from all 56 compounds found in the bighorn sheep. Metabolic pathways with impact values greater than 0.1 are considered important, along with a p-value greater than 0.05. The p-values were corrected with the Holm-Bonferroni correction to reduce the error of multiple comparisons. The metabolic pathways that are significantly affected by the compounds identified are indicated on the right.*

biomarkers that are also found in significantly important pathways (Table 2) are creatinine found in arginine/proline metabolism and asparagine found in alanine, aspartate and glutamate metabolism. In March, the potential biomarkers identified in all herds were creatinine, dimethyl sulfone, and carnitine. The only potential biomarker also found in a significantly important pathway (Table 2) is creatinine, which is found in arginine/proline metabolism.

In summary, NMR metabolic profiling may have the potential to serve as a tool for evaluating management decisions for herds of bighorn sheep. Through the identification of potential biomarkers, it is possible to discriminate herds from each other within and between seasons. These biomarkers represents a potential panel of metabolites that may be used for assessing nutritional status, environmental stress, and herd health through the identification of significantly important metabolic pathways related to energy and protein balance. However, these results represent a single "snapshot" at a given time, making the predictive value of these biomarkers as this early stage of development low. In order to improve our understanding and have more confidence in these predictive values of identified biomarkers it is necessary to incorporate samples over a period of time from the same herds. This next year, a sample of animals from 3 bighorn herds that were repeatedly captured and sampled in fall and again in late winter/early spring for 3 consecutive years, 2015-2017, will be processed, profiled and analyzed using NMR metabolic profiles. This will give researchers a longitudinal view of the use of NMR metabolic profiling as a predictive tool for managing bighorn sheep populations. In addition to the longitudinal sampling we intend to explore opportunities for controlled short-term studies with captive bighorn sheep maintained at various research facilities. An important extension of this research would also be obtaining samples from bighorn sheep at various stages of respiratory disease or in other ways known to be physiologically compromised to begin developing biomarkers for accessing disease.

Objective # 4: *Collect first deployment of GPS collars and begin spatial analysis of data.*



As part of the sampling objectives for each population, a subset of 15 adult females, was fitted with paired GPS and VHF radio collars equipped mortality sensors (Models: TGW4400 [GPS] and MOD400 [VHF], Telonics Inc, Mesa, Arizona). The GPS collars were programmed to transmit a VHF signal and record location information every four hours for a period of approximately 21 months, storing the data internally. Each collar was equipped with a CR-2A release mechanism programmed to release the collar from the animal on a scheduled date, at which point field crews would be able to navigate to the dropped collar via telemetry and retrieve the stored data for analysis.

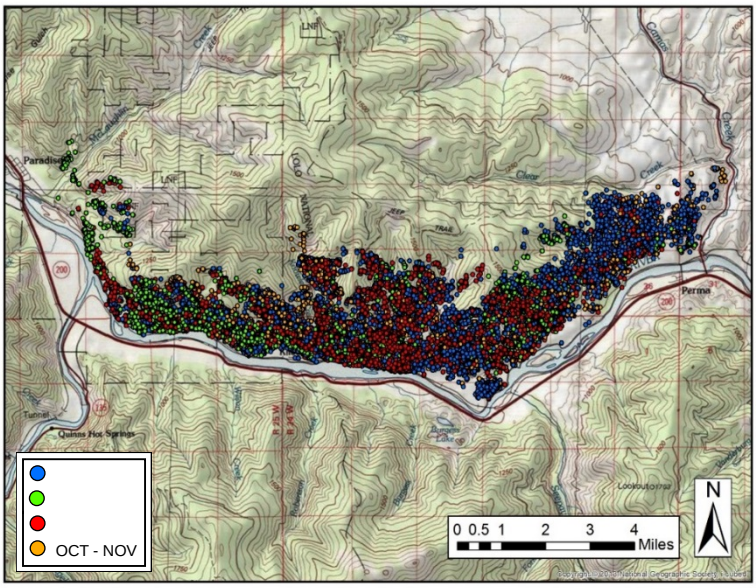
To date, a total of 180 animals have been fitted with GPS collars. Of these collars, data from 88 has been retrieved and 20 more are scheduled to be recovered by July, 2017. Three collars are currently considered unrecoverable. During December 2016, 52 store-on-board collars were deployed among the herds as well as 17 Iridium satellite-linked radio collars (Model TGW570). The Iridium collars report real time mortality updates and location information every two days for an estimated 5 years providing researchers and managers with a valuable tool for understanding and monitoring bighorn sheep populations.

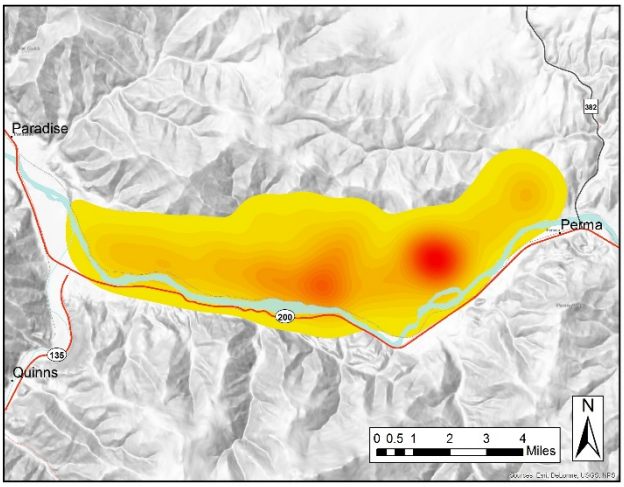
Spatial analysis has begun for six of the eight study herds and will continue throughout the remainder of 2016. Initial compilation of the data is displayed below for each herd, and includes successful GPS fixes and kernel density estimates for June to September (yellow) and December to March (blue) plotted for each herd.

Figure 16. *From left to right: FWP biologist Kristina Smucker recovering collar from the Castle Reef herd, MSU volunteer Collin Peterson using telemetry locate a released GPS collar in the Paradise herd, and collar from Paradise herd showing triggered CR-2A release mechanism .*

Paradise:

Of the original 15 collars deployed, 14 have been recovered. The remaining collar failed to release from the animal though may be recoverable via ground darting in the future. Collars from this herd had an overall GPS fix success of 99% and an adjusted fix success of 96% after censoring inaccurate fixes. A total of 57,032 useable locations were collected between December 3, 2014 and August 10, 2016 (846 days). An additional 10 collars were deployed December 18, 2016 and are scheduled for recovery in the spring of 2018. Paradise bighorn sheep are nonmigratory, living year-round on the same low-elevation slopes directly above the river.





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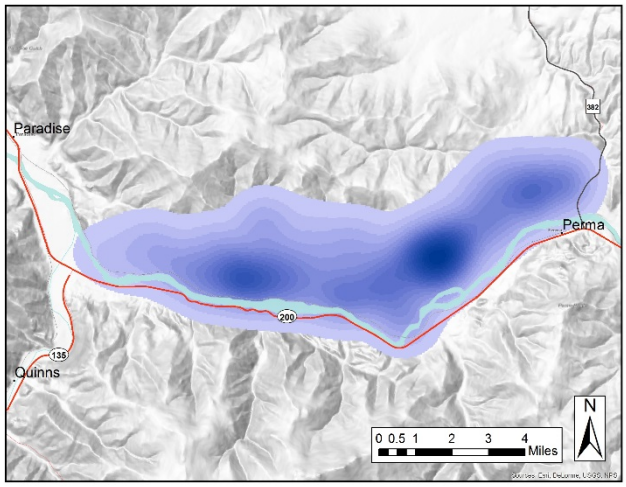
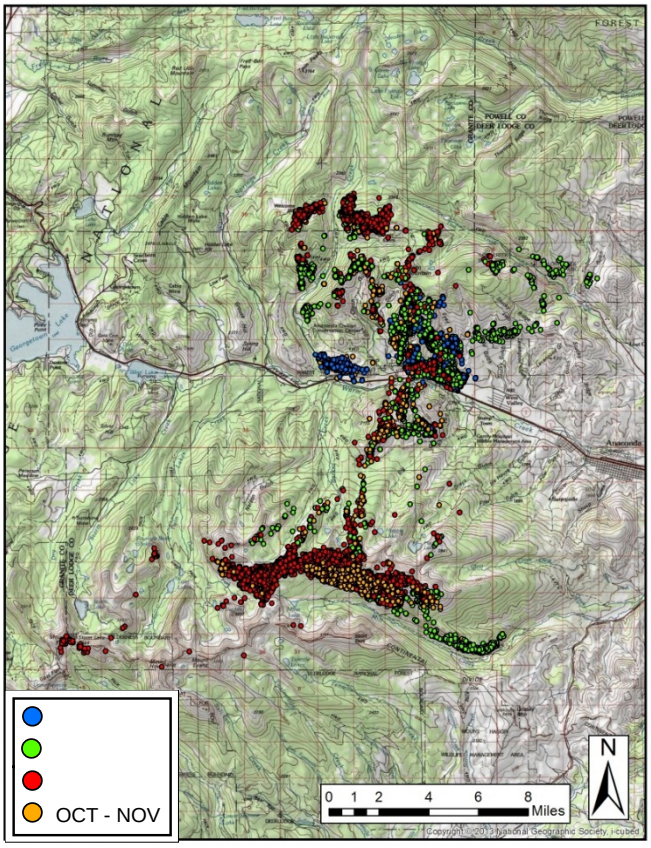
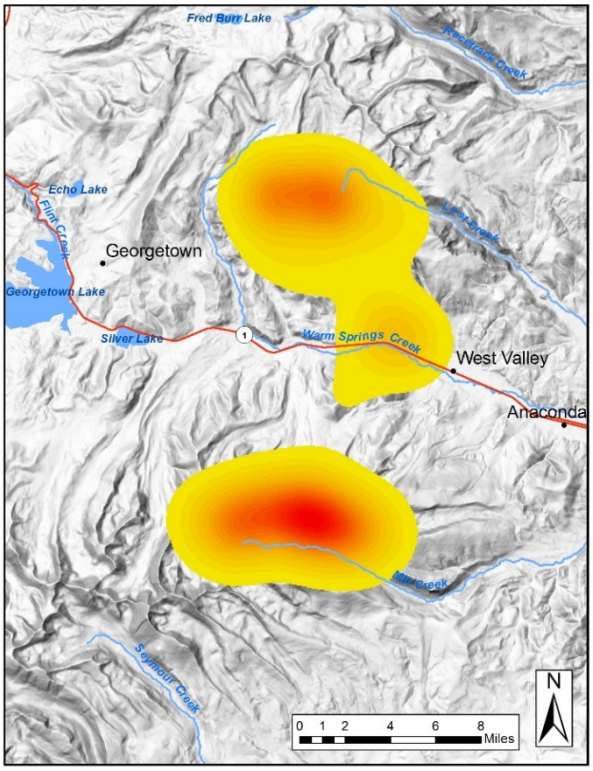


Winter

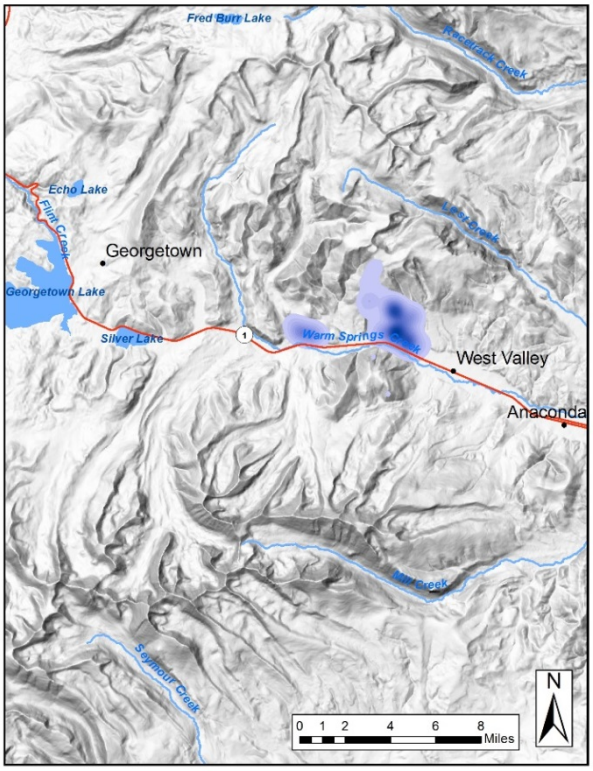
Lost Creek:

Data from 14 of 18 collared animals has been collected. Four more collars, deployed December 2015, are scheduled to be recovered in April, 2017. Collars in this herd had an overall GPS fix success of 99% and an adjusted fix success of 97% after censoring inaccurate fixes. A total of 43,867 useable locations were collected between January 3, 2015 and Dec 1, 2016 (699 days). Nine additional collars were deployed December 21, 2016 and are scheduled for recovery in the spring of 2018. Lost Creek bighorn sheep are primarily seasonal migrants with two distinct high-elevation summer ranges, one north and one south of Warm Springs Creek.





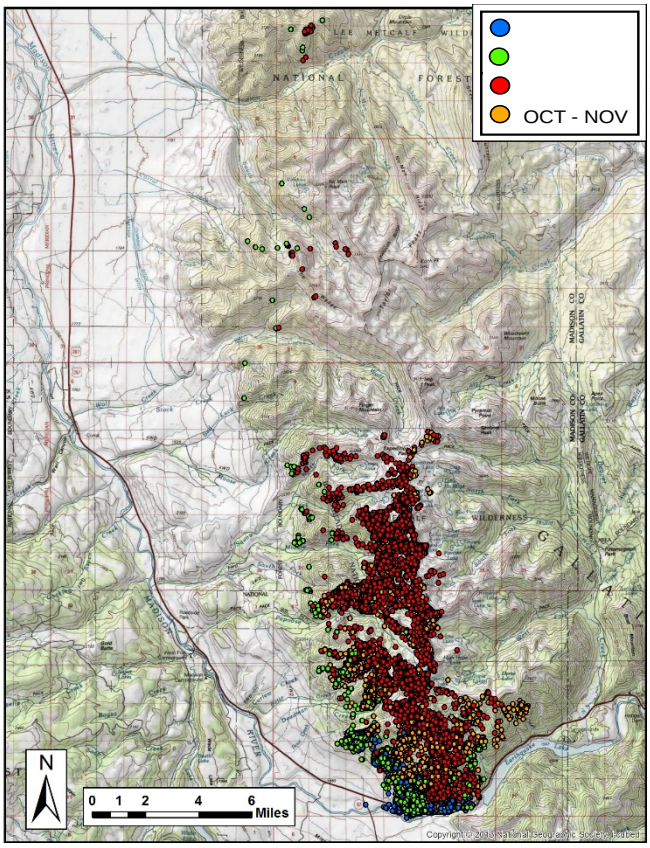
Summer

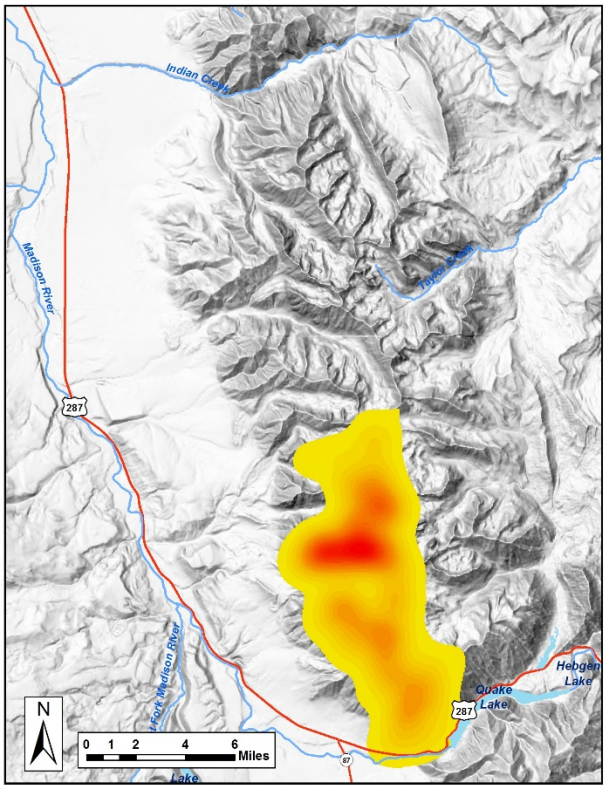


Winter

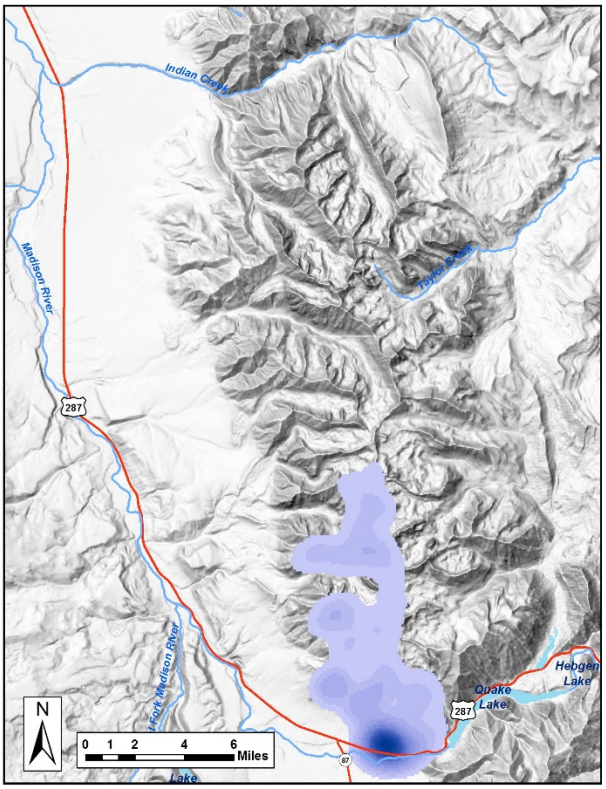
Hilgard:

All 15 originally deployed collars were recovered successfully. Collars had an overall GPS fix success of 100% and an adjusted fix success of 96% after censoring for inaccuracy fixes. A total of 58,019 useable locations were collected between December 18, 2013 and May 1, 2016 (866 days). Ten Iridium satellite-linked GPS collars were deployed December 18, 2016. Hilgard bighorn sheep are seasonal migrates with summer range on the high-elevation slopes and ridges of the southern Madison Range north of the winter range.





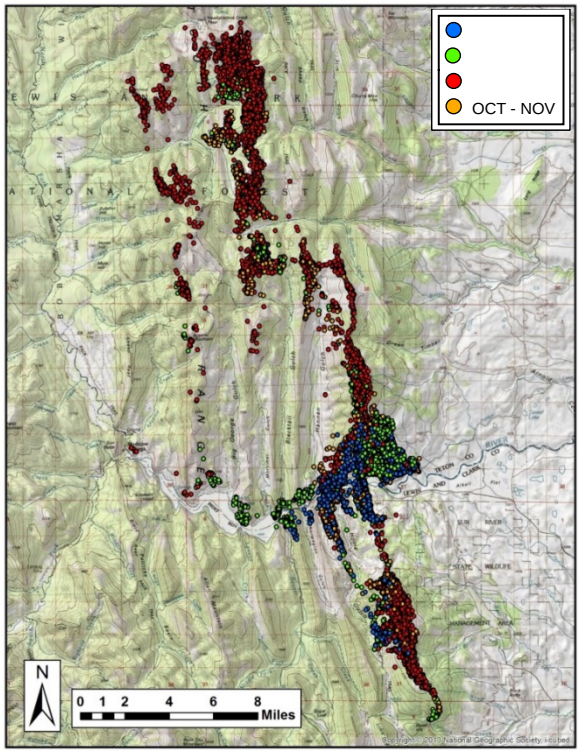
Summer

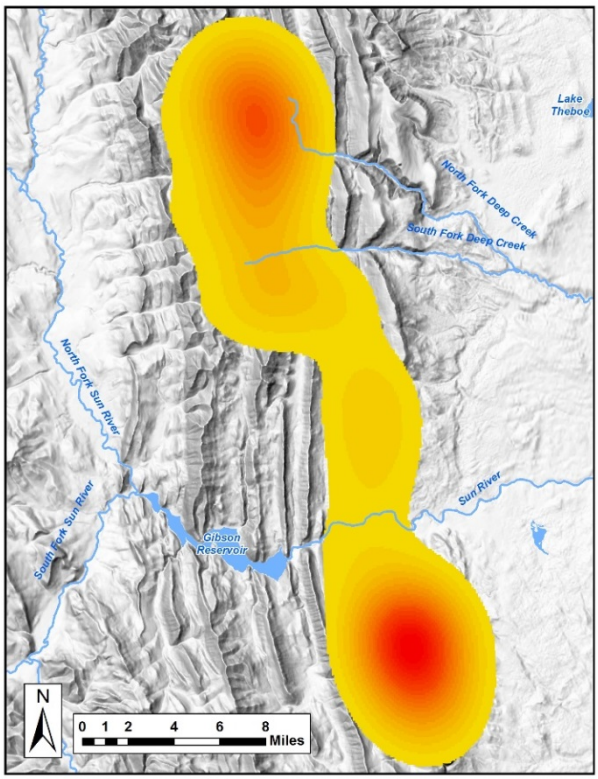


Winter

Castle Reef:

Of the 17 animals initially collared, data from 15 has been recovered. One of the remaining collars, redeployed after a mortality event, is scheduled to release March 25, 2017. The VHF beacon in the remaining GPS collar failed and was unable to be collected after releasing from the animal. Collars collected from the Castle Reef herd had an overall fix success of 99% and an adjusted fix success of 94% after censoring inaccuracy fixes. A total of 52,349 usable locations were collected between December 12, 2014 and August 10, 2016 (608 days). Seven Iridium satellite-linked GPS collars were deployed December 2016 and an additional four collars (3 Iridium RECON-4560-3, and 1 TGW4400) are scheduled to be deployed via helicopter and ground darting capture operations in February 2017. Castle Reef bighorn sheep are seasonal migrants, summering at higher elevations to the north and south of the winter range.





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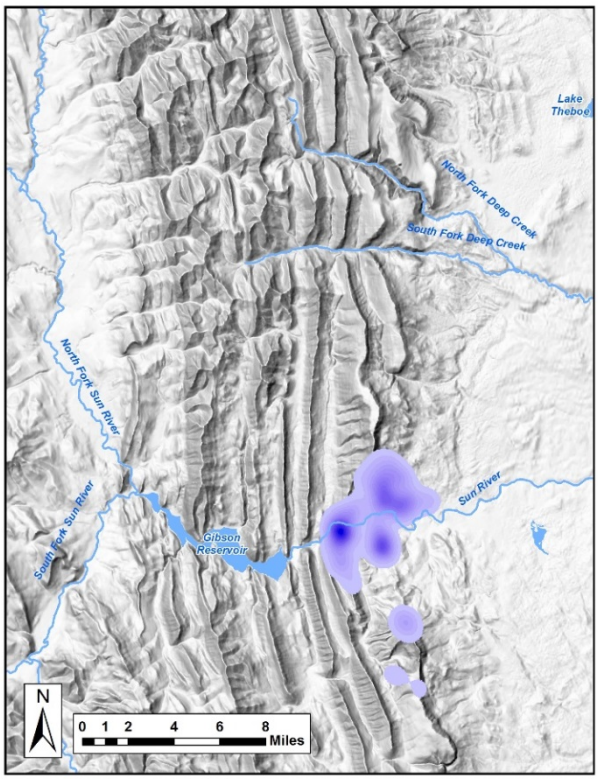
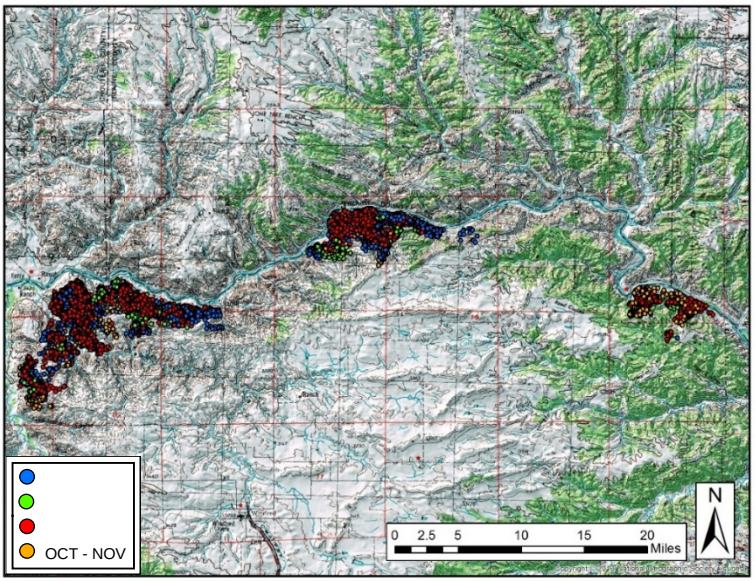
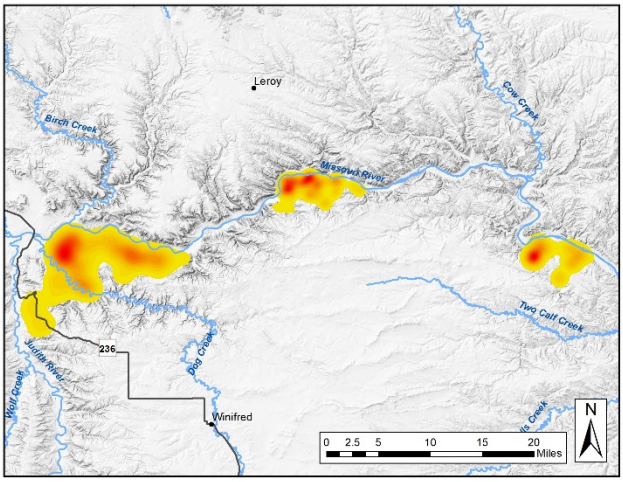


Winter

Fergus:

All 15 originally deployed collars were recovered successfully. Collars in the Fergus herd had an overall GPS fix success of 100% and an adjusted fix success of 97% after censoring inaccurate fixes. A total of 62,173 usable locations were collected between December 6, 2014 and July 10, 2016 (583 days). An additional ten collars were deployed December 13, 2016 and are scheduled for recovery in the spring of 2018. Fergus bighorn sheep are non-migratory.





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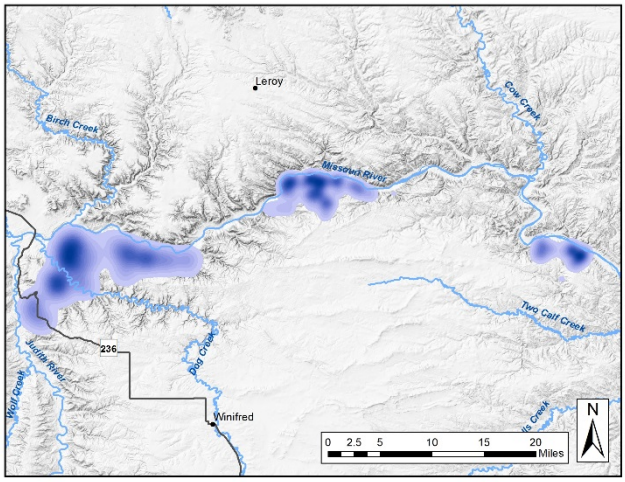
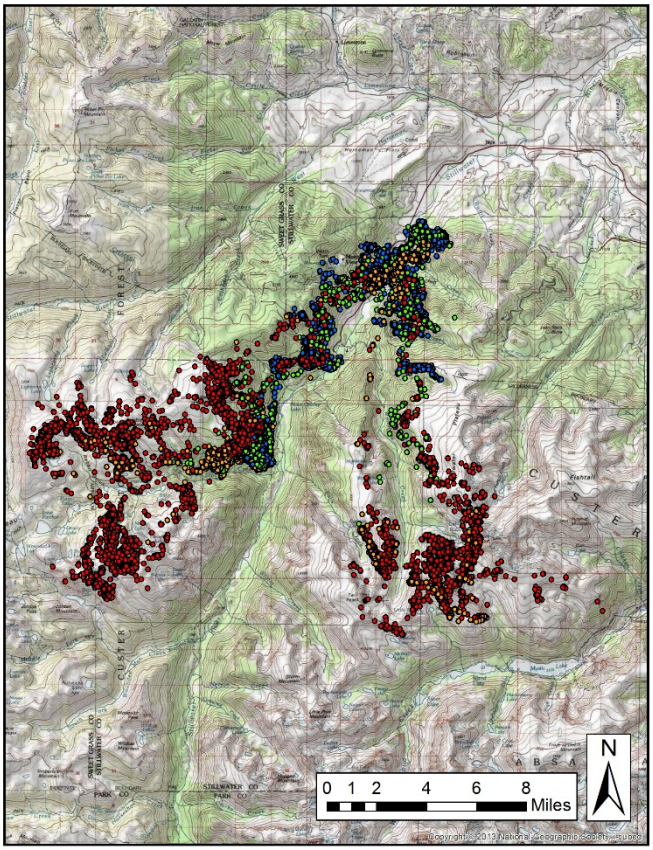
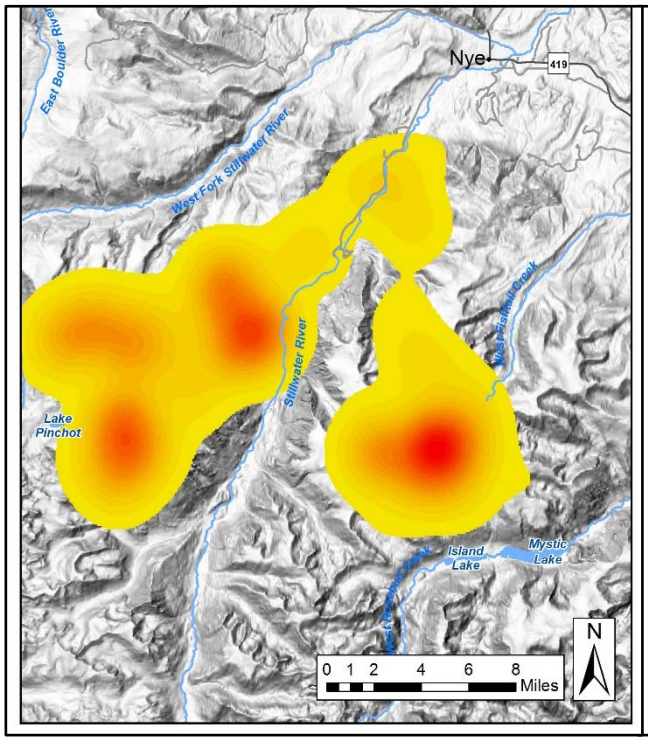


Winter

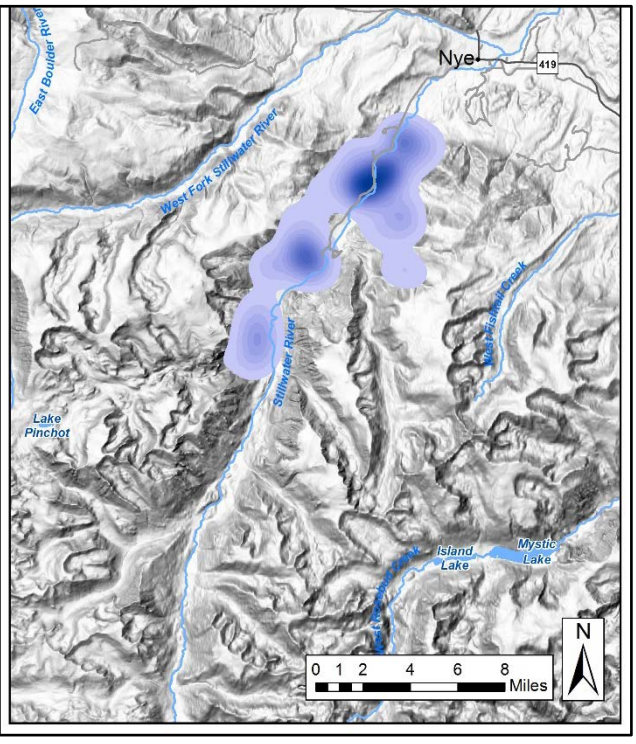
Stillwater:

Of the 16 initially collared animals, data from 15 has been recovered. The release location of the remaining collar was inaccessible and therefore unrecovered. The collars that were recovered had an overall GPS fix success of 100% with an adjusted fix success of 88% after censoring inaccurate fixes. A total of 51,154 usable locations were collected between December 19, 2014 and November 22, 2016 (705 days). Due to limited animal availability and the challenges of ground-darting operations, only five new collars are scheduled to be deployed during 2016 resampling efforts. As of January 31, 2017 three of the five GPS collars have been deployed and are scheduled to release spring of 2018. Stillwater bighorn sheep are seasonal migrants with two distinct high-elevation summer ranges, one east and one west of Stillwater River.



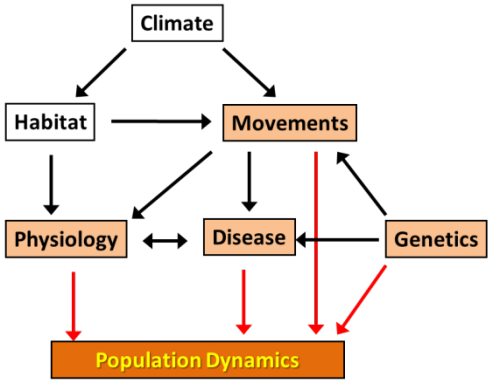


Summer



Winter

Objective # 5: *Monitor demographic rates in instrumented populations*



Accurate estimates of population size and demographic vital rates of wildlife populations are fundamental to guiding management actions because they elucidate demographic health and predict future population dynamics. Population growth is explicitly described by several vital rates: adult survival, fecundity, juvenile survival, immigration, and emigration. Reliable estimates of these vital rates allow for inference of population growth or decline independently from the use of sequential population estimates (Eberhardt 2002, DeCesare *et al.* 2012). Knowledge of the relative contribution of different vital rates to dynamics of wildlife populations is imperative to identifying mechanistic drivers of population dynamics. Accordingly, accurate estimates of vital rates are fundamental for implementing both effective research and management programs of wildlife populations. An important objective of the Montana Bighorn Sheep Study is to develop a simple, cost effective monitoring program that wildlife managers will be able to adopt as part of routine management activities, and use this program to estimate population size, adult female survival, and annual recruitment.

5.1 Adult Female Survival

Adult female survival is being monitored in the eight study populations by use of VHF and store-on-board GPS radio-collars equipped with mortality sensors which allow for known fate survival estimation. Survival of radio-collared animals is generally monitored at least once every three months, though the instrumented animals are often checked more frequently. The wide survival monitoring intervals often precluded determining cause of death, however; date of death was inferred from GPS collar data

To date, 208 adult females from all eight study populations have been radio-collared and monitored for survival (Table 1), 25 of which have died. Animals instrumented with radio-collars in winter 2016/2017 (n=69) are not included in the following discussion, however all were found to be alive in follow up survival checks in January 2016. Causes of death have included hunter-harvest (n=4), cougar predation (n=5), trauma (n=1), vehicle collision (n=1), and disease (n=1), however; the cause of most mortalities were undetermined (Table 3). In 2016, two mortalities occurred January-February, two occurred March-April, 4 occurred May-June, and one occurred in October. An additional female died January 3, 2017. The percentage of instrumented adult females captured in the study populations in winter 2014/2015 or previous that entered 2016 alive and survived to present, ranged from approximately 56% at Castle Reef to approximately 90% at Fergus. Herd specific summaries are presented below.

Table 3. *Cause of death for mortalities of adult female bighorn sheep in the seven study populations which have been monitored since winter 2014/2015. Middle Missouri is not shown due to the very recent implementation of survival monitoring.*

CAUSE OF DEATH	STUDY POPULATION							TOTAL
	Fergus	*Paradise*	*Hilgard*	*Stillwater*	*Castle Reef*	*Lost Creek*	*Petty Creek*	
Hunter Harvest	3	0	1	0	0	0	0	**4**
Disease	0	0	0	0	0	1	0	**1**
Trauma/Accident	0	0	1	0	0	0	0	**1**
Roadkill	0	1	0	0		0	0	**1**
Predation	0	1	1	0	2	1	0	**5**
Undetermined	0	2	2	2	5	2	0	**13**
TOTAL	**3**	**4**	**5**	**2**	**7**	**4**	**0**	**25**

Paradise:

A total of 4 collared adult females have died in this population. Two of the 15 adult females originally radio collared in 2014 died in 2015, and another two in 2015 leaving 73% of the originally collared animals remaining in the population.

Mortality Date	*Cause of Death*
3/16/2015	Predation
10/4/2015	Undetermined
4/28/2016	Undetermined
10/4/2016	Roadkill
TOTAL	**4**

Lost Creek:

A total of 4 collared adult females have died in this population. Three of the 12 animals that were radio-collared in January and March 2015 died in 2015, and another died May 2016, leaving 65% of these animals alive and in the population.

Mortality Date	*Cause of Death*
4/15/2015	Disease
5/23/2015	Undetermined
12/25/2015	Predation
5/30/2016	Undetermined
TOTAL	**4**

Hilgard:

Nineteen radio collared females captured in January 2012 (n=5) and December 2013 (n=15) remained in the population in 2016. Prior to the start of the study, a single adult female (radio-collared January 2012) died April 9th 2014. On January 6th, 2015, fifty-two animals were translocated out of the population, including two of the remaining 2012 radio-collared adult females, but both returned to the population before the start of the next winter along with 5 of the Lotek™ transplant collared animals. In 2015, two of the 19 originally collared females died and another died April 1st 2016 leaving 84% of these animals remaining. In addition, one of the

females collared in January 2015 died from predation in October of that year resulting in a total of 5 mortalities among 27 collared adult females in the population.

Mortality Date	Cause of Death
4/9/2014	Trauma
5/18/2015	Undetermined
10/4/2015	Predation
11/21/2015	Hunter Harvest
4/1/2016	Undetermined
TOTAL	**5**

Castle Reef:

Seven of the 16 adult females that were radio-collared December 2014 - March 2015 have died, leaving 56% of these animals remaining in the population.

Mortality Date	Cause of Death
4/29/2015	Undetermined
1/31/2016	Predation
2/24/2016	Undetermined
5/2/2016	Undetermined
5/22/2016	Undetermined
6/21/2016	Undetermined
1/3/2017	Undetermined
TOTAL	**7**

Fergus:

Three of the 30 adult females that were radio-collared in December 2014 died in 2015, leaving 90% of these animals alive and in the population. No other mortalities have occurred to date. All three mortalities were due to hunter harvest.

Mortality Date	Cause of Death
Unknown	Hunter Harvest
10/31/2015	Hunter Harvest
11/3/2015	Hunter Harvest
TOTAL	**3**

Stillwater:

Two of the 15 adult females that were radio-collared December 2014 - March 2015 died in 2015, leaving 87% of these animals alive and in the population. To date, no additional mortality of collared animals has occurred. One died April 1st and the second died at an unknown date, but likely in June. The cause of both mortalities remains unknown.

Mortality Date	Cause of Death
4/1/2015	Unknown
7/6/2015	Unknown
TOTAL	**2**

Petty Creek:

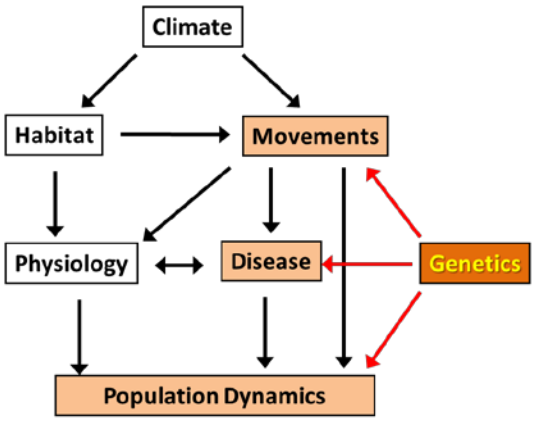
To date, none of the 15 adult females collared Feb 2, 2016 have died.

5.2 Pregnancy

Pregnancy rates of adult female animals (≥ 1.5 years old) in study populations as well as numerous populations in Wyoming were assessed using serum assays that measure serum concentrations of pregnancy specific protein "B" (PSPB) and progesterone (P4). PSPB concentrations indicate whether an animal is or recently was pregnant, however, require up to a month following fertilization to reliably indicate pregnancy. P4 concentrations indicate whether the animal is cycling (reproductively active) and capable of becoming pregnant (if sampled during the breeding season) or is pregnant (if sampled after the breeding season). For animals sampled in December (near the end of the breeding season) PSPB cannot reliably assess pregnancy and P4 can reliably indicate whether or not an animal is cycling, but not whether it has been successfully bred. The combination of PSPB and P4 concentrations can be used to infer aborted pregnancies when PSPB concentrations are indicative of pregnancy but P4 concentrations are not.

For animals sampled in December (n=10), the pregnancy rate was 80%. For animals sampled from January-March (n=134), the overall pregnancy rate was estimated at 90%. This pattern of very high pregnancy rate across all sampled herds is similar to results from our initial sampling during the first two years of the study and corroborates findings from previous studies that bighorn sheep pregnancy rates are consistently high and not likely an important factor limiting lamb recruitment (Singer *et al.* 2000, Cassirer and Sinclair 2007, Stephenson *et al.* 2012). Despite the evidence for overall high pregnancy rates, there still was some variability in the test results among populations and interesting idiosyncrasies that may indicate ecological differences among populations which will be explored further toward the end of the study.

Objective #6: *Collect and provide samples for bighorn sheep genetics and complete preliminary genomic analyses*



Genetic investigations were added to the Montana Bighorn Sheep Study project in 2016 as an integral component of a comprehensive research program to address potential limiting factors in bighorn sheep restoration, conservation, and management. For example, genetic consequences of inbreeding in small populations can impact recruitment and local adaptations can influence translocation success. Comparing genetics of different bighorn sheep herds could potentially provide information to describe genetic connectivity and diversity of examined herds, as well as discover links between herd demography and genetics. Genetics research may also serve to inform evaluation of genetic diversity in current or previously small populations, aid in selection of potential source populations for augmentation or reestablishment projects, determine what populations have low genetic diversity and might benefit from augmentation, discover what populations are genetically unique, and examine potential links between genetics and population history of respiratory diseases.

The Ovine array is a new genetic analysis technique originally developed for domestic sheep that provides considerable promise for advancing bighorn sheep genetics research. The Ovine array contains approximately 700,000 single nucleotide polymorphisms (SNPs), with approximately 24,000 markers that are informative for Rocky Mountain bighorn sheep (Miller *et al.* 2015). This technique represents a significant advancement in genetic analysis of bighorn sheep, as most previous studies have used microsatellites and less than 200 genetic markers. In addition, the Ovine array provides the potential to map informative SNPs to genomic areas of known function. The Ovine array provides the capability to conduct whole genome genotyping of bighorn sheep and can serve to increase understanding of population genetics.

6.1 Collection of genetic samples

We have over 800 high-quality bighorn sheep genetic samples from different populations across Montana and Wyoming available for genomic analysis (Table 4). Samples are available due to past capture efforts coordinated by Montana Fish, Wildlife and Parks, Wyoming Game and Fish Department, the Greater Yellowstone Area Mountain Ungulate Project, Yellowstone National Park, Glacier National Park, and USGS. During capture efforts by MSU and Montana Fish, Wildlife and Parks from November 2016 to February 2017, we collected 298 genetic samples from 190 different bighorn sheep. These genetic samples were collected from the Castle Reef, Fergus, Lost Creek, Middle Missouri Breaks, Paradise, Taylor-Hilgard, and Stillwater herds. We collected multiple types of genetic samples, including gene cards, biopsy ear punches, whole

blood, and nasal swabs. Collection using gene cards involves placing 2-4 drops of whole blood directly from the syringe onto each of the four circles of filter paper on an FTA Classic gene card. To obtain DNA of greater quality than gene cards can provide, we also collected biopsy ear punches, whole blood, and nasal swabs. Biopsy punches were obtained from ear cartilage during ear tagging and stored frozen in diluted ethanol. Genetic nasal swabs were used on a small number of individuals as a pilot project to explore a new and simpler method for managers to obtain high quality genetic samples during capture. In addition to the samples collected this year, Montana Fish Wildlife and Parks has been collecting DNA using gene cards since 2004.

Herd	Management Agency	Samples currently available	Samples currently assayed	Minimum no. samples to be assayed
Castle Reef[rh]	Montana FWP	48	0	15
Fergus[rh]	Montana FWP	59	30[c]	0
Grave Creek[rh] (Petty Creek)	Montana FWP	16	0	15
Lost Creek[rh]	Montana FWP	37	0	15
Middle Missouri Breaks[rh]	Montana FWP	35	0	15
Paradise[rh]	Montana FWP	44	0	15
Stillwater[rh]	Montana FWP	22	16[a]	0
Taylor/Hilgards[rh]	Montana FWP	96	30[c]	0
Clark Fork Cut-off	Montana FWP	4	0	0
Galton	Montana FWP	30	0	15
Highlands	Montana FWP	16	0	15
North Clark Fork	Montana FWP	1	0	0
Tendoys	Montana FWP	18	14[a]	1
Wild Horse Island	Montana FWP	27	2[a]	13
Glacier National Park	NPS	98	25[b]	0
Mount Everts-Yellowstone NP	NPS	5	0	0
Rocky Boy Reservation	Tribal	22	0	0
Absaroka Metapopulation	Wyoming F&G	224	87[a]	0
National Bison Range	USFWS	21	0	0
Totals		823	204	119

[rh] Herds in Montana state-wide research project
[a] Analysis of these samples was funded by the Wild Sheep Foundation, Holly Ernest at the University of Wyoming, and Gray Thornton from the Wild Sheep Foundation.
[b] Analysis of these samples was funded by the National Geographic Society.
[c] Analysis of these samples was funded by Montana Fish, Wildlife and Parks.

Table 4. *High quality genetic samples (gene cards, ear biopsy punches, tissue, nasal swabs, and/or DNA extractions) from different animals currently available for bighorn sheep genomic analyses from Montana and Wyoming. Herd units not managed by Montana FWP are shaded in gray.*

6.2 Extraction of genetic samples and assessment of DNA quality

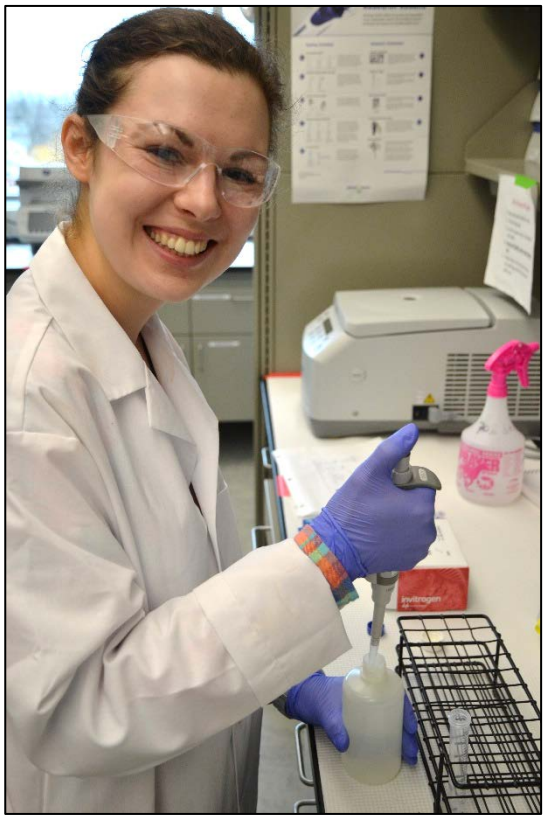


Figure 17. *Graduate student Elizabeth Flesch conducting an assessment of DNA quality of bighorn sheep samples in the Animal and Range Sciences laboratory based in the College of Agriculture at MSU.*

During extraction of bighorn sheep genetic samples at MSU, we gained information regarding the quality of DNA that can be extracted from different types of bighorn sheep genetic samples in our lab. While gene cards provide a relatively low-cost method to store genetic samples at room temperature over long periods of time, we found that there are some limitations to their use for genomic analysis. Older gene cards that have not been stored with desiccant in foil pouches over long periods of time provided extractions with lower overall quality and occasionally required multiple extraction attempts to achieve suitable quality for SNP genotyping. More recently collected gene cards that were stored in foil pouches provided higher quality DNA extractions than the older cards. However, these samples were not sufficiently high quality to consider sequencing uses with currently available technology. In addition, despite thorough assessment of DNA quality and quantity in our lab prior to genotyping, a small number of the gene card extractions provided low quality SNP genotyping results.

Thus, during capture efforts from November 2016 to February 2017, we also collected ear punch and whole blood samples for genomic analysis. Ear punches were collected using a single use biopsy punch tool to capture ear cartilage prior to ear-tagging and stored frozen in 90% ethanol. Ear punch extractions generally provided greater quality and concentrations of extracted DNA than gene card extractions. We also collected whole blood samples for a limited number of captures that can provide extractions suitable for sequencing when extracted within days of capture. In addition, we extracted DNA from tissue sampled from hunter-harvested animals that provided high quality extractions. Whole blood samples are often stored frozen for long-term storage, but DNA can degrade during freezer storage and blood sample thawing, however, we were able to successfully extract DNA from previously frozen blood samples. In addition, we conducted a pilot effort to collect genetic nasal swabs and assess quality of extractions to potentially provide managers with an alternative user-friendly way to collect genetic samples during capture. Initial assessments of nasal swab extractions indicated high DNA quality and concentrations, and we plan to further quantify DNA quality of these extractions through application of PCR.

6.3 Preliminary genomic analysis results

6.3.1 Genomics Pilot Study

From 2015 to 2016 we conducted a pilot study that quantified genetic attributes of bighorn sheep populations with a range of different herd histories in Montana and Wyoming to investigate genetic similarity and differences, genetic heterogeneity, and genetic distance. In addition, we sought to evaluate the utility of the Ovine array and assess the quality of DNA that could be recovered from recent and archived bighorn sheep samples. We analyzed approximately fifteen individuals from each of four different populations that we hypothesized would differ in genetic characteristics, due to population attributes that potentially impacted their genetics, including origin (native/reintroduced), population size, bottleneck history, degree of connectivity, augmentation history, and geographic separation. We selected four populations that provided a spectrum of these herd attributes, including the Tendoys, Stillwater, and Glacier National Park in Montana and the northeastern Greater Yellowstone Area in Wyoming. We opportunistically genotyped two bighorn sheep from Wild Horse Island that had been translocated to the Tendoys in 2012 and were harvested in 2015.

Pilot Study Herd Characteristics and Genetic Hypotheses

In general, genetic connectivity among herds through geographic proximity and augmentation can increase genomic similarities. However, the extent of connectivity can vary based on landscape features and the success rate of past augmentations. Thus, we sought to evaluate the following hypothesis:

- Hypothesis #1: Bighorn sheep in geographic proximity or with shared translocation histories will have more similar genomics detected by the Ovine array than those herds that are not in geographic proximity or with shared translocation history.

We also examined population attributes that may impact genetics of each examined herd to predict genomic results detected using the Ovine array (Figure 18). First, we expected native and reintroduced herds to have differing genetics, because initial genetic composition and diversity of founders in a newly established herd can have a strong impact on the population genetics. This "founder effect" can result in low genetic diversity and subsequent genetic drift, because the herd was founded by a small number of individuals. In contrast, native herds are more likely to contain more genetic diversity and adaptations to their local environment. Secondly, we expected population size to impact herd genetics. Small population size can result in lower likelihood of herd persistence, limited adaptive potential, and increased susceptibility to inbreeding, which can impact the overall herd. For our pilot study, we categorized herds into three different population sizes: "small" (on average less than 100 individuals), "medium" (100-200 individuals), and "large" (greater than 200 bighorn sheep).

Thirdly, we expected that past bottlenecks (a severe reduction in population size at a point in time) in herd history could impact population genetics. Bottlenecks can result in a decrease in genetic variation, an increase in inbreeding, and greater frequency of detrimental alleles, which can all negatively impact probability of herd persistence. We classified three categories of potential bottlenecks, including "mild" (large populations with no record of past bottlenecks), "moderate" (possible past bottlenecks), and "strong" (known past bottlenecks). Finally, connectivity with other bighorn sheep herds can impact population genetics, as isolation and

consequent lack of gene flow can cause a decline in genetic diversity. Lack of gene flow in isolated herds has been cited to promote strategic genetic augmentation of bighorn sheep. We classified herd connectivity as "high" when a herd was a part of a known, large metapopulation of bighorn sheep, "some" when limited connectivity with other herds was suspected, and "isolated" when no known connectivity (other than augmentation) occurred. Genetic connectivity in the Tendoys herd is currently uncertain, as this population may potentially experience genetic connectivity with the Lemhi herds in Idaho. We used this information regarding herd histories to evaluate the following hypothesis:

- Hypothesis #2: Native, large herds with mild potential for past bottlenecks and high connectivity will have greater heterozygosity than reintroduced, small herds with strong potential for past bottlenecks and little to no connectivity.

By combining herd attributes to predict their genetic impacts, we ranked potential genetic concern for each herd to address hypothesis #2 (Figure 18), from low (East GYA and Glacier National Park), moderate (Stillwater), to high (Tendoys and Wild Horse Island), and we expected these differences could be detected using the Ovine array. As potential genetic concern increases, there was a greater expected probability to observe higher inbreeding and lower heterozygosity in a herd. Herds with low genetic concern were predicted to have lower inbreeding coefficient values, whereas herds with high genetic concern were predicted to have higher inbreeding coefficient estimates. Based on a synthesis of these herd history characteristics, we expected heterozygosity to be highest in the East GYA and Glacier National Park, lower in the Stillwater, and lowest in the Tendoys and Wild Horse Island herds.

Pilot Study Results

We analyzed SNP genotypes for 57 total bighorn sheep in Montana and Wyoming, including 14 from the East GYA, 11 from Glacier National Park, 16 from Stillwater, 14 from the Tendoys, and 2 from Wild Horse Island. We were not able to reach our target number of 15 samples for three of the herds, as SNP genotypes from the Ovine array failed quality control standards for three samples (1 from the East GYA and 2 from Glacier National Park). In addition, three samples necessary to reach the target of 15 per herd were not available for the pilot study (1 from the Tendoys and 2 from Glacier National Park). As an alternative to these three samples, we opportunistically included two samples from Wild Horse Island and one additional sample from Stillwater. In general, we confirmed that approximately 24,000 SNPs from the Ovine array were informative for bighorn sheep (Miller et al. 2015). We further filtered the dataset to include 12,726 relevant, unlinked SNPs, in order to remove potential bias that can be introduced by SNPs that are close together on the genome and highly linked.

Hypothesis 1: Genomic similarities and differences

To address our first hypothesis, we conducted a principle component analysis (PCA), which is a multivariate analysis that summarizes variation of included SNPs and can provide insight into relationship patterns and clusters of individuals. We conducted the PCA using 12,726 relevant, unlinked SNPs for all bighorn sheep in the pilot study. The top two eigenvectors are displayed in Figure 19.

Herd Attribute	East GYA	Glacier National Park	Stillwater	Tendoys	Wild Horse Island
Native or Reintroduced	Native	Native	Native	Introduced	Introduced
Population Size	Large	Large	Small	Small	Medium
Potential Bottlenecks	Mild	Mild	Moderate	Strong	Strong
Connectivity	High	High	Some	Isolated?	Isolated
Potential genetic concern	Low				High

Figure 18. *Herd attributes of five bighorn sheep herds analyzed in the genomics pilot study. There was a range of attributes among herds that were predicted to cause different herd genetics.*

The results of the PCA illustrated that SNP genotyping has the potential to provide information to identify genetically unique herds. Clustering of SNP genotypes correctly grouped herds known to have mixed based on geographic proximity and management history, as well as separated groups not known to have mixed. The Glacier National Park population appeared to be highly unique in comparison to other herds examined in the pilot study, as the eigenvector values for these bighorn sheep did not overlap with any other herds. The East GYA and Stillwater herds clustered together in the PCA, likely due to the geographic proximity of these herds, as the bighorn sheep occupying the Beartooth and Absaroka ranges are suspected to represent a large metapopulation complex. In general, the amount of genomic data included in the PCA can impact the amount of differentiation observed in multi-variate space. The observed differentiation using genomic data from the Ovine array demonstrated our capability to distinguish differences among herds using SNP genotyping. However, we can only evaluate genomic differences among herds included in the genomic analysis. We can further evaluate if the differences among herds are meaningful by using association analysis to determine if herd attributes of interest correlate to certain markers.

To continue to assess if we could uniquely identify herds, we conducted a trial association analysis using herd identification as the phenotype and determined that approximately 30 markers were correlated to herd. This preliminary result suggested that the Ovine SNP array detected sufficient variation in the 57 samples to enable future association analyses between markers and phenotypic traits of interest. There was noticeable genetic variation among individuals within each population, which also provided significant potential for future genetic research in relating individual, diverse genotypes to differing physical characteristics

(phenotypes) of bighorn sheep. In addition, we mapped the hybrid genome map built by Miller et al. 2015 to the observed SNPs, which approximates chromosomal location of bighorn sheep markers based on the Ovine genomic map. To assess coverage of unlinked SNPs across the entire bighorn sheep genome, we plotted the result of the association analysis between genotype and herd by chromosome location. Wide spread in SNP locations across the known genome suggested that the Ovine SNP array has good coverage across chromosomes. In addition, detected variation in correlation significance levels indicated that there is sufficient variation detected by the Ovine array, such that these data are likely usable for association analyses and may provide significant genomic correlations in relation to traits of interest.

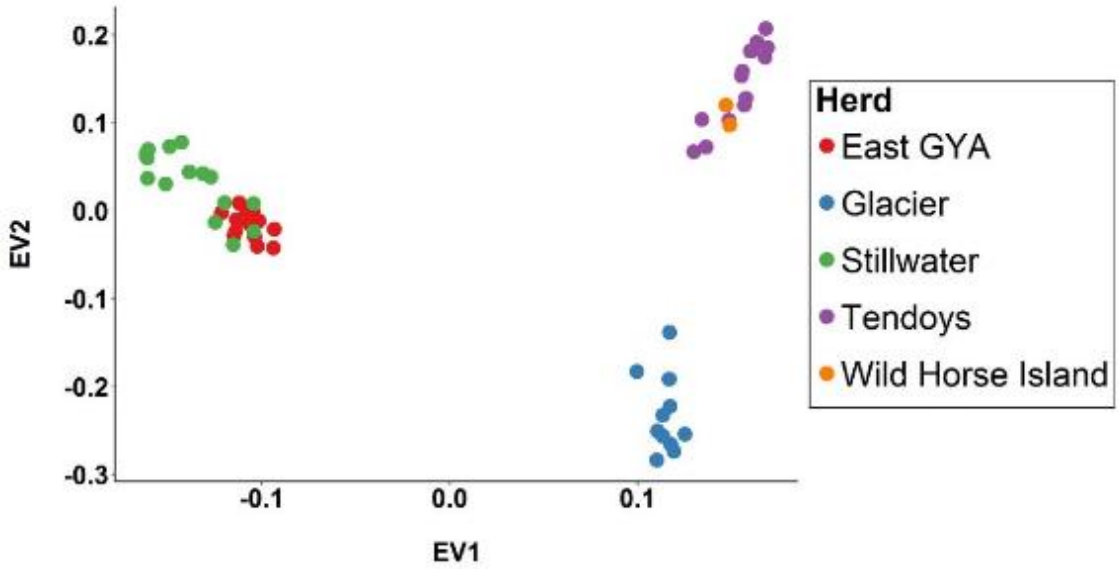


Figure 19. *Principle component analysis of variation bighorn sheep genomic data analyzed in the pilot study. Axes are labeled with the highest two eigenvectors. Herds that are separated by more space are more genetically different, and herds that overlap are genetically similar. Genetic variation is also apparent among individual bighorn sheep.*

The PCA also demonstrated that SNP genotypes could potentially be used to inform selection of source populations for augmentation. For example, the PCA indicated that signatures of past augmentations could potentially be detected through SNP genotyping, as the Wild Horse Island bighorn sheep clustered with the Tendoys. This overlap may be due to multiple alternative explanations, as there was an augmentation of 49 Wild Horse Island bighorn sheep in 2012. The clustering may indicate: 1) multiple Wild Horse Island animals were harvested in the Tendoys and were not necessarily identified as Wild Horse Island animals upon sample collection; 2) Wild Horse Island animals genetically contributed to the Tendoys population after the introduction of 49 bighorn sheep from Wild Horse Island to the Tendoys in 2012; and/or 3) genetic lineages of the two herds were similar, since both herds were reintroduced and had source populations from the Rocky Mountain Front. We hope to further explore and narrow these possibilities through further research to assess genetics of each herd and identify unique genetic signatures of each population. In addition, we plan to use additional population genetic metrics to compare more herds and further evaluate genomic similarities and differences. This information can be useful for translocation planning, as managers could determine if there are

genetic differences among populations prior to identifying optimal source and recipient herds for augmentation.

Hypothesis 2: Heterozygosity and inbreeding coefficient estimates
To address our second hypothesis, we examined the distributions of inbreeding coefficients for each herd, which were also informative and demonstrated the potential of SNP genotyping to address numerous objectives regarding bighorn sheep genetic characteristics (Figure 20). Inbreeding coefficient estimates are a metric that can be used to examine genetic diversity using genome-wide SNP data. This test assumed that homozygous genotypes for an individual occurred either by chance or because both alleles were from the same ancestor. We first estimated allele frequencies using all pilot study genotypic data and then estimated the number of homozygous genotypes expected in each sample. From that, we calculated the estimated inbreeding coefficient for each bighorn sheep sample. Coefficient of inbreeding is often used to evaluate livestock populations, and it is well-documented that for every 0.1 increase in positive inbreeding coefficient for a domestic animal, there is a 10% decrease in overall growth and reproductive performance. Inbreeding coefficients below 0 suggest that the individual was not impacted by inbreeding.

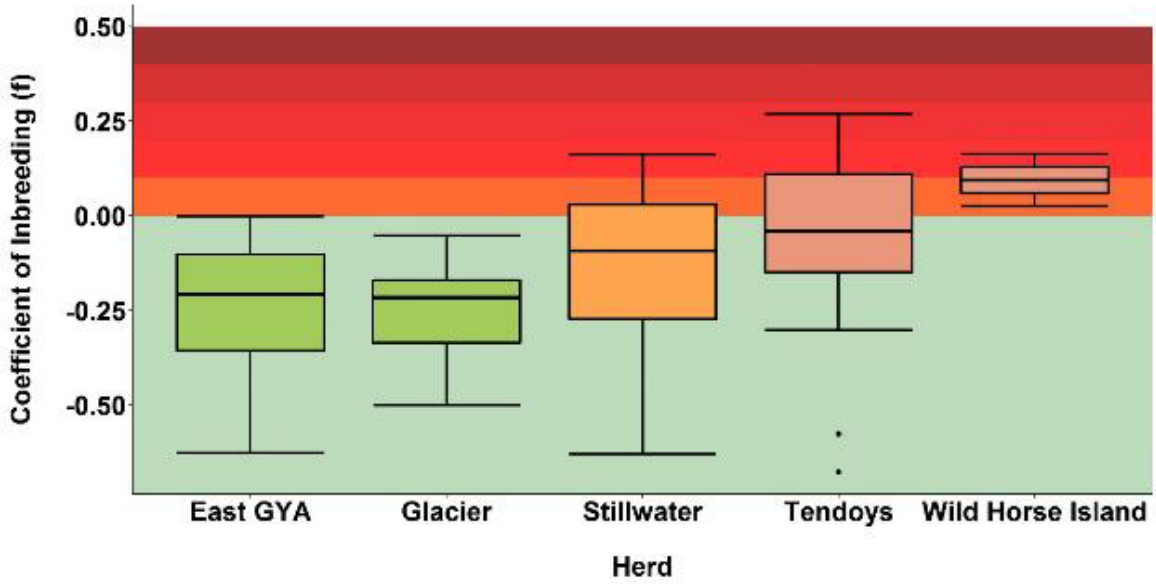


Figure 20. *Inbreeding coefficients of bighorn sheep sampled in the pilot study. Boxes are color-coded based on the potential genetic concern of each herd, predicted based on herd attributes. Inbreeding coefficients less than 0 did not exhibit inbreeding.*

However, there can be multiple different causes and interpretations of inbreeding values, and quantification of inbreeding coefficient values alone cannot definitively determine if a herd experienced problematic inbreeding depression. In general, inbreeding coefficient information for a population can serve as a helpful piece of information to consider along with other factors in order to evaluate herd health. We do not yet understand potential effects of inbreeding on bighorn sheep performance, but we quantified inbreeding coefficients of bighorn sheep in the pilot study to explore the distribution and differences in heterozygosity among herds. As expected based on herd attributes, East GYA and Glacier National Park bighorn sheep exhibited no inbreeding in contrast to the other herds examined. This is likely because these herds are large, native populations with high connectivity and low potential for past bottlenecks, based on

known herd history. The Stillwater contained a distribution of bighorn sheep with and without inbreeding, which may have been a consequence of the small herd size, past history of bottlenecks, and possible local adaptation, with the potential for genetic connectivity with the East GYA, as evidenced in the PCA.

Some Tendoys and both Wild Horse Island animals had high inbreeding coefficient values, and the Tendoys herd contained the three most inbred bighorn sheep in the pilot study. Therefore, inbreeding assessment results were consistent with the ranking of populations from low to high genetic concern based on their herd histories and attributes (Figure 18). However, individuals from the Tendoys that had the highest inbreeding coefficient values were closely related to the Wild Horse Island animals. Thus, this observed result could be due to the fact that Wild Horse Island animals with potentially high inbreeding coefficients were relatively recently introduced to the Tendoys. (Potential explanations for their genetic similarities are listed in the PCA description.) In general, the inbreeding results (Figure 20) suggested that additional genetic objectives can be addressed through SNP genotyping. Specifically, SNP genotypes can be used to evaluate founder and bottleneck potentials in populations, as differences in inbreeding coefficient distributions among herds were predicted based on herd attributes. Wild Horse Island is an example of a population expected to have evidence of inbreeding, as it was established in 1939-1947 with 8 bighorn sheep and was only augmented once in 1987 with two individuals. In general, SNP genotyping was able to detect that Wild Horse Island had greater inbreeding than a large, outbred population, such as the East GYA. Therefore, the SNP genotype work can serve to accurately identify low heterozygosity herds, and this approach could be applied to identify relative differences in inbreeding among herds suspected to have low heterozygosity. In general, it is important to recognize that the coefficient of inbreeding metric used in this pilot study can be impacted by the overall heterozygosity of the animals and markers included in the calculations. While the coefficient of inbreeding served as a helpful preliminary assessment of overall heterozygosity, we plan to use additional genomic metrics to further address this research question.

Summary

Our pilot project results suggested that our genetic predictions based on management history were accurate, in that detected genomic differences and inbreeding coefficient distributions among herds were estimated as we predicted, based on herd attributes. We determined that SNP information can be useful in identifying genetic differences among populations. This may be informative for translocation planning, as managers could compare herd genetics prior to identifying optimal source and recipient herds for augmentation. We also assessed inbreeding coefficients for each herd, which demonstrated the potential of SNP genotyping to address questions regarding heterozygosity of individual bighorn sheep and herds. Specifically, SNP genotypes can be used to evaluate founder and bottleneck potentials in populations and serve to accurately identify low heterozygosity herds. This approach could be applied to estimate differences in inbreeding among herds that are suspected to have low heterozygosity. The Ovine array detected sufficient variation in 57 analyzed samples to enable future association analyses between markers and phenotypic traits of interest. There was noticeable genetic variation among individuals within each population, which suggested that there is significant potential for future genetic research in relating individual, diverse genotypes to differing physical characteristics (phenotypes) of bighorn sheep. Since our pilot study results suggest that the Ovine array is informative for genomic analysis of bighorn sheep herds, we plan to continue further genomic research of additional herds to help inform bighorn sheep management.

6.3.2 Ancient DNA

Anthropologists working in the high alpine environments in the Greater Yellowstone Area have recovered bighorn sheep skull caps, horn cores, and bones that have been dated to pre-European settlement from receding ice patches. We developed a partnership with an anthropologist that has a number of bighorn sheep specimens that were collected on the Beartooth Plateau along the Montana-Wyoming border in the northeast GYA that have been radiocarbon dated (2200-3800 yr BP). To take advantage of the potential of these samples to better understand the genetics of contemporary bighorn sheep populations in the region we are collaborating with Dr. Beth Shapiro (Univ. Calif. Santa Cruz) and her team, who are experts in the extraction and analyses of ancient DNA. Dr. Shaprio's team successfully recovered adequate DNA from three of the ice patch specimens. Due to DNA degradation of these ancient samples, we do not plan to use the Ovine HD SNP genotyping array for ancient DNA analysis, but instead will implement mitochondrial DNA analysis for comparison with the extant bighorn sheep population occupying the Beartooth-Absaroka ranges of the GYA. We plan to compare 26 contemporary bighorn sheep mitochondrial DNA genomes from the Beartooth-Absaroka complex with the three ancient samples (Figure 21). We expect preliminary results from this effort by June 2017.

Figure 21. *An example of an ancient bighorn sheep specimen radiocarbon dated to pre-European settlement that was recovered from a receding high-elevation ice patch located on the Beartooth Plateau in the northeast GYA near the Montana-Wyoming border.*

This represents an exciting opportunity to compare the genome of the bighorn populations that existed in the GYA prior to contact with domestic sheep and their associated respiratory pathogens that were introduced to the region at the time of European settlement. We can expect the genome of the pre-settlement bighorn sheep to represent the historic condition of native bighorn sheep when their populations were both numerous and robust. The introduction of exotic respiratory pathogens into the naive GYA bighorn populations when domestic sheep were initially introduced to the region undoubtedly resulted in catastrophic mortalities and strong selection for bighorn that could mount a successful immunological defense against the

pathogens. Recent sampling of bighorn sheep populations in the region indicate that these exotic pathogens are present in nearly all population segments, suggesting that the current bighorn populations have likely been under continuous selection pressure for resilience against the exotic pathogens since they were introduced approximately 150 years ago. Current and historical population sizes, as well as past bottlenecks can be successfully detected by comparing mitochondrial DNA genomes. Thus, we expect significant differences in the genetic characteristics of pre-settlement bighorn populations of the eastern GYA and the populations that occupy the region today that should provide significant biological insight for the conservation and management of bighorn sheep.

6.3.3 Upcoming Research Efforts

In 2017 we plan to evaluate sample size required for genetic characterization of herds, explore the potential of targeted sequencing to address disease research questions, and plan additional genomic analyses. We are currently conducting an assessment to estimate the number of bighorn sheep necessary to sample from a herd to adequately characterize the genetic attributes of each herd. The literature provides little insight into this issue and while we had a target of 15 animals per herd in the pilot study, a formal evaluation of sample size requirements will aid in generating the highest quality data for the resources invested. Sample size may impact genetic inference, as genetic uniqueness, genetic distance, and inbreeding could be assessed differently, depending on the sampling scheme and the total number of bighorn sheep evaluated. Thus, we plan to determine the optimal number of animals to sample from each herd for genetic analyses. Information regarding optimal sample size would serve to maximize genetic insight for management and limit costs associated with genetic sample collection, processing, and analysis. To address this question, we will conduct a simulation of the consistency and bias of genetic metrics (herd uniqueness, genetic distance, and genetic diversity) via a simulation where we would take 1,000 random sub samples of 5, 10, 15, 20, 25, and 30 individual bighorn sheep.

We have extracted and genotyped 30 samples from two Montana herds, including the Fergus and Taylor Hilgard herds, and one Wyoming herd, the Beartooth-Absaroka complex. We expect these herds to have differing levels of genetic diversity due to their diverse herd histories, and these attributes will be quantified for the sampling simulation. The Fergus herd represents a large herd 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 successful reintroduction that has experienced both bottlenecks and augmentations. The Taylor Hilgard herd represents a native population that has experienced multiple augmentations and all-age die-offs, but has recovered to a moderate size. In addition to these Montana herds, we plan to analyze a total of 86 bighorn sheep across east Greater Yellowstone Area in Wyoming (Wyoming hunt units of 1-5 and 22). The Beartooth-Absaroka metapopulation will serve as a baseline comparison of a large, native herd with high anticipated connectivity and genetic diversity. Thus, assessment of sample size necessary for genetic characterization of these herds with differing herd histories will serve to provide valuable insight on how to optimize sampling for genetic research and management in Rocky Mountain bighorn sheep. Using this simulation, we will optimize the sample size required for genetic characterization to both minimize cost and provide reasonable confidence in genetic conclusions.

In addition to the simulation work, we plan to further develop a bioinformatics workflow and additional options for our bighorn sheep genomic research. In general, we plan to employ and

evaluate different genomic analysis techniques to assess population genetics of Montana herds and the Beartooth-Absaroka complex in Wyoming. In March 2017, graduate student Elizabeth Flesch will participate in the 6th Programming for Evolutionary Biology course in Leipzig, Germany, to gain valuable bioinformatics skills from international experts that she will apply to the bighorn sheep genomics dataset. The course will not only benefit analysis of our bighorn sheep SNP genotype dataset, but will also serve to inform planning of future research efforts. We are interested in exploring the use of next generation sequencing for certain areas of the genome, and the course modules regarding high-throughput sequencing data will allow us to consider these research options. We plan to explore the potential of targeted sequencing of the major histocompatibility complex (MHC) to address disease susceptibility questions.

Deliverables

Annual Reports

R.A. Garrott, K.M. Proffitt, J.J. Rotella, C.J. Butler. 2014, 2015. The role of disease, habitat, individual condition, and herd attributes on bighorn sheep recruitment and population dynamics. Annual Reports, Federal Aid in Wildlife Restoration Grant W-159-R1.

R. Garrott, K. Proffitt, J. Rotella, J. Berardinelli, J. Thompson, C. Butler, E. Lula, E. Flesch, R. Lambert. 2016. The role of disease, habitat, individual condition, and herd attributes on bighorn sheep recruitment and population dynamics. Annual Reports, Federal Aid in Wildlife Restoration Grant W-159-R1.

Thesis

C.J. Butler. 2017. Assessing respiratory pathogen communities and demographic performance of bighorn sheep populations: a framework to develop management strategies for respiratory disease. M.S. thesis, Montana State University, Bozeman.

Professional Presentations

C.J. Butler, R.A. Garrott, J.J. Rotella. 2014. Correlates of recruitment in Montana bighorn sheep populations. Montana Chapter of the Wildlife Society 52nd Annual Conference, Bozeman, MT.

R.A. Garrott, J.J. Rotella, K.M. Proffitt, J. Ramsey, C.J. Butler. 2014. Montana's new statewide bighorn sheep research initiative. Montana Chapter of the Wildlife Society 52nd Annual Conference, Bozeman, MT.

R.A. Garrott, J.J. Rotella, K.M. Proffitt, J. Ramsey, C.J. Butler. 2014. Montana's new statewide bighorn sheep research initiative. 19th Biennial Northern Wild Sheep and Goat Council Symposium, Fort Collins, CO.

C.J. Butler, R.A. Garrott, H. Edwards, J. Ramsey, D. McWhirter, N. Anderson. 2014. A collaborative regional initiative to correlate respiratory pathogens demographic attributes of bighorn populations. 19th Biennial Northern Wild Sheep and Goat Council Symposium, Fort Collins, CO.

C.J. Butler, R.A. Garrott, K.M. Proffitt, J.J. Rotella. 2015. One year progress report for the Montana Statewide Bighorn Sheep Research Project. Montana Chapter of the Wildlife Society 53rd Annual Conference, Helena, MT.

R.A. Garrott, C.J. Butler, J. Ramsey, K.M. Proffitt. 2015. Approaches initiated to gain insight into respiratory disease in Montana's bighorn sheep herds. Montana Chapter of the Wildlife Society 53rd Annual Conference, Helena, MT.

C.J. Butler, R.A. Garrott, J.J. Rotella, D. McWhirter, H. Edwards, P.J. White, E. Almberg, J. Ramsey, K.M. Proffitt. 2015. Northern Rockies collaborative bighorn sheep research initiative. West-wide, Adaptive Disease Management Venture Oversight Committee Meeting, Salt Lake City, UT.

C.J. Butler, and R.A. Garrott. 2016. What does it all mean? Interpreting respiratory pathogen survey results for bighorn sheep management. Montana Chapter of the Wildlife Society 54nd Annual Conference, Missoula, MT.

E.P. Flesch, J.M. Thomson, R.A. Garrott, and T.A. Graves. 2016. An initial assessment of the potential of genomic analysis to help inform bighorn sheep management. Montana Chapter of the Wildlife Society 54nd Annual Conference, Missoula, MT.

M. R. Herrygers, J.R. White, J.M. Thomson, C.J. Butler, D.E. McWhirter, W.H. Edwards, K. Monteith, R.A. Garrott, and J.G. Berardinelli. 2016. Pregnancy rates, metabolites and metabolic hormones in bighorn sheep during and after the breeding season. Montana Chapter of the Wildlife Society 54nd Annual Conference, Missoula, MT.

J.R. White, M.R. Herrygers, J.M. Thomson, V. Copie, B. Tripet, C.J. Butler, D.E. McWhirter, K. Monteith, R.A. Garrott, and J.G. Berardinelli. 2016. Developing physiological profiles using nuclear magnetic resonance spectroscopy to inform bighorn sheep (*Ovis canadensis*) management. 2016. Montana Chapter of the Wildlife Society 54nd Annual Conference, Missoula, MT.

M.R. Herrygers, J.G. Berardinelli, J.R. White, V. Copie, B. Tripet, C.J. Butler, D.E. McWhirter, W.H. Edwards, K. Monteith, and R.A. Garrott. 2016. Pregnancy rates, metabolites, metabolic hormones, and application of nuclear magnetic resonance spectroscopy of metabolic profiles for assessing physiological status in bighorn sheep (*Ovis canadensis*). 20th Biennial Northern Wild Sheep and Goat Council Symposium, Moscow, ID.

R.A. Garrott, P.J. White, D.E. McWhirter, W.H. Edwards, K. Proffitt, J. Ramsey, M. Wood, E. Almberg, and J.J. Rotella. 2016. The Montana-Wyoming collaborative bighorn sheep research program. 20th Biennial Northern Wild Sheep and Goat Council Symposium, Moscow, ID.

E.P. Flesch, J.M. Thomson, R.A. Garrott, and T.A. Graves. 2016. An initial assessment of the potential of genomic analysis to help inform bighorn sheep management. 20th Biennial Northern Wild Sheep and Goat Council Symposium, Moscow, ID.

C.J. Butler, and R.A. Garrott. 2016. What Does It All Mean? Interpreting respiratory pathogen survey results for bighorn sheep management. 20th Biennial Northern Wild Sheep and Goat Council Symposium, Moscow, ID.

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