THESIS

GENETIC NATURAL RESISTANCE TO BRUCELLOSIS IN YELLOWSTONE NATIONAL PARK BISON (BISON BISON): A PRELIMINARY ASSESSMENT

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ABSTRACT

GENETIC NATURAL RESISTANCE TO BRUCELLOSIS IN
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As a nationally recognized symbol of perseverance and survival, the American bison (*Bison bison*) continues to be the focus of many conservation efforts. This is particularly true in Yellowstone National Park (YNP) where genetic diversity and population dynamics are threatened by the presence of *Brucella abortus* infection, the causative agent of brucellosis. To further complicate management efforts, the potential for transmission of *B. abortus* to livestock surrounding the park could have effects on human and animal health in addition to negative economic impacts.

Genetic natural resistance to infectious diseases such as brucellosis has been examined in bison and other species. Consideration of using genetic natural resistance as a management tool within YNP relies on its applicability to the population and effectiveness in identifying susceptible or resistant animals. The aim of this study was to further investigate genetic natural resistance to brucellosis in bison using the prion protein gene (PRNP). This could provide a genetic management tool for future populations by identifying resistant and susceptible genotypes to brucellosis that governing agencies could screen when selecting animals for removal.

Animals used in this study were part of the Bison Quarantine Feasibility Study (BQFS), which was conducted by state and federal government agencies to explore alternative options for bison that leave the protection of the park's perimeters as part of the Interagency Bison

Management Plan (IBMP). After completing the study, the bison involved will be released into areas of their historic range and managed as a conservation herd. In addition to investigating genetic natural resistance, other genetic aspects were analyzed in this quarantine herd.

We evaluated genetic diversity at 42 microsatellite loci representing each of the nuclear chromosomes in the bison genome. These markers allowed us to determine parentage which helped quantify the genetic diversity contained in the conservation herd. It was also important to understand the genetic diversity of the herd and to determine if any genetic characteristics such as cattle DNA introgression or low genetic diversity may threaten the protected status of this herd. Previously developed mitochondrial and nuclear DNA microsatellite markers were applied to determine if any introgression of domestic cattle DNA is present.

PRNP examination provided conflicting results from previous bison PRNP studies, justifying further research into the importance of PRNP in genetic natural resistance. As a conservation herd, this group of bison demonstrates high retention of unique YNP genetic diversity, high percentage of adult animals contributing offspring, and no evidence of cattle DNA introgression. This study explains support for using YNP bison as stocking animals for future satellite herds under the condition that BQFS protocol is followed. Results of these genetic analyses provide a thorough description of a quarantined herd of YNP bison destined for release to brucellosis-free public lands.

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DEDICATION

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CHAPTER I

INTRODUCTION

I. Background

Founded in 1872, Yellowstone National Park (YNP) was established as the first national park in the world (Haines 1977). A variety of wild fauna reside in Yellowstone National Park, including elk, bighorn sheep, grizzly bears, and, perhaps most notably, bison (*Bison bison*). The YNP herd is the oldest and largest federal bison herd in the United States and, as such, it receives both national and international public attention. This free-ranging herd is the only herd in the U.S. and one of two in North America that have descended from a continuously free-ranging wild herd (Halbert 2003). Extensive population management has contributed to a successful recovery, but also has resulted in artificial hybridization with cattle, domestication, and unnaturally isolated populations across North America. Despite these unforeseen genetic consequences, preserving the integrity of the bison genome is crucial to conservation of the species.

The YNP bison herd represents a germplasm, a collection of genetic precursors and resources for an organism, that maintains some portion of genetic diversity remaining from pre-settlement herds. It is also one of the only herds in North America that are not yet known to be affected by cattle DNA introgression where domestic cattle genes are integrated into the bison genome. Therefore, this herd has been targeted as the best source for developing satellite herds, i.e., herds that originate from parent herds that are managed outside of YNP to conserve the genetic integrity of a species compared to herds managed for production purposes. The greatest

concern with using bison from YNP for satellite herds, however, is their exposure to *Brucella abortus* and potential to spread the pathogen outside the park to domestic livestock.

Brucella abortus is the causative bacterial agent for bovine brucellosis, an infectious disease that can infect several species, including cattle and humans. Due to intense eradication efforts in the U.S., brucellosis has been eradicated from the rest of the country except in YNP. Yellowstone National Park bison that migrate out of the park in search of food may interact with livestock around the boundaries of parks and wildlife reserves. Cattle owners in Montana, Wyoming, and Idaho have heightened concern for *B. abortus* transmission to cattle when bison leave the borders of the park (Meyer and Meagher 1995).

Management plans for bison leaving the park have been controversial and debated for years because culling is a critical component of these plans. Culling has been considered the most cost-effective method to control bison interactions outside YNP as compared to other methods such as vaccination or test-and-slaughter methods. To evaluate another potential control measure, a cooperative study among several state and federal agencies was initiated in 2005 as part of the Interagency Bison Management Plan (IBMP; Clarke *et al.* 2005). The Bison Quarantine Feasibility Study (BQFS) aims to ascertain the efficacy of a program quarantining and testing YNP bison that leave the park in order to generate a herd that qualifies as free of brucellosis. This herd can be used to populate areas where bison have been extirpated.

Bison populations have been recovering from near extinction since the late 19th century. Conserving this herd is important to protecting the remaining genetic diversity of North American bison populations. Special precaution should be considered with satellite herds formed from YNP due to the potential for *B. abortus* transmission and developing brucellosis while also conserving maximum genetic diversity. The following sections discuss the major genetic and

disease issues that should be considered when developing a satellite herd formed from YNP animals.

II. Brucellosis

Bovine brucellosis is primarily a reproductive infectious disease caused by the bacteria *Brucella abortus*. This chronic, untreatable disease affects both livestock and wildlife species and is easily transmitted between and among mammalian species, including humans. Bovine brucellosis has spread to both domestic and non-domestic animals in the last century from cattle originating in Europe and western Asia (Meagher and Meyer 1994). Cattle, sheep, goats, pigs, elk, bison, and buffalo are all at high risk for developing this disease through infection with various species of *Brucella*.

Bovine brucellosis is manifested by abortions, birth of weak or nonviable offspring, and lower milk production in female bison and cattle. Abortions in YNP bison can occur into the second trimester (about mid-December) to the end of gestation (between mid-April and mid-June; Meyer and Meagher 1995). Brucellosis can potentially cause infertility in males and females in bison and cattle. It is transmitted through oral contact with aborted tissues (i.e., fetus, placenta, or discharges), blood, infected milk, feces, and contaminated environments (Peterson *et al.* 1991; Williams *et al.* 1993; Meyer and Meagher 1995; Cheville *et al.* 1998; Rhyan *et al.* 2009). Infected females can continue to shed the bacteria in their milk for months after infection and in subsequent pregnancies. Antibodies are also passed vertically to nursing calves until about five months of age, but maternal antibodies are not enough to protect against brucellosis in young animals (Dobson and Meagher 1996; Rhyan *et al.* 2009). Scavengers may be able to spread the pathogen by carrying aborted fetuses over long distances. Infected fetuses can harbor

the bacteria for up to 100 days after being aborted, though direct sunlight can shorten that period to between 20 and 30 days (Clarke *et al.* 2005). That the bacteria can survive in the environment and within an infected animal for long periods makes it difficult to prevent *B. abortus* transmission.

Brucellosis poses health and economic problems throughout the world. Human cases of brucellosis have been attributed to contact with infected wildlife (Davis 1990). However, the greater concern is humans contracting infection through *B. abortus* transmission and developing brucellosis from cattle that have been infected through contact with infected wildlife (Cheville *et al.* 1998). Annual losses from brucellosis in livestock and dairy industries in the United States peaked in 1952 totaling $400 million (U.S. Department of Agriculture 2011). Today in the U.S., the economic impacts of *Brucella abortus* infection have dropped to less than $1 million annually due to the efforts of the National Brucellosis Program that has brought brucellosis under control and virtually eliminated the infection in domestic cattle. Since 1934, over $3.5 billion have been spent by federal, state, and private organizations to promote vaccination against brucellosis and attempted eradication of the disease in domestic livestock (Cheville *et al.* 1998). Almost every U.S. state has been declared bovine brucellosis free, with the exception of the remaining reservoir of infected animals found in the Greater Yellowstone Area (GYA; Roffe *et al.* 1999a) and some sporadic occurrences. Cattle herds declared brucellosis free are allowed to participate in interstate movement which provides significant economic benefits to a state's cattle industry (Genho 1990; Kilpatrick *et al.* 2009). Therefore, considerable economic loss results when an animal within a cattle herd is found seropositive, as the entire herd can be potentially quarantined and a state's brucellosis status may be reclassified (Keiter 1997).

Bison were the first recorded wildlife species in the world to contract brucellosis (Davis 1990). Mohler discovered the first serological evidence in YNP bison in 1917 (Mohler 1917; Peterson *et al.* 1991; Dobson and Meager 1996; Cheville *et al.* 1998; Rhyan *et al.* 2009). Bison calves raised on *B. abortus* infected domestic cows are the probable source of brucellosis into YNP (Meagher and Meyer 1994). Positive agglutination reactions from sera of two Yellowstone cows that had aborted were recorded along with one cow that had not aborted and tested negative (Meyer and Meagher, 1995). The bacterium was not isolated until 1930, when it was discovered in the testicle of an infected male bison from the National Bison Range, Moiese, Montana (Creech 1930).

Because bison are gregarious animals and constantly moving in nomadic fashion and melding with other bison herds (Meagher and Meyer 1994), animals infected with *B. abortus* can easily spread the pathogen. They can also contract the infection from other species harboring the pathogen (Davis 1990). Some herds of bison and elk will migrate to human-assisted feeding grounds in the National Elk Refuge near Jackson, Wyoming (Dobson and Meagher 1996). This situation creates increased potential for elk and bison to come into contact with cattle and each other.

It appears that brucellosis virulence differs between host species and is dependent on intensity of exposure, but there is little evidence that the disease increases mortality in bison or elk (Dobson and Meagher 1996). The main area of concern with brucellosis in bison involves interspecific transmission to cattle and elk and the economic impacts of *B. abortus* transmission. Though no recent infection between domestic cattle and bison has occurred, the concern of possible *B. abortus* transmission to domestic cattle and their developing brucellosis remains very high (Meyer and Meagher 1995; Kilpatrick *et al.* 2009).

Females of both species that are pregnant when exposed to *B. abortus* and seroconvert tend to show increased effects of the disease, including abortions and reduced birth rates (Fuller *et al.* 2007). During the second pregnancy after infection, the rate of abortion drops considerably (Peterson *et al.* 1991). Subsequent pregnancies tend to be normal as naturally acquired resistance to the bacteria develops. Infected seropositive cows likely remain seropositive and infected for a prolonged time. Antibody has not been shown to be protective as bison have the potential to seroconvert at any age, regardless of antibody level (Roffe *et al.* 1999a; Rhyan *et al.* 2009).

Although animals are able to clear infection by *B. abortus*, bacteria may lie dormant inside the host's body for years. Titers in YNP bison remain high for *Brucella* spp. antibodies, yet few animals show distinguishable clinical signs (Meyer and Meagher 1995). Young bison could pose a transmission risk if they are harboring a latent infection from an exposure as calves. The disease then manifests at sexual maturity when the animals experience classic signs of brucellosis, including abortions, shedding organisms in body fluids, or developing antibodies to *B. abortus* (Rhyan *et al.* 2009).

It is estimated that approximately 10% of the Yellowstone National Park's bison herd are actually infected with *B. abortus*, while 30-50% of bison test seropositive (Meagher and Meyer 1994; Cheville *et al.* 1998; Rhyan *et al.* 2009). Bacterial culture tests provide the 10% estimate, suggesting that brucellosis prevalence based on sero-reactors demonstrate a considerable overestimate of the true level of infection (Dobson and Meagher 1996). Serological blood tests detect the presence of antibodies as a result of *Brucella* spp. infections such as with *B. abortus* and classify an animal as seropositive or seronegative for *Brucella* spp. Seropositive animals have been exposed to the bacteria via infection or vaccination, but may not be infectious. High antibody levels cannot be considered indicators of resistance to *B. abortus* infection, only

exposure to the bacteria (Roffe *et al.* 1999b). However, seronegative results do not confirm the absence of infection, because some of these animals in chronically infected herds are carrying live *B. abortus* but have not begun to show clinical signs of the disease (Cheville *et al.* 1998). Serological tests vary in specificity and sensitivity which should be considered in classification of infected animals. The tests do not indicate whether the potential hosts actively carry the pathogen (Dobson and Meagher 1996, Berger and Cain 1999).

Bacterial cultures are used to determine the presence of bacteria in tissue samples. Cultures of the bacteria will indicate an infection but cannot distinguish the level of infection. If bacteria are missing from the clinical sample or the sample contains low levels of the bacteria, a false-negative culture outcome could result. Simulations of *B. abortus* infection in YNP have shown that the threshold host population for the bacteria to establish itself is low, between 200-300 animals (Dobson and Meagher 1996). Sustained infection requires the population to be over this threshold population. This low number of host animals suggests that to eradicate the pathogen and subsequent disease from YNP, detrimental population reductions would need to be made to the YNP bison herd.

Host responses to infectious diseases are variable and complex. Bison and cattle demonstrate biological differences in immunity such as antibody production, lymphocyte performance, and macrophage or susceptibility genes (Cheville *et al.* 1998). Though the two species are genetically similar, their immunologic responses to the same pathogen are remarkably different. For instance, bison reactions to bovine brucellosis do not mimic that of cattle. Vaccines for bovine brucellosis that has been developed in cattle show varying degrees of success when administered to bison. For instance, the strain 19 vaccine (S19) which is developed using the smooth phenotype of *B. abortus*, is not as effective in bison as cattle when used as a

calfhood vaccine and may be pathogenic, causing a high percentage of bison calves to become infected (Roffe *et al.* 1999a; Meyer and Meagher 1995; Olsen *et al.* 2003). In addition, serology tests cannot distinguish between bison vaccinated with S19 and bison infected naturally with *B. abortus* which could pose problems for serology surveys post-vaccination. There is further evidence that this vaccine may be pathogenic, whereby inducing abortion in adult pregnant bison at a significantly higher rate than in pregnant cattle (Meyer and Meagher 1995; Roffe *et al.* 1999a; Olsen *et al.* 2003). In contrast, the live *B. abortus* strain RB51 vaccine stimulates antibody production that can be distinguished from natural infection due to its use of the rough strains of *Brucella spp.*, though it may induce abortions in pregnant bison when vaccinated mid-gestation (Treanor *et al.* 2010). Early gestation vaccination limits the abortive risk when using RB51, but the efficacy of repeated vaccinations to bison is undetermined.

Differences in immune response and disease susceptibility between bison and cattle may be explained by external factors such as environment and management practices, or by internal factors such as nutrition and genetics. Exploring the role of genetics in disease susceptibility and immune response in various species is of continued interest in livestock and wildlife management.

III. Bison genetics

Phylogeny

The Bovidae (Order: Artiodactyla) includes several subfamilies including various antelope, cattle, goat, sheep, and bison species in the world. The number of subfamilies has been debated for years, with the most recent estimate being nine subfamilies (Grubb 2001). The American and European bison (genus *Bison*) and their closest relatives, domestic cattle (*Bos*) and

water buffalo (*Bubalus*), reside within one of the three major clades in the subfamily Bovinae (Ritz *et al.* 2000; Hassanin and Ropiquet 2004; Fernández and Vrba 2005). The *Bison* genus most recently diverged from and is most closely related to *Bos*. This split occurred around 1 million years ago (Loftus *et al.* 1994), about 3 million years after the *Bos*-*Bubalus* divergence (Ritz *et al.* 2000). Such a recent divergence between *Bison* and *Bos* genera may explain the hybridization capability between both bison species with domestic cattle and other members of the genus *Bos* (see below; Boyd 1914; Goodnight 1914; Meagher 1986).

Mitochondrial DNA (mtDNA) is maternally inherited in vertebrates and is used to determine taxonomic relationships and differences among populations within species (Frankham *et al.* 2002). Mitochondrial DNA analyses found that the genera *Bison* and *Bos* are more closely related to each other than either group is to *Bubalus* (Miyamoto *et al.* 1989; Hassanin and Ropiquet 2004). Further phylogenetic analyses were completed using microsatellite markers, which are variable numbered short tandem repeats across the nuclear genome and are used to measure genetic diversity and relatedness within and among populations (Blouin *et al*. 1996; Ritz *et al*. 2000). These analyses found *Bubalus* to be the most divergent lineage in the bovine cladogram (Ritz e*t al.* 2000). However, studies using microsatellites did not sufficiently resolve whether *Bison* were more closely related to *Bos* or *Bubalus*.

The phylogenetic relationship determined by mitochondrial DNA and nuclear DNA analyses is supported when considering the 3' untranslated region (UTR) sequences of the natural resistance associated macrophage protein gene (*NRAMP1*) which has been studied extensively in various species as a predictor of natural resistance to bacterial diseases (see *Genetic Natural Resistance* subsection). Bison and cattle are seen to have more shared monomorphic sites (presence of only one allele at a locus; compared to a polymorphic site which

has multiple alleles at one site) and similar number of $(GT)_n$ insertions than between either cattle and water buffalo or bison and water buffalo (Hořín *et al.* 1999). Further comparisons of the *NRAMP1* gene demonstrate that bison have 99.1% sequence identity with the same gene in cattle (Ables *et al.* 2002). Based on this similarity, markers from the cattle genome have been used to map polymorphisms in the bison genome due to their homology and close evolutionary relationships (Mommens *et al.* 1998; Schnabel *et al.* 2000; Halbert and Derr 2008).

Genetic diversity and parentage analysis

The American bison is a prime example of conservation success in North America. Until the 1870's, tens of millions of bison roamed the Great Plains (Freese *et al.* 2007; Halbert 2003; Hedrick 2009). This population was decimated to near extinction by the mid-1880s through massive slaughters aimed at collecting hides and meat. Additionally, widespread habitat loss and expanding human activities such as agricultural development (i.e., domestic animal grazing and farming) have forced bison into much smaller ranges. Bison populations have been reduced as habitat has been diminished to geographically isolated areas within national parks, reserves, other public lands, and private ranches (Meagher 1986; Sanderson *et al.* 2008). Since the early 1900's, with protective management from U.S. and Canadian governments along with private ranchers and conservationists, bison numbers have rebounded to more than 500,000 bison at present (Boyd 2003; Halbert 2003). However, this recovery may mask consequences of a demographic bottleneck (i.e., rapid reduction of population size) that may have had negative impacts on the genetic diversity and germplasm of this species.

Genetic diversity, often described as heterozygosity, is necessary for species to combat environmental change and increase evolutionary potential (Nei *et al.* 1975; Franklin 1980; Lacy

1987; Wilson and Strobeck 1999; Frankham *et al.* 2002). Reduction in genetic diversity can result in genetic drift, inbreeding, loss of fitness, increased mortality or susceptibility to disease, or reduced ability of individuals within a population to adapt to stochastic events (Nei *et al*. 1975; Lacy 1987; Frankham *et al.* 2002; Primack 2004; Templeton 2006). The effects of genetic drift, i.e., the random process of allelic frequencies changing from one generation to the next, are amplified in small populations. Smaller populations are more susceptible to genetic drift than larger populations due to the decreased number of individuals in the population and therefore decreased allelic variation to pass on to offspring (Nei *et al.* 1975; Franklin 1980; Lacy 1987; Wilson and Strobeck 1999; Frankham *et al.* 2002). In addition, low frequency or rare alleles, including detrimental or beneficial ones, have a higher chance of being lost or becoming fixed due to chance in small populations with each subsequent generation, thus reducing genetic diversity and leading to population differentiation.

When populations are drastically reduced, as has happened with Yellowstone bison, it can result in a genetic bottleneck which causes the loss of alleles (especially rare alleles), reduced genetic diversity, and random changes in allele frequencies due to the effects of genetic drift (Nei *et al*. 1975; Lacy 1987; Wilson and Strobeck 1999; Frankham *et al.* 2002; Primack 2004; Templeton 2006). The consequences of a genetic bottleneck depend on how rapidly the population declines, the change in population size, the length of the bottleneck, and the rate of population growth following the bottleneck event (Nei *et al.* 1975). Extensive genetic analyses of the American bison have shown a remarkable conservation of genetic diversity despite the severe population decline over a century ago (Mommens *et al.* 1998; Wilson and Strobeck 1999; Boyd 2003; Halbert 2003; Halbert and Derr 2008). Stringent monitoring of bison populations and translocation of animals to maintain overall genetic diversity have enabled the preservation of

remaining bison genetic diversity. This has not been without consequences, which will be examined in the next section.

To monitor the effects of genetic bottlenecks, genetic drift, inbreeding depression, and other factors affecting genetic diversity, the use of microsatellite DNA has become a powerful tool. Microsatellite DNA has been utilized to determine levels of heterozygosity in populations as well as determine parentage (Blouin *et al*. 1996; Mommens *et al.* 1998; Schnabel *et al.* 2000; Halbert and Derr 2008). They can be effective measures of genetic diversity in a satellite herd or small population where effects of genetic drift are heightened. Inbreeding depression, caused by inbreeding and subsequent decreased reproduction and survival within the population, is an effect of genetic drift that is difficult to prevent in small populations (Frankham *et al.* 2002; Templeton 2006), but can be monitored using such techniques as microsatellite markers.

Parentage analysis has been used in conservation or captive-bred populations to minimize inbreeding effects and determine the effective size of populations (Blouin *et al*. 1996; Frankham *et al.* 2002). Identifying paternal and maternal genetic contributions in the population can be pertinent to determining mating systems and breeding behaviors that may influence which animals are allowed or able to breed and contribute to future generations. In small populations where the effects of inbreeding depression are amplified, it is not beneficial to have multiple offspring from a few sires because inbreeding and loss of genetic diversity may be expedited. In a satellite herd, it may be advantageous to determine if multiple males are siring offspring so that genetic diversity can be maximized in the offspring. Also, parentage analysis could aid in decisions to translocate animals to the herd if only certain lineages are being represented and more genetically diverse animals need to be introduced to supplement the herd.

Creating a microsatellite DNA inventory of the founder population's allelic diversity could guide future management and conservation strategies. For instance, by using genetic considerations when choosing animals to translocate from the founding population to a satellite herd, management teams could increase the chances of retaining genetic diversity, minimizing inbreeding, and maximizing genetic variability in future generations by introducing animals with rare or polymorphic alleles. Preserving as much genetic variation as possible within satellite herds will prove vital to the preservation of the bison germplasm.

Several genetic analyses of the bison germplasm have been completed in recent years. Domestic cattle microsatellite, nuclear, and mitochondrial DNA markers have been proven effective measures of bison genetic diversity (Mommens *et al.* 1998; Ward *et al.* 1999; Ritz *et al.* 2000; Schnabel *et al.* 2000). These markers have demonstrated that herds differ in genetic diversity due to differences in founder population size, source of translocated animals, relative genetic contribution of founding individuals, difference in culling strategies, and effective population sizes (Wilson and Strobeck 1998; Primack 2004; Halbert and Derr 2008). Varying genetic diversity between bison herds in North America is important to consider when choosing source populations for satellite herds. Analyses on YNP bison have substantiated its higher genetic variation and lacking evidence of cattle DNA introgression (Halbert 2003; Halbert *et al.* 2005; Freese *et al.* 2007; Halbert and Derr 2007). Further considerations in bison conservation genetics concern the amount of cattle DNA introgression present in populations that may be source herds, which may have damaging effects on conserving this species in the future.

Cattle DNA introgression

Another conservation issue in bison genetics concerns the hybridization of bison with domestic cattle. Hybridizations generally occur through purposeful introduction of two species by humans or through habitat modification so the species are in contact (Rhymer and Simberloff 1996; Ward *et al.* 1999). Historically, the practice of crossing bison and cattle had been used to improve the genetic traits of domesticated livestock (Boyd 1914; Goodnight 1914; Ward *et al.* 1999; Freese *et al*. 2007). However, within a satellite herd, genetic consequences of hybrid animals need to be considered to preserve species integrity as well as genetic diversity.

Interspecific hybridization has been a consequence of the fragmentation of the bison's habitat, attempts of domesticating bison, and indiscriminate crossbreeding with domestic cattle to improve domestic stocks (Polziehn *et al.* 1995; Ward *et al.* 1999, Freese *et al.* 2007). Anthropogenic hybridization between bison and cattle began in the late 1890's when private ranchers experimented with hybrids in order to improve their cattle stock with commercially favorable traits found in bison (Jones 1907; Boyd 1914; Goodnight 1914). Livestock production has benefited from the genetic similarities between bison and cattle that allow them to crossbreed. Cattalo, the offspring between the two species, are noted for increased meat production, leaner meat product, increased growth rates, and higher tolerance of extreme temperatures (Jones 1907; Boyd 1914; Goodnight 1914; Dary 1974).

Hybridization between these two species does not occur naturally and crossing the species has proved difficult. Successful crosses have only occurred between bison bulls and domestic cows. If they survived to breeding age, the resulting hybrid F_1 offspring, mostly female, were able to backcross to bison males. Few male hybrid offspring survive to adulthood and were usually sterile due to a morphological and structural difference in the sex Y chromosome of the

offspring (Steklenev and Yasinetskaya 1982; Hedrick 2009). In the past century, these human-controlled crossings of North American bison with domestic cattle have resulted in the introgression of domestic cattle genes into several bison populations, some of which have contributed to the founder stock of some existing bison herds.

Domestic cattle DNA has been identified in the wild bison herds (i.e., introgression) through analysis of both mitochondrial (Polziehn *et al.* 1995; Ward *et al.* 1999) and nuclear DNA markers (Halbert *et al.* 2005). Due to their clonal pattern of inheritance (i.e., clonal inheritance of mtDNA from mothers to daughters without recombination), mitochondrial markers in some cases have been effectively used to differentiate between closely related species and identify interspecific hybridization (Ward *et al.* 1999). Four distinct cattle mtDNA haplotypes have been identified in several bison herds of North America (Ward *et al.* 1999).

Though mtDNA markers can be a powerful tool in identifying cattle DNA introgression, it is possible that a herd with known hybridization would contain no mtDNA evidence of the introgression due to the mitochondrial genome only being inherited through the dam (Halbert *et al.* 2005). Thus, nuclear introgression using microsatellite markers was examined in addition to mtDNA markers to ensure identification of domestic cattle DNA introgression (Halbert *et al.* 2005, Halbert and Derr 2007). With a relatively high mutation rate, microsatellite markers are able to identify any cattle DNA introgression that may have occurred in recent history (Ellegren 2000; Halbert *et al.* 2005). Nuclear introgression of domestic cattle DNA was not identified in YNP samples in previous studies (Halbert *et al.* 2005; Halbert and Derr 2007), further supporting the absence of cattle gene introgression in the YNP herd and the inclusion of this herd in stocking satellite herds.

Examination of cattle DNA introgression has shown that at least five public bison populations in North America currently have no evidence of either mitochondrial or nuclear domestic cattle introgression. Non-introgressed herds include two herds that have descended from a constant wild herd, Wood Buffalo National Park in Canada and Yellowstone National Park in the United States (Halbert 2003). It is estimated that out of 500,000 plains bison in the U.S. and Canada, less than 1.5% are likely free of domestic cattle gene introgression (Freese *et al.* 2007). With high levels of introgression, conserving any populations that are genetically pure wild bison is a high priority. These herds are important sources of "pure" bison germplasm that are invaluable to the conservation of the species.

Identifying individuals or populations with hybrid ancestry is important to prevent the loss of genetic integrity and bison genetic variation when establishing a satellite herd. The consequences in having hybrid animals in a satellite herd may result in legal changes to their protective status (O'Brien and Mayr 1991; Hill 1993). It also can lead to different management strategies when dealing with these hybridized populations. Halbert (2003) suggested that since the YNP population is one that contains both high levels of genetic variation and no evidence of domestic cattle introgression, it should be used to stock satellite herds.

Genetic natural resistance

Natural resistance associated macrophage protein (*NRAMP1*)

Genetically controlled disease resistance is a polygenic trait as found by studies of resistance/susceptibility in mice; however, single genes have been observed to have a major effect on immune mediated resistance in mice in a wide range of infectious diseases (Schurr *et al.* 1990; Templeton and Adams 1990; Beckers *et al.* 1995; Vidal *et al.* 1995). Specifically,

NRAMP1 has been studied extensively (Templeton and Adams 1990; Malo *et al.* 1994; Vidal *et al.* 1995; Feng *et al.* 1996; Templeton *et al.* 1996; Hořín *et al.* 1999; Barthel *et al.* 2001; Ables *et al.* 2002; Coussens *et al.* 2004; Caparelli *et al.* 2006 & 2007; Paixao *et al.* 2007; Ganguly *et al.* 2008). Polymorphisms within the *NRAMP1* gene were first researched in mice and have been associated with resistance or susceptibility to various infectious diseases, including several *Mycobacterium* species, *Salmonella typhimurium*, and *Leishmania donovani* (Templeton and Adams 1990; Malo *et al.* 1994; Vidal *et al.* 1995; Feng *et al.* 1996; Coussens *et al.* 2001). Humans have shown a strong correlation between *NRAMP1* polymorphisms in both coding and non-coding regions with susceptibility or resistance to tuberculosis (Hořín *et al.* 1999; Coussens *et al.* 2001).

This gene induces an immunological response using antimicrobial activity of macrophages against intracellular parasites during early infection (Feng *et al.* 1996). Homologs of the murine *NRAMP1* gene have been found in a number of mammalian species including cattle, bison, elk, red deer, water buffalo, sheep, goats, and swine (Templeton *et al.* 1996). Mammalian *NRAMP 1* genes are generally expressed in phagocytic cells like macrophages or leukocytes.

Early research on *NRAMP1* gene in cattle suggested that a polymorphic microsatellite located in the 3' untranslated region (3' UTR) was strongly associated with natural resistance to brucellosis and determined the resistant or susceptible phenotype (Hořín *et al.* 1999; Barthel *et al.* 2001; Coussens *et al.* 2001). The length of a repeat at this site, $(GT)_n$ where n is 13, 14, 15, or 16, determined susceptibility or resistance. Specifically, the genotypes of homozygous $(GT)_{13}$ allele and heterozygous $(GT)_{13}$ genotype demonstrate greater resistance as compared with allelic combinations with other $(GT)_{n\neq13}$ sequences. Recently, however, Paixão *et al.* used a live study

with experimentally infected pregnant and nonpregnant cattle to show that no apparent linkage was observed between polymorphisms in the 3' UTR region and resistance to *B. abortus* infection and developing brucellosis (2007).

Other studies have attempted to determine if there are other possible portions of the bovine *NRAMP1* gene that confer natural resistance (Adams *et al.* 1996; Hořín *et al.* 1999; Coussens *et al.* 2001; 2004). A particularly interesting polymorphism was noted by Coussens *et al.* (2004) within intron X of the bovine *NRAMP1* gene. Comparing intron X sequences of different individual cattle revealed the insertion of 3 "G" nucleotides at positions 37, 40, and 98. Animals showed both homozygosity and heterozygosity for this locus. However, association with resistance has not been confirmed by recent unpublished studies.

Natural resistance to brucellosis is not only an unconfirmed possibility in the cattle world, but also within herds of water buffalo (*Bubalus bubalis*). However, more convincing evidence for natural resistance to this disease is found in water buffalo than cattle. Using the $(GT)_n$ nucleotide insertions in the 3' UTR region of the *NRAMP1* gene in water buffalo, analogous to that in cattle, researchers have been able to confirm correlations to resistant and susceptible phenotypes (Borriello *et al.* 2006; Capparelli *et al.* 2006; 2007). To date, no such correlation between natural resistance to brucellosis and polymorphisms within the *NRAMP1* gene has been examined in bison.

Prion protein gene

Investigating natural resistance using the prion protein gene *(*PRNP*)* is a novel approach to identifying apparent resistance or susceptible genotypes to various bacterial diseases such as brucellosis (Schläpfer *et al.* 1999; Hills *et al.* 2001; Seabury *et al.* 2004). The PRNP encodes the

prion protein (PrP), which is more commonly identified by its role in the pathology of neurodegenerative diseases such as transmissible spongiform encephalopathies (TSE) or prion diseases (Prusiner 1998; Goldmann 2008). These diseases include bovine spongiform encephalopathy in cattle, Creutzfeldt-Jakob disease in humans, scrapie in sheep and goats, and chronic wasting disease in deer and elk. Variants in the PRNP have been associated with the onset or timing of diseases in various animals, most notably in TSE (Goldmann 1992; Prusiner 1998).

The normal or cellular form of the prion protein (PrP^{C}) is expressed in certain parts of the body, including the central nervous system, lymphatic tissue, and at neuromuscular junctions as a glycosylated cell-surface protein held *in situ* by a glycolipid anchor (Schläpfer *et al.* 1999; van Rheede *et al.* 2003). Prion diseases are expressed when the PrP^{C} in the host is misfolded and converted to the diseased form (PrP^{Sc}) during the posttranslational process. The conformation of PRNP will change depending on which isoform (PrP^{C} or PrP^{Sc}) is present (Prusiner 1998; Jackson and Clarke 2000). In addition to PRNP conformation, polymorphisms within the PRNP may be responsible for differences in incubation time or susceptibility to prion diseases (Seabury and Derr 2003; Sander *et al.* 2005; Saunders *et al.* 2007).

In species other than cattle, it has been shown that expression of TSE is dependent on polymorphisms in PRNP (Schläpfer *et al.* 1999; Goldmann 2008). The polymorphisms are termed non-synonymous because their base substitutions cause amino acid replacements that could potentially change the shape and function of a particular protein (Frankham *et al.* 2002). Such amino acid replacements encoded by the PRNP have been associated with expression of transmissible and hereditary spongiform encephalopathies in mammalian species (Schläpfer *et al.* 1999; Hills *et al.* 2001; Seabury *et al.* 2004).

Susceptible phenotypes and genotypes to bacterial diseases may also be dependent on PRNP polymorphisms. As indicated in mice, the host cellular prion protein may be a cell surface receptor or aid transport of *B. abortus* (Schläpfer *et al.* 1999; Hills *et al.* 2001; Seabury *et al.* 2004). Successfully identifying these polymorphisms could potentiate control and eradication efforts of certain diseases.

Single nucleotide polymorphisms (SNPs) are abundant in mammalian genomes and useful for many purposes including identity of portions of the genome associated with response to infection. SNPs are nucleotide sites that are polymorphic, alternative bases at the same site, and may include a change in nucleotide or insertion or deletion of a single base nucleotide. Most SNPs are the result of rare mutation events and are assumed to occur randomly. The mutations arise from DNA replication errors or through repair of DNA damage due to environmental mutagens (Whitaker and Banfield 2005). These mutations may or may not be expressed in the phenotype. However, in structural genes such as PRNP, these polymorphisms can have a dramatic effect on the biology of the whole organism.

One particular SNP has demonstrated evidence of determining susceptibility or resistance to brucellosis in bison. In this species, PRNP exon 3 alleles were found to possess six octapeptide nucleotide repeats and were identical in sequence with exception of one non-synonymous SNP at nucleotide position 50 (T50C; Seabury *et al.* 2005). This predicted polymorphism caused the amino acid methionine to be converted to threonine. Seabury *et al.* (2005) noted the genotype (C/C) resulting in this amino acid substitution was significantly correlated with the *Brucella* spp. seropositive animals collected from YNP and suggested that this non-synonymous substitution may have functional implications in PrP^C biogenesis or entry of *Brucella* spp. into bison host cells based on susceptible or resistant genotypes. Examining

other bison herds that were not exposed to *B. abortus* supported this theory as the T allele was significantly more abundant in these herds and thus considered the resistant allele. Utilizing this polymorphism to screen *Brucella* spp. exposed bison for resistant genotypes could aid management decisions in bison herds that are chronically infected with *B. abortus*.

IV. Yellowstone National Park

Management and control of brucellosis infected bison

Managing wild populations and their diseases within the boundaries of national parks and other protected areas is challenging. Brucellosis is a prime example. Even though host organisms such as bison or elk are generally restricted to YNP and the surrounding GYA by anthropogenic regulations, *B. abortus* bacterial agents are not limited by those boundaries. In some instances, such as brucellosis, human intervention may be justifiable in order to protect native populations, domestic animals, and humans from becoming infected with the pathogen. Significant management concerns are stimulated by some diseases in wildlife due to increasing interactions between wildlife and domestic animals (Aguirre and Starkey 1994).

Bison roam in large herds searching for food and water. In years when grass and forage are abundant, herds will generally stay within the boundaries of YNP. Since the early 1980's, the population of bison in Yellowstone has fluctuated between 2000 to over 4000 animals depending on the season, winter severity, and management actions (Clarke *et al.* 2005; Fuller 2006) without any subsidizing from outside herds.

Bison will spend most of the year at higher altitudes within the park but may graze at lower altitudes outside the park when grass is in high demand during the winter (Kilpatrick *et al.* 2009). An agreement, the Interagency Bison Management Plan (IBMP), was made between the

Montana Department of Livestock, Montana Department of Fish, Wildlife, and Parks, U.S. Department of the Interior, National Park Service, USDA Forest Service, and USDA Animal and Plant Health Inspection Service concerning the management of YNP bison as they cross park boundaries (Clark *et al.* 2005). Under the IBMP, state and federal agencies work to prevent interspecific transmission of brucellosis to domestic cattle through various management methods including: hazing, shooting, or capturing bison; or applying test-and-slaughter methods to the *B. abortus*-infected bison that migrate beyond the park's boundaries (Morris and McBeth 2003; Clarke *et al.* 2005). These efforts have been implemented to control the spread of brucellosis from within YNP borders.

When determining animals to be removed, the animals are selected based on consensus results of several serology tests determined by Uniform Methods and Rules (UM&R) for Brucellosis Eradication (U.S. Department of Agriculture 2003; see Gall *et al.* 2000 and Nielsen 2002 for further details). The screening carries the risk that some animals with antibodies present that have been exposed but are not necessarily infectious may be slaughtered as well (Bienen and Tabor 2006). Varying sensitivity and specificity of the tests allow for inconsistent management actions depending on the interpretation of the results. For instance, previously tested bison with low titers of *Brucella* spp. antibodies have been shown to still harbor the intracellular bacteria that could eventually cause the bison to become infectious (Rhyan *et al.* 2009). The animal could be chosen for slaughter since it may become infectious, or it may not be chosen due to its low titer and thus low chance of developing the disease. However, seropositive animals are removed without regard to age, sex, or genetic makeup. These management strategies are now being developed to decrease transmission of *B. abortus* in bison and thereby lower prevalence of brucellosis in the park.

Genetic composition of YNP bison and their impact on brucellosis control strategies

Yellowstone National Park's bison herd was started around the turn of the 20th century with 46 animals (Meyer and Meagher 1995). Today, after years of monitoring and protecting this population, summer herds of bison in Yellowstone number over 3500 animals including adults and calves. As noted previously, the genetic composition of this herd is invaluable and unique, being one of the only bison herds that demonstrate no detectable introgression by cattle DNA (Halbert 2003). Because the YNP herd's genetic contribution (i.e., genetic diversity) to the overall U.S. bison population is higher relative to other federal herds, the genetic make-up of potential satellite herds created by YNP animals will be able to offset the effects of genetic drift better than the genetic contribution of founder animals from other herds.

The original breeding stock of YNP consisted of 18 females from the Pablo-Allard herd in Montana and three males from the Goodnight herd in Texas being imported in 1902 and being added to the native herd of about 25 animals (Meyer and Meagher 1995). Presently, the bison population in YNP consists of two genetically distinguishable breeding groups or subpopulations, the central and northern (Halbert 2003). Though the herds were considered spatially distinct before the 1980's, recent evidence suggests potential genetic interchange between the two herds (Gates *et al.* 2005). The extent of genetic interchange has yet to be determined.

V. Study objectives

The overall aim of this study is to contribute to current management methods in controlling the spread of brucellosis caused by *B. abortus* from YNP bison to other species. First, genetic natural resistance to brucellosis will be assessed using specific polymorphisms found

within the bison PRNP gene. Specifically, this project will investigate whether variation within PRNP is significantly associated with the presence of *Brucella* spp. antibodies in YNP bison. Results could support the use of genetic natural resistance as a selection and control technique within the YNP bison herd.

Secondly, because this herd has been quarantined under the rules of the BQFS and is anticipated to be reintroduced as a satellite herd, it is necessary to attempt to understand the initial genetic constitution and potential impacts of this study on the future of these select YNP individuals from a genetic perspective. Parentage and genetic diversity of captured groups of Yellowstone bison will be determined in order to quantify the potential genetic diversity available to begin a satellite herd. This will allow estimates of the total genetic diversity within the herd which will be compared to the total genetic diversity of YNP bison as a whole. Additionally, because smaller satellite herds are being re-established, it is important to ensure that these founder populations are genetically pure and free of domestic cattle DNA introgression. To test this, previously developed mitochondrial and nuclear DNA microsatellite markers will be applied to confirm the absence of cattle DNA introgression. These genetic analyses will provide a preliminary genetic assessment of satellite herds from Yellowstone National Park and a model for reintroducing a native species to areas of its historic range.

As the American bison continues to be a national symbol of North American wildlife, it is ever important to conserve the genetic integrity of the species for future generations. Animals from YNP, though a valuable source of genetic diversity and free of cattle DNA introgression, are afflicted by *B. abortus* exposure. Satellite herds founded by animals originating in the YNP herd are under much more regulations due to this bacterial disease. As such, it is important for managers of these satellite herds to be able to incorporate these genetic, epidemiological, and

ecological aspects into their conservation strategies. The following chapters aim to provide an initial examination of genetic factors important to a YNP satellite herd of bison.

In Chapter II, we investigate the association between polymorphisms within the bison PRNP and individual animal's innate resistance to brucellosis as determined by serology and culture tests. Chapter III addresses genetic diversity, hybridization of bison and cattle, and other genetic aspects that should be considered when using YNP bison as stocking animals for a satellite herd created for conservation purposes. The final chapter provides management suggestions for this satellite herd originating from YNP and for future satellite herds.

CHAPTER II

GENETIC NATURAL RESISTANCE TO BRUCELLOSIS IN A YELLOWSTONE NATIONAL PARK BISON CONSERVATION HERD: PRELIMINARY INVESTIGATION

Summary

The potential for identifying genetic natural resistance to brucellosis in American bison (*Bison bison*) was described by Seabury *et al.* (2005). They found a significant association between nucleotide variation within the prion protein gene (PRNP) exon 3 and *Brucella* spp. seropositivity in bison. Specifically, bison possessing the C/C genotype were regarded as having a positive correlation with presence of *Brucella* spp. antibodies. In the current study, the variations within the PRNP were examined in bison from Yellowstone National Park (YNP; n = 107) that were captured and quarantined as part of a study implemented by the Interagency Bison Management Plan. In contrast to the findings by Seabury *et al.* (2005), no distinct relationship between seropositive animals and genotype ($p = 0.649$) or sex ($p = 0.297$) was determined. Further, no significant difference was found between genotype ($p \geq 0.896$) and allelic frequencies ($p \geq 0.894$) between the quarantined YNP bison and YNP bison used in Seabury *et al.* 2005, suggesting that the difference was not caused by a dissimilarity in sampled allelic distribution between the two study populations. Significant variations in seroprevalence and sex ratios between the two studies ($p < 0.001$) provide evidence that the differences in natural resistance may be dependent on ecological and/or other genetic factors that were not examined in either study. In summary, there is inconclusive evidence to indicate that PRNP variation is correlated to natural resistance to brucellosis in bison.

Introduction

Bovine brucellosis is an infectious reproductive disease caused by the bacteria *Brucella abortus*. This chronic, untreatable disease affects both livestock and wildlife ungulate species and the pathogen is easily transmitted intraspecies and interspecies. In cattle (*Bos taurus*) and bison (*Bison bison*), the disease is characterized by abortions, birth of weak or nonviable offspring, and retained placentas (Williams *et al.* 1993; Rhyan *et al.* 1994; Thorne 2001). The bacteria are transmitted through oral contact with aborted tissues (i.e., fetus, placenta, or discharges), blood, infected milk, feces, and contaminated environments (Peterson *et al.* 1991; Williams *et al.* 1993; Meyer and Meagher 1995; Rhyan *et al.* 2009).

Brucellosis in domestic cattle has resulted in tremendous economic impacts in the United States (US) over the past century. Billions of dollars have been spent by federal, state, and private organizations to promote vaccination against brucellosis in an attempt to eradicate the disease in domestic livestock (Cheville *et al.* 1998). Herds found to be brucellosis free are allowed to participate in interstate travel which provides significant economic benefits to a state's cattle industry (Genho 1990; Kilpatrick *et al.* 2009). Considerable economic loss results when an animal within a herd is found to be seropositive with *Brucella* spp. antibodies, as the entire herd is quarantined and a state's brucellosis status may be reclassified (Keiter 1997).

Much of the US has been declared bovine brucellosis free, with the exception of the remaining reservoir of brucellosis-infected animals in the Greater Yellowstone Area (GYA; Meyer and Meagher 1995; Roffe *et al.* 1999a) and a few sporadic occurrences. Brucellosis caused by *B. abortus* occurs naturally in free-ranging bison and elk (*Cervus canadensis*) in the GYA and has the potential to be transmitted to domestic cattle. Brucellosis in YNP was initially reported by Mohler in 1917 and *B. abortus* has since been documented in free-ranging bison of

Yellowstone National Park (YNP; ID, MT, WY, USA; Peterson *et al.* 1991; Meagher and Meyer 1994; Dobson and Meager 1996; Cheville *et al.* 1998; Rhyan *et al.* 2009). Based on serologic exposure, *B. abortus* prevalence in YNP bison is estimated around 30-50%, and this may threaten the disease status of cattle herds that are located adjacent to the park's boundaries (Meagher and Meyer 1994; Cheville *et al.* 1998; Rhyan *et al.* 2009).

To protect cattle from *B. abortus* transmission from infected wildlife and developing brucellosis, many human-initiated methods of management have been implemented, such as vaccinating cattle and bison against brucellosis, hazing wandering bison back into YNP boundaries, culling of bison that have left the park boundaries, and others (Stevens *et al.* 1994; Olsen *et al*. 2003; Clarke *et al.* 2005). These methods have had varying degrees of success: vaccinating cattle and bison is the most effective measure when administered as calves, but must be given yearly in bison for maximum protection which is not cost effective or logistically feasible; restricting bison to within the boundaries of the park removes the immediate threat of transmission of *B. abortus* to cattle, but does not guarantee the animals will remain there; and removing animals from the population may have negative impacts on herd genetic diversity. None of these management methods have been proven to resolve the threat of *B. abortus* transmission from wildlife to cattle.

To increase efficacy of these techniques, additional control measures are being considered for use in reducing transmission of *B. abortus* to domestic stock. Genetically controlled disease resistance is an identifiable polygenic trait as found by studies of resistance/susceptibility in mice; however, single genes have also been observed to have a major effect on immune mediated resistance in mice in a wide range of infectious diseases (Schurr *et al.* 1990; Templeton and Adams 1990; Beckers *et al.* 1995; Vidal *et al.* 1995). Such diseases

include tuberculosis, legionellosis, as well as brucellosis. Identification of a gene or genes that may be associated with natural resistance brucellosis could provide another means of controlling the disease, i.e., preventing infection from occurring by removing animals that lack the gene(s) that confer natural resistance. In areas with *B. abortus* infected bison herds such as the GYA, screening for genes that control natural resistance may introduce another screening and management tool for minimizing *B. abortus* transmission to domestic cattle.

Investigating natural resistance using the prion protein gene (PRNP) is a novel approach to identifying apparent resistance or susceptible genotypes to various bacterial diseases such as brucellosis (Schläpfer *et al.* 1999; Hills *et al.* 2001; Seabury *et al.* 2004). The PRNP encodes the prion protein (PrP), which is more commonly identified by its role in the pathology of neurodegenerative diseases such as transmissible spongiform encephalopathies (TSE) or prion diseases (Prusiner 1998; Goldmann 2008). Prion diseases are expressed when the PrP^{C} in the host is misfolded and converted to the diseased and protease resistant form (PrP^{Sc}) during the posttranslational process. The conformation of PRNP will change depending on which isoform (PrP^{C} or PrP^{Sc}) is present (Prusiner 1998; Jackson and Clarke 2000). In addition to PRNP conformation, polymorphisms within the PRNP may be responsible for differences in incubation time or susceptibility to prion diseases and bacterial diseases (Seabury and Derr 2003; Sander *et al.* 2005; Saunders *et al.* 2007).

As indicated in mice, the host cellular prion protein may be a cell surface receptor or aid transport of *B. abortus* through interactions with the heat shock protein Hsp60 (Schläpfer *et al.* 1999; Hills *et al.* 2001; Seabury *et al.* 2004). As *B. abortus* is being internalized by phagocytic cells such as macrophages, the Hsp60 on the bacterial surface promotes PrP^{C} aggregation on the macrophage, which induces membrane ruffling (Watarai *et al*. 2003; Watarai 2004). This

ruffling encourages ingestion of the bacteria via pinocytosis by the macrophage. Once internalized, *B. abortus* functions to block phagolysosome fusion and thereby establish chronic, intracellular infection within the host's macrophages.

Single nucleotide polymorphisms (SNPs) are abundant in mammalian genomes and useful for many purposes including identifying portions of the genome associated with response to infection. Most SNPs are the result of rare mutation events and are assumed to occur randomly. The mutations arise from DNA replication errors or through repair of DNA damage due to environmental mutagens (Whitaker and Banfield 2005). These mutations may or may not be expressed in the phenotype. In structural genes such as PRNP, however, these polymorphisms can have a dramatic effect on the biology of the whole organism.

One particular PRNP SNP has been identified as the determinant for susceptibility or resistance to brucellosis in bison (Seabury *et al.* 2004; 2005). In this species, alleles within PRNP exon 3 were found to possess six octapeptide nucleotide repeats and were identical in sequence with the exception of one non-synonymous SNP at nucleotide position 50 (T50C; Seabury *et al.* 2005). This polymorphism caused the amino acid methionine to be converted to threonine. Seabury *et al.* (2005) noted the genotype (C/C) resulting in this amino acid substitution was significantly correlated with *Brucella* spp. seropositive animals collected from YNP. It was suggested that this non-synonymous substitution may have functional implications in PrP^C biogenesis or entry of *Brucella* spp. into bison host cells based on susceptible or resistant genotypes. Previous research has indicated that mutations and amino acid substitutions in the signal sequence of PrP^C can significantly alter the ratio of three topological forms of PrP^C which are synthesized in the endoplasmic reticulum (Watarai *et al.* 2003; Watarai 2004). This action could potentially lead to increased risk for neurodegenerative disease due to overproduction of a

specific topological form (^{Ctm}PrP) which has been associated with accumulated PrP^{Sc}. The ^{Ctm}PrP form contains an uncleaved N-terminal signal peptide which has been shown in mice to be cell-penetrating and capable of transporting large hydrophilic cargoes through the cell membrane, which may be important for infection (Lundberg *et al.* 2002).

Examination of other bison herds that were not exposed to *B. abortus* supported this theory as the T allele was significantly more abundant in these herds and thus considered the resistant allele. Utilizing this polymorphism to screen *B. abortus* exposed bison for resistant genotypes could aid management decisions for bison herds that are chronically infected with *B. abortus* such as the YNP herd.

The purpose of this study was to further investigate the role of the PRNP gene in genetic natural resistance to brucellosis in bison as identified by Seabury *et al.* (2005). Using bison collected from YNP as part of a cooperative study initiated by the Interagency Bison Management Plan, we investigated whether this SNP variation within the bison PRNP exon 3 is significantly associated with *Brucella* spp. antibodies tested through serology. This was accomplished by analyzing the frequencies of allelic variations and SNP genotypes of PRNP exon 3 from bison sampled for this study and comparing them with observed values for previously captured and tested YNP animals (Seabury *et al.* 2005).

Methods and Materials

Animal selection and sampling

Animals in this study were part of the Bison Quarantine Feasibility Study (BQFS) that was started in spring 2005 by the Interagency Bison Management Plan (IBMP) (Zaluski *et al*. 2010). The BQFS is a cooperative study involving the Montana Department of Livestock, Montana Department of Fish, Wildlife, and Parks, U.S. Department of the Interior, National Park Service, USDA Forest Service, and USDA Animal and Plant Health Inspection Service. The study was initiated to explore alternative options for bison that leave the protection of the park's perimeters. Usually the animals are eradicated to avoid contact between bison and cattle outside the YNP boundaries. The goal of the BQFS is to determine the feasibility, efficacy, and associated risk of utilizing a protocol for bison quarantine set forth in the Uniform Methods and Rules (UM&R) for Brucellosis Eradication and to qualify bison from Yellowstone National Park (YNP) as free of brucellosis.

The animals in the BQFS were selected for management actions because they moved out of the park boundaries near West Yellowstone, MT and therefore they were eligible for slaughter according to the IBMP. The study's aim was to evaluate the feasibility of isolating bison in a quarantine setting and testing them over time to qualify individual bison as free of brucellosis. If the adults and calves did not develop a positive titer or bacterial culture to *Brucella* spp. by the following winter, the intent was to reintroduce this group of bison to private pieces of land, i.e., Native American lands or other national parks not including YNP.

Yearling calves were initially captured with other bison migrating out of YNP near West Yellowstone in 2005 and Gardiner, MT, in 2006 and 2008. The animals were then transported to state designated pastures in Montana for the purpose of quarantine. Only animals captured in

2008 (n=107; Table 1) were considered in this study. To qualify for quarantine, yearling animals were to test seronegative on brucellosis tests performed at the trap, which included the card test for rapid screening and the fluorescent polarization assay (FPA) for confirmation (Nielsen *et al.* 1996; Gall *et al.* 2000; Schumaker *et al.* 2010). Both tests are completed in the field and do not require laboratory conditions. The FPA has higher sensitivity for detecting *Brucella* spp. infection than the standard card test, but is less cost-effective (Schumaker *et al.* 2010). Thus, both tests were used in the screening to ensure proper classification of the animals. Once included in the study, the animals underwent a monitoring program where blood was drawn and tested for *Brucella* spp. antibodies throughout the year. After one year of isolation, about half of the animals were chosen regardless of serological outcome or sex and sent to slaughter. Blood, urine, and tissues of slaughtered animals were subsequently tested and bacterial cultured for any positive brucellosis results. Remaining members of the population that tested negative on serological tests for brucellosis were bred in late summer, calved out in spring, and continually serologically tested for antibodies to *Brucella* spp.. All tissue samples for DNA extraction were collected prior to selection for slaughter (n=107).

Serology testing

Whole and heparinized blood samples were collected from live animals for serology and bacterial culture at scheduled collection periods throughout the study. Necropsy and specimen collection followed guidelines developed by the Greater Yellowstone Interagency Brucellosis Committee (GYIBC) for brucellosis management and research sampling of bison (Aune *et al.* 1995). The sampling protocol designated for the slaughtered BQFS bison included collection of whole and heparinized blood, urine, ileum, kidney, liver, spleen, whole lymph nodes

(supramammary (female), superficial inguinal (male), popliteal, prefemoral, superficial cervical (pre-scapular), internal iliac, hepatic, mesenteric, bronchial, mandibular, parotid, and retropharygeal), and ovaries, uterus, testicle, epididymis, seminal vesicles where appropriate. Tissues were placed in sterile Whirl-Pak® bags and kept frozen for shipment to National Veterinary Services Laboratories (NVSL, Ames, Iowa, USA) for bacterial culture, using the methods of Alton *et al.* (1988). Whole-blood specimens (centrifuged, collected, and aliquoted), plasma, and sera was shipped to NVSL for a panel of nine serologic tests: standard card, standard plate (SPT), standard tube (STT; Anonymous 1965a), rivanol, buffered acidified plate antigen (BAPA; Anonymous 1965b), complement fixation (CF; Anonymous 1993), particle concentrate fluorescence immunoassay (PCFIA; IDEXX Laboratories, Westbrook, Maine, USA), and FPA. Because specificity and sensitivity of tests used for detecting *Brucella* spp. infection vary (Schumaker *et al.* 2010), serological decisions were based off of a consensus of positive results from multiple tests. Samples were considered seropositive if serum reactions were observed on two or more tests, with at least one of these tests being quantitative. Defined positive levels for quantitative tests are determined by the UM&R for Brucellosis Eradication (U.S. Department of Agriculture 2003).

Due to the variable sensitivity and specificity of individual serology tests in bison, only samples with consensus positive serological results on multiple tests were considered a diseased animal. Animals with suspect status (i.e., weakly positive serological results for *Brucella* spp. antibodies) were therefore not euthanized or cultured for bacteria in further testing events. Only reactors (i.e., high serologic titers in one or more tests) to *Brucella* spp. infection were removed from the quarantine population. Animals that test seropositive are either harboring current

infections, have recovered from previous infection, or have been exposed to *B. abortus*, but are resistant (Dobson and Meagher 1996).

DNA Extraction

Tissues were collected in the field by performing a biopsy punch from the ear of each animal. Biopsies were placed in Whirl-Pak® bags and kept frozen until DNA extraction could be completed. Genomic DNA from 107 tissue samples was extracted using a DNeasy Tissue Extraction Kit (Qiagen, Valencia, CA) and following the manufacturer's protocol. The final extraction product was kept at -25°C until it was used in the polymerase chain reaction.

Sequencing

Amplification of the 795 basepair fragment of the bison PRNP gene was performed using flanking primers SAF1 and SAF2 (Prusiner *et al.* 1993) to sequence exon 3 of the PRNP gene. Polymerase chain reaction (PCR) reactions and thermal cycling parameters followed Seabury *et al.* 2005. Resulting fragments were co-loaded into a single injection for separation on an ABI 3600 (Applied Biosystems) using an internal size standard (Mapmarker LOW; Bioventures, Inc., Murfreesboro, TN, USA).

Amplification, amplicon sequencing, and single nucleotide polymorphism (SNP) detection followed previously published methods (Seabury and Derr 2003; Seabury *et al.* 2004 and 2005). PRNP exon 3 SNP variations were determined using Sequencher Version 4.2.2 (Gene Codes Corporation, Ann Arbor, MI, USA).

Statistical analyses

Data for bison PRNP exon 3 for the BQFS bison were subdivided into either seronegative or seropositive animals as determined by the combined serology tests. Allelic frequencies and genotypic differentiation in the bison PRNP were determined using the probability test and Hardy-Weinberg equilibrium values were calculated using GenePop 4.1 (Rousset 2008). We evaluated the hypothesis of no difference between the seropositive and seronegative individuals in terms of serology using logistic regression. Results for suspect versus seronegative animals were compared using logistic regression. If no difference was observed, then suspect and seronegative animals were combined into single group for the remainder of the analyses (Dohoo *et al.* 2003; StataCorp 2007). Serological outcome association with genotype was assessed to ascertain which genotypes may have contributed to natural resistance or susceptibility while sex-specific associations were considered to identify any degree of natural resistance in either males or females. The Z_{max} test, followed as in Seabury *et al*. (2005), was also used to determine the statistical associations between seropositive and seronegative groups within the individual genotype classes (Ewens *et al*. 1992; Lange 2002; El Galta *et al.* 2008). Associations between and among animals from the BQFS cohort and the population studied in Seabury *et al*. 2005 were calculated using Pearson's chi-square test to determine any significant variance in genotypic and allelic frequencies between seronegative and seropositive groups (Dohoo *et al.* 2003). Various characteristics of the study populations' composition, such as proportions of males and females within seropositive and seronegative classes, were also compared using Pearson's chi-square tests. Test results were considered statistically significant when $p < 0.05$. Power analysis (80%) was completed to determine the appropriate sample size to determine an association between genotypes (Win Episcope 2.0, CLIVE, Edinburgh, Scotland).

Results

Of the 107 bison tested, 23 (21.5%) were seropositive and 35 (32.7%) were suspect positive animals, for a combined antibody prevalence of 54.2% (Table 1). There were 49 (45.8%) animals that did not exhibit any antibody response to *Brucella* spp. antigens. Only four samples, three females and one male, (17.39%) were culture positive for *Brucella* spp. and all were seropositive reactors.

For this SNP, the BQFS population did not deviate from Hardy-Weinberg expectations. Genotypic and allelic frequencies of seropositive animals, seronegative animals (Table 2), and overall frequencies between BQFS bison and the group of YNP animals studied in Seabury *et al.* 2005 (n = 117) were compared using Pearson's chi-square test of association (Table 3). Three SNP genotypes were analyzed: homozygous T (T/T), heterozygous (T/C or Y), and homozygous C (C/C). No associations were found to be significant for genotypic frequencies ($p \geq 0.896$ for all comparisons, df = 2) or allelic frequencies ($p \geq 0.894$ for all comparisons, df = 1). There were 105 samples sequenced bidirectionally and two samples sequenced in one direction using SAF1 primer only. A clear genotype could not be produced for one individual (sample 26-08), therefore it was removed from further analysis. Because suspect (or weakly positive) animals were grouped with the seropositive reactors from the BQFS test group as determined by NVSL, it was necessary to assess any significant association between suspect animals and seronegative reactors or if the groups were indeed significantly different from one another.

There was no statistically significant difference between these two groups with regard to serology and genotype ($p = 0.497$; Fisher's exact test; two-sided) or sex ($p = 0.629$). Thus, suspect animals were combined with the seronegative reactors for the subsequent analyses.

When comparing seronegative and seropositive reactor BQFS bison, no statistical significance was found between serology and SNP genotype ($p = 0.649$) or sex ($p = 0.297$). The results of the Z_{max} test also did not find any significant association between serology and genotypic classes (Z_{max} $p = 0.132$). Since previous studies (Seabury *et al.* 2005) within the YNP population determined a positive correlation between serology and genotype, an analysis between study populations from both studies was conducted to identify any information that would explain the difference in results.

There were significant differences in various population characteristics between both study groups (Table 3). The BQFS population had significantly fewer seropositive animals and significantly more seronegative animals overall than the population studied by Seabury *et al.* ($p < 0.001$; 2005). The numbers of males and females also differed significantly between the groups for the overall populations and for the seropositive subgroups ($p < 0.001$).

Discussion

Endemic diseases such as brucellosis pose an important health and economic concern for livestock, wildlife, and humans throughout the world. Human cases of brucellosis have been attributed to contact with infected wildlife and consumption of contaminated food sources to allow *Brucella* spp. transmission (Davis 1990). Guarding the livestock industry from potential contamination benefits the animals within the food chain and future consumers. Within the U.S., bovine brucellosis in particular has had a significant impact on the cattle industry in the past century, with the greatest concern coming from the detection of *Brucella* spp. antibodies in cattle that have been infected through contact with infected wildlife (Cheville *et al.* 1998).

Prevention of such contact is an integral part to managing wildlife in the GYA. However, the difficulty in preventing contact between wildlife and livestock arises with regulations placed as to the proper precautions in handling the movement of wildlife in and around YNP by state and federal agencies. Much research has been focused on predicting bison movement patterns outside of the park but this may not be a reliable measure of protection due to random bison movement around the park's boundaries and yearly fluctuations in food availability (Clarke *et al.* 2005; Gates *et al.* 2005; White *et al.* 2011). The practicality of separating these two species involves the cooperation of those agencies monitoring the bison movement and also the livestock owners that graze animals around YNP, which could be difficult due to conflicting goals for the area (Gates *et al.* 2005; White *et al.* 2011). Utilizing a tool such as the ability to detect natural resistance to brucellosis would enable management teams to change their focus towards efforts on removal of susceptible animals from the population in addition to manipulating the herd's movements to the boundaries of the park.

Polymorphisms within the PRNP gene have shown some association with genotypes susceptible to bacterial diseases in various species (Schläpfer *et al.* 1999; Hills *et al.* 2001; Seabury *et al.* 2004). Seabury *et al.* (2005) found significant associations between PRNP SNP polymorphisms and susceptible and resistant serotypes to *Brucella* spp. within bison. This relationship was not mirrored in the BQFS population; however this result could be attributed to various genetic or environmental factors.

It is generally accepted that seroprevalence of *Brucella spp.* antibodies in bison within the YNP is between 30-50% and are caused by *B. abortus* specifically (Meagher and Meyer 1994; Cheville *et al.* 1998; Rhyan *et al.* 2009). Sero-agglutination titers of bison are neither reflective nor diagnostic of the status of infection of bison. In our study, quarantined animals were

monitored for significant titers that resulted in a combined antibody prevalence of 54.2%, which was calculated using both weakly positive and seropositive animals and is consistent with the general seroprevalence estimate for YNP. Seroreactivity does not guarantee a bacterial culture positive result, as seen in a quantitative study conducted by Roffe *et al.* (1999b) which determined that 46% of seropositive bison were actually culture positive. Only four of the 23 BQFS seropositive samples, however, were culture positive (17.39%). In the study carried out by Seabury *et al*. (2005), there were significantly more seropositive animals, but specific information regarding the selection of seropositive animals (i.e., if the study protocol combined weakly positive with high titer results) and bacterial culture results were not available. Discrepancies in classification of seropositive animals between the two studies could have accounted for the vast difference in the number of seropositive animals.

Further, the bison population in YNP consists of two genetically distinguishable breeding groups or subpopulations, the central and northern (Halbert 2003). In the BQFS and Seabury *et al.* studies, the animals were collected from West Yellowstone and Gardiner, MT, respectively. It is possible that migration patterns between the collection years could have changed the genetic disposition of the study populations, meaning that animals from one or both herds may be represented in the studies. However, allelic or genotypic differences between the two study groups were not detected. In the future, it may be beneficial to analyze the serology and PRNP genotypes between the central and northern herds to discern any differences in seroprevalence and genotypic frequencies.

Seabury *et al.* (2005) found the C/C genotype to be significantly associated with seropositive animals collected from YNP. Therefore, proportions of this genotype within seropositive and seronegative groups of animals from the BQFS group were used to calculate the

sample size needed to detect a significant difference between the serological groups. Percentages of the homozygous C/C genotype of seronegative (15.48%) and seropositive (13.04%) in the BQFS group estimated that 3222 animals from each serology group would be required to determine a statistically significant difference in the percent of homozygous C/C animals between the two serology groups. Under this assumption, it could be argued that the sample sizes in either study were still not representative of the entire YNP population due to constantly changing demographics due to disease, season, and other factors. If it was cost effective, collecting more samples may increase the power of the findings. This is difficult, though, based on the regulations that surround collection of samples from animals residing within YNP and also due to the disease status of this particular bison herd.

Though our analyses presently fail to support the hypothesis that PRNP can be used as a screening tool for bovine brucellosis susceptible genotypes, the potential for polymorphisms within the bison PRNP being correlated to natural resistance to brucellosis cannot be ruled out. The PRNP may act as part of an unknown genetic cascade to protect against infectious disease, but that relationship is not currently understood. Management of this disease based on genetic screening would require more extensive genetic studies of a multitude of genes in the bison genome that would include sample collections for more than one year and a more representative sample of both herds and with equally distributed sex and serology ratios. The herd dynamics of YNP bison are constantly changing due to natural and anthropogenic factors. To better understand the role of PRNP in natural resistance to brucellosis in YNP bison, more research is needed to understand other factors affecting the genetic and disease status, including demographics, oscillation of antibody prevalence, and ecology of the YNP bison herd.

Tables

Table 1. Distribution of Bison Quarantine Feasibility Study bison samples by sex, *Brucella* spp. serological status, and genotype.

		Genotype			
	Total	T/T	T/C (Y)	C/C	Unknown [1]
Female seronegative	59	19	30	10	0
Female seropositive	19	4	12	3	0
Male seronegative	25	8	13	3	1
Male seropositive	4	1	3	0	0
Total	107	32	58	16	1

[1] Unknown genotype resulted from indistinguishable prion protein gene sequencing. This sample was not included in the analysis.

Table 2. Observed PRNP exon 3 allelic and genotypic frequencies in individuals from Bison Quarantine Feasibility Study. The group was further subdivided by *Brucella* spp. serological phenotypes into seronegative and seropositive classes.

		Allele [1]		**Genotype** [1]		
	n	T	C	T/T [2]	T/C [2]	C/C [3]
Seronegative	83	0.584	0.416	0.325	0.518	0.157
Seropositive	23	0.543	0.457	0.217	0.652	0.130
Total	106 [4]	0.575	0.425	0.302	0.547	0.151

[1] Frequencies rounded to three decimal places.
[2] Genotypes T/T and T/C code for the amino acid methionine.
[3] Genotypes C/C code for the amino acid threonine.
[4] One unknown genotype resulted from indistinguishable prion protein gene sequencing. This sample was not included in the analysis.

Table 3. Comparison of various herd dynamics with associated p-values between the Bison Quarantine Feasibility Study herd and the Yellowstone National Park herd, which was studied by Seabury *et al.* 2005.

	BQFS	YNP	p-value [3]
Seronegative	83	44	**p < 0.001**
Female	59	~ [2]	~
Male	24	~ [2]	~
Seropositive	23	73	**p < 0.001**
Female	19	37	**p < 0.001**
Male	4	36	**p < 0.001**
Total	106 [1]	111	

[1] One unknown genotype resulted from indistinguishable prion protein gene sequencing. This sample was not included in the analysis.
[2] Information not provided in Seabury et al. (2005). Therefore, no tests of association were completed.
[3] Pearson's chi-square test of association was used to calculate p-values.

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CHAPTER III

GENETIC ANALYSIS OF A QUARANTINED YELLOWSTONE NATIONAL PARK BISON HERD

Summary

As part of the Bison Quarantine Feasibility Study (BQFS) implemented by the Interagency Bison Management Plan (IBMP), 89 bison (*Bison bison*) from Yellowstone National Park (YNP) were quarantined and tested to qualify as free of brucellosis. These animals were to be reintroduced to areas of their historic range as satellite herds of YNP bison for conservation purposes. It is important to understand the genetic diversity of the herd and to determine if any genetic characteristics such as cattle DNA introgression or low genetic diversity may threaten the protected status of this herd. We evaluated genetic diversity at 42 microsatellite loci representing each of the nuclear chromosomes in the bison genome. We found no detectable evidence of cattle DNA introgression in this herd through nuclear markers and mitochondrial DNA analysis. Parentage analysis of the BQFS herd indicated that the majority of mature adults were actively breeding and contributing offspring to the herd. Genetic diversity levels in the quarantined herd were high and comparable to the YNP parent herd, suggesting a low risk of inbreeding in the near future. Based on these analyses, the genetic diversity currently available within the BQFS herd will provide a strong foundation for bison satellite herds and for the preservation of the species.

Introduction

The American bison (*Bison bison*) is a prime example of conservation success in North America. Until the 1870s, tens of millions of bison roamed the Great Plains (Freese *et al.* 2007; Halbert and Derr 2008; Hedrick 2009). This population was decimated to near extinction by the mid-1880s through massive slaughters aimed at collecting hides and meat. Extensive genetic analyses of American bison have shown a remarkable conservation of genetic diversity despite the severe population decline over a century ago (Mommens *et al.* 1998; Wilson and Strobeck 1999; Boyd 2003; Halbert 2003; Halbert and Derr 2008). Stringent genetic monitoring of bison populations and translocation of animals has enabled the preservation of the remaining bison genetic diversity.

Re-establishing satellite herds of bison in areas of their historic range has been recommended as part of the effort to preserve the wild bison genetic diversity (Freese *et al.* 2007; Sanderson *et al*. 2008). Conserving this genetic diversity, however, is compromised by various factors such as small herd size, introgression of cattle genes, intensive management and culling practices, and infectious diseases (Freese *et al.* 2007; Sanderson *et al.* 2008). Due to small herd sizes, satellite or re-established herds can suffer from inbreeding depression and risk extinction, such as with the Texas State Bison Herd that has been fraught with problems including a small founder population and evidence of genetic drift and inbreeding due to extremely low levels of genetic diversity (Halbert *et al*. 2004). Introgression of cattle DNA into bison herds has been detected in some bison herds and different management strategies are needed when dealing with these hybridized populations. Having hybrid animals in a satellite herd may even result in legal changes to their protective status (O'Brien and Mayr 1991; Hill 1993). Finally, indiscriminate culling of animals from bison herds due to proximity to livestock or fear

of spreading an infectious pathogen potentially threatens to reduce genetic diversity via mortality, which could act as a population bottleneck and threaten the evolutionary potential of a herd (Halbert 2003).

The Yellowstone National Park (YNP) bison herd retains a small portion of genetic diversity from pre-settlement herds (Halbert 2003). This free-ranging herd is the only herd in the US and one of two in North America that have descended from a continuously free-ranging wild herd. Free-ranging, in this case, is defined as the population being maintained without fences and without supplemental feed since 1967. A free-ranging population also must be subjected to natural population size management such as predation, resource competition, and natural mortality (Halbert 2003). YNP's bison herd was initiated around the turn of the 20th century with 46 animals (Meyer and Meagher 1995). Today, after years of monitoring and protection, summer herds of bison in Yellowstone include over 3500 animals including adults and calves. Presently, the bison population in YNP is believed to consist of two genetically distinct breeding groups or subpopulations: the central and northern subpopulations (Halbert 2003; Halbert *et al.* 2012). Recent evidence suggests potential genetic interchange between the two herds (Gates *et al.* 2005), but the extent of genetic interchange has yet to be determined. Remarkably, genetic analyses have determined that despite their population reduction the YNP genetic contribution (i.e., genetic diversity) to the overall U.S. bison population is higher relative to other federal herds (Halbert 2003; Halbert and Derr 2008; Hedrick 2009) such that the YNP herd has been proposed for propagating satellite herds. The presence of cattle DNA introgression in bison herds restricts the use of those herds in future conservation planning. Out of ~500,000 plains bison currently in the U.S. and Canada, fewer than 1.5% are likely free of domestic cattle genes (Freese *et al.* 2007). The YNP population has been found to contain both high levels of genetic

variation and no evidence of domestic cattle introgression thus these animals are preferred stock for satellite herds (Halbert 2003; Halbert *et al.* 2005; Freese *et al.* 2007; Halbert and Derr 2007).

The presence of bovine brucellosis and its causative agent *Brucella abortus* within the YNP herd raises concerns about using these animals for satellite herds. This chronic, untreatable disease affects both livestock and wildlife ungulate species and the pathogen is easily transmitted within and among species. In cattle (*Bos taurus*) and bison (*Bison bison*), the disease is characterized by abortions, birth of weak or nonviable offspring, and placenta retention (Williams *et al.* 1993; Rhyan *et al.* 1994; Thorne 2001). Much of the US has been declared bovine brucellosis free, with the exception of a remaining reservoir of brucellosis-infected animals, predominantly bison and elk (*Cervus canadensis*), in the Greater Yellowstone Area (GYA; Meyer and Meagher 1995; Roffe *et al.* 1999) with a few sporadic occurrences elsewhere. Initially reported by Mohler in 1917, *B. abortus* has since been documented in free-ranging bison of YNP (US; Peterson *et al.* 1991; Meagher and Meyer 1994; Dobson and Meager 1996; Cheville *et al.* 1998; Rhyan *et al.* 2009). Bovine brucellosis occurs naturally in free-ranging bison and elk in the GYA and *B. abortus* has the potential to be transmitted to domestic cattle. Based on serosurvey data, *B. abortus* prevalence in YNP bison is estimated between 30-50%. This high prevalence indicates a high probability of transmission to cattle located adjacent to the park's boundaries and threatens the disease status of those cattle herds, which would result in large economic losses for producers (Meagher and Meyer 1994; Cheville *et al.* 1998; Rhyan *et al.* 2009).

To protect cattle from *B. abortus* transmission from infected bison, several management approaches have been implemented, such as vaccinating cattle and bison against brucellosis, hazing wandering bison back into YNP, and permanent removal of bison that have left the park

(Stevens *et al.* 1994; Olsen *et al*. 2003; Clarke *et al.* 2005). Animals in this study were part of the Bison Quarantine Feasibility Study (BQFS) that was started in spring 2005 by the Interagency Bison Management Plan (IBMP) (Zaluski *et al*. 2010). The BQFS is a cooperative investigative study involving the Montana Department of Livestock; Montana Department of Fish, Wildlife, and Parks; U.S. Department of the Interior; National Park Service; USDA Forest Service; and USDA Animal and Plant Health Inspection Service (USDA-APHIS). The study was initiated to explore alternative options for bison that leave the protection of the park's perimeters.

The goal of the BQFS is to determine the feasibility, efficacy, and associated risk of utilizing a protocol for bison quarantine set forth in the Uniform Methods and Rules (UM&R) for Brucellosis Eradication in order to qualify some YNP bison as free of brucellosis. Under this protocol, the quarantined herd must complete consecutive negative herd blood tests once the seroreactors are removed from the herd and slaughtered, with serological tests being conducted every 30-45 days until all animals are declared negative. Special consideration is given to female bison which must complete two calvings within the quarantine and maintain seronegative status (U.S. Department of Agriculture 2003). These parameters, if followed completely, will result in animals that could contribute to a brucellosis-free satellite herd.

Future management of the BQFS herd as a satellite herd could benefit from DNA analyses aimed at identifying paternal and maternal genetic contributions in the population (Blouin *et al.* 1996; Frankham *et al.* 2002; Bowyer *et al.* 2007). In small populations, such as satellite herds, where the effects of inbreeding depression are amplified, it is not beneficial to have multiple offspring from a few sires since inbreeding and loss of genetic diversity may be expedited. Also, parentage analysis could aid in decisions to translocate animals to the herd if

certain lineages are overrepresented and more genetically diverse animals need to be introduced to supplement the herd.

The purpose of our study is to inventory the current genetic diversity of the BQFS quarantine herd using previously described microsatellite, mitochondrial, and nuclear DNA markers to: 1) determine the degree, if any, of cattle DNA introgression within the BQFS herd; 2) determine the portion of allelic diversity within the BQFS herd relative to the YNP parent herd; and 3) investigate sire contribution to genetic diversity within the captive herd. From a broader perspective, this work may provide an important tool in tracking bison genetic diversity as brucellosis-free satellite herds are re-established throughout the U.S.in years to come and aid in management and breeding plans for the satellite herds.

Methods and Materials

Animal selection and sampling

Yearling calves were initially captured with other bison migrating out of YNP near West Yellowstone in 2005 and Gardiner, MT, in 2006. Individuals in this study (n=89) were obtained from bison targeted for slaughter because they moved to the park boundaries near West Yellowstone, MT, according to management actions to protect cattle enacted according to the IBMP. The yearling animals were then entered into the BQFS and transported to state-mandated pastures in Montana. To qualify for quarantine, yearling animals tested seronegative on brucellosis tests performed at the trap, including the card test for rapid screening and the fluorescent polarization assay (FPA) for confirmation (Gall *et al.* 2000). Once included in the study, the animals underwent a monitoring program where blood was drawn and tested using a panel of nine serologic tests including the card test and FPA for *Brucella* spp. antibodies

throughout the year as determined by the UM&R for Brucellosis Eradication (U.S. Department of Agriculture 2003). After one year of isolation, about half of the animals (n = 61) were chosen randomly without any prior criteria and sent to slaughter. Blood, urine, and tissues of slaughtered animals were subsequently tested and bacterial cultured for any seropositive results for *Brucella* spp. antibodies. Remaining members of the population (n = 43) were bred in late summer of 2007, calved out in spring, and continually serologically tested monthly for antibodies to *Brucella* spp. for the remainder of the study period.

Tissue samples were collected for DNA extraction from animals that remained after the random slaughter selection. In the fall of 2007, females were divided according to pregnancy status while males were randomly chosen for two groups (Appendix I). The first group (Group A) consisted of heifers (n = 21) that were confirmed pregnant plus six bulls (n_{GroupA} = 27). Females that did not conceive in the fall of 2007 (n = 14) were placed in a separate quarantine pen with two bull bison (Group B; n_{GroupB} = 16). Both groups were exposed to bulls in a second breeding season in fall of 2008 during the quarantine period. Offspring born from the two calving seasons in 2008 and 2009 were also included in the genetic analyses of the current study (n_{2008}=16; n_{2009}=30; Table 4).

Sample collection and DNA extraction

Tissue samples were collected in the field under the guidance of USDA-APHIS Veterinary Services personnel by collecting tissue from an ear of each animal. Tissues were placed in Whirl-Pak® bags and kept frozen until DNA extraction could be completed at the USDA-APHIS Wildlife Genetics Laboratory at the National Wildlife Research Center (Fort Collins, CO). Genomic DNA from 107 tissue samples was extracted using the DNeasy Tissue

Extraction Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Recovered DNA was kept at 25°C until molecular analysis.

Mitochondrial DNA sequencing and analysis

A mitochondrial DNA (mtDNA) screen was used to detect evidence of domestic cattle introgression in the bison studied (Ward *et al.* 1999). The mitochondrial genome contains a segment from the highly variable control region from which domestic cattle haplotypes can be sequenced if they are present. This screen was performed with minimal exceptions as in Halbert *et al.* (2007) by laboratory personnel at the DNA Technologies Core Laboratory (Texas A&M University, Bryan, TX). Positive and DNA-free negative controls were used in every polymerase chain reaction (PCR) run.

Genotyper 3.6 software (Applied Biosystems) was used for allele size identification and comparison and for the presence of domestic cattle alleles.

Microsatellite amplification

Fourteen nuclear markers were used as a second screen to determine whether domestic cattle DNA introgression existed in this study population (Halbert and Derr 2007). The specific nuclear markers were chosen and developed because they were not shared between domestic cattle and bison but were instead specific to domestic cattle. These markers were amplified by laboratory personnel at the DNA Technologies Core Laboratory (Texas A&M University, Bryan, TX) using the same parameters as Halbert and Derr (2007).

Breeding system analyses in the BQFS herd was performed using 11 previously developed microsatellite markers described by Schnabel *et al.* (2000) that were validated for

parentage testing in bison. These were divided into two multiplex reactions based on non-overlapping allele size ranges and dye type: PRTG 1 and 2 (Table 5). These markers were also used to assess genetic diversity along with 17 additional highly polymorphic bovine microsatellite markers found throughout the cattle genome described by Halbert (2003) which were run as multiplexed into three mixes: 80, 82, and 85 (Table 5). All multiplexes and PCR conditions were followed as described by Halbert (2003).

All PCR products were analyzed using an ABI 3600 DNA Analyzer (Applied Biosystems, Foster City, CA) with a Mapmarker 400 internal size standard (Mapmarker LOW, Bioventures, Inc., Murfreesboro, TN).

Microsatellite data analysis

To assess genetic diversity in the BQFS herd, expected (unbiased genetic diversity, H_E; Nei 1987) and observed heterozygosities (H_O), as well as mean number of alleles per locus (N_A), were obtained by using the Microsatellite Toolkit for Microsoft Excel (Park 2001). Unbiased genetic diversity was assessed as a measure of expected heterozygosity in the populations based on allele frequencies, which reduces effects due to sample size variations compared to observed heterozygosities. The genetic diversity estimate from the BQFS population was compared to previously collected genetic data from its parent population (YNP; Halbert and Derr 2007).

Evaluating genetic differentiation determines how genetically distinct populations are from one another. Genetic differentiation was investigated through examination of pairwise F_{ST} or θ (Weir and Cockerham 1984) values to assess the degree of genetic differentiation (if any) occurring between the BQFS herd and its parent YNP population. If genetic differentiation was detected then it would suggest that the BQFS herd did not sample the YNP bison genetic

diversity sufficiently. θ was estimated using the program FSTAT 2.9.3 (Goudet 2002) which was also used to test for Hardy-Weinberg equilibrium. Deviations from Hardy-Weinberg equilibrium can indicate inbreeding or population fragmentation in a population, sampling bias, or problems with the microsatellite markers used (Frankham *et al.* 2002; Templeton 2006). We assessed departures from Hardy-Weinberg equilibrium using FSTAT 2.9.3 and GenePop 4.1 (Rousset 2008).

Genetic characteristics of the BQFS herd were compared to previously tested bison from seven established North American bison populations (Halbert and Derr 2007; Table 6). In addition, STRUCTURE 2.3.3 (Pritchard *et al.* 2000) was used to assess the relationship of BQFS herd individuals to these known bison populations by grouping individuals into genetic clusters with *a priori* population information using a Bayesian approach. The goal of this analysis was to confirm whether the BQFS herd clustered entirely into the YNP herd or if admixture with any other herd was detected. If admixture from a non-YNP herd was observed, the concern for genetic contribution from mixed lineages or potential cattle DNA introgression would prompt further genetic testing and could compromise conservation management decisions for the BQFS herd.

In STRUCTURE, we used an admixture model to assess the fraction of a BQFS herd individual's genome that was attributable to the known populations. The correlated alleles option was used in all tests along with 20,000 burn in replicates and 40,000 Markov chain permutations. Individuals in the BQFS herd needing assignment were given a value of zero in the Popflag column, and all individuals from defined herds were given a value of 1, which allowed repeated updating of allele frequencies of all groups except the individuals in the targeted herd. Because seven clusters (BNP, FN, NBR, TRN, TRS, WC, and YNP) were expected based on previous

work by Halbert *et al.* (2007), only six to eight (K = 6-8) inferred clusters were assessed. The data was analyzed using the LOCPRIOR model within STRUCTURE, which uses the sampling locations of individuals to assist the clustering process. Six tests for each value of *K* were performed. All other settings used default parameters (Pritchard *et al.* 2010). Results from all runs were summarized and inspected using STRUCTURE Harvester v0.6.7 (Earl 2011) where the best estimates of *K* were inferred by examining averages and standard deviations of the log of the probability of the data (Ln *P*(*D*)). The clusters were aligned using the program CLUMPP 1.1.2 (Jakobsson and Rosenberg 2007) using the *Greedy* option and 1000 repeats of randomized input order. Consequent cluster designations were visualized using the program Distruct 1.1 (Rosenberg 2004).

Parentage analyses were completed for each breeding season. In the first breeding season (2007), all males (n = 8) were considered potential sires for the offspring born in 2008. We conducted separate analyses completed for both groups (Group A and Group B) for the second breeding season (2008) as males were not intermixed between groups. Parentage was determined using birthdates of each animal and multilocus microsatellite genotype matches to assign candidate parents to juveniles using the program PARENTE (Cercueil *et al.* 2002) to assess paternity. Approximate ages and genotypes from the offspring, known dams, and potential sires were used in the program. Known dam and calf pairings were based on daily observations and monitoring by field researchers (Rebecca Frey, personal communication). We were able to assume nearly 100% sampling as all animals within the herd were sampled and included in the analyses with the exception of one calf that died without a tissue sample being collected; therefore no genetic analyses were completed on this individual (field tag 86-82). Because all animals were sampled, the probability of excluding the sire from the analysis is low as compared

to a wild population where all adults may not be sampled. A scoring error rate of 1% was assumed, based on recommendations of previous parentage studies to account for genotyping error, mutations, or null alleles (Marshall *et al.* 1998; Schnabel *et al.* 2000; Cercueil *et al.* 2002; Halbert *et al.* 2004). A minimum parentage probability of 80% was considered acceptable for the correct identification of parental assignment. All individuals aged three years or older were considered as potential parents since sexual maturity is most commonly reached between two and four years (Meagher 1986).

Results

Using mtDNA screening, no domestic cattle haplotypes were present in any of the BQFS bison samples. In addition, the nuclear marker screen showed no domestic cattle alleles were present in our study herd. These results demonstrated that there was no detectable cattle DNA introgression in the BQFS herd.

A single monomorphic microsatellite marker was detected in the BQFS herd as in the YNP herd (Y-chromosome marker INRA189; Table 5). Values for number of alleles per locus, size ranges, and heterozygosity were comparable between the BQFS population and its parent population (YNP; Table 5). The number of alleles ranged from one to six per locus in the BQFS herd, which is less than the one to seven alleles per locus reported for the YNP population. The number of alleles surviving in the BQFS study herd represents a 7.11% loss of alleles from the YNP parent population. Average heterozygosity ranged from 0.20 to 0.91 in the BQFS herd as compared with 0.20 to 0.85 in the total YNP population (not significantly different, $p = 0.52$).

Pairwise F_{ST} values averaged 0.008 across both the BQFS and YNP populations demonstrating low differentiation between the herds. None of the loci within the BQFS herd

violated Hardy-Weinberg equilibrium ($P > 0.05$, all $p \geq 0.11$). Linkage disequilibrium was observed in 4.3% of the pairwise marker combinations in the BQFS population (nominal $p = 0.01$); no significant deviation from linkage equilibrium was noted in the YNP population (Halbert and Derr 2007).

Bayesian clustering strongly supported seven genetic clusters (Figure 1). Although the Ln $P(D)$ increased slightly with K = 6 clusters, the smaller standard deviation around K = 7 clusters suggested that animals from the BQFS population were not genetically distinct from the established YNP herd (Figure 2). All runs were similar as animals in the BQFS cluster were more than 98.97% associated with the same cluster as YNP for all of the simulations. This confirms that the BQFS animals used were indeed representatives of the YNP population and not migrants from another herd.

We were able to unambiguously identify both sire and dam for 15 (88.24%) of 17 calves born in the spring of 2008 and all 17 calves born in spring of 2009 for Group A. For one of the 2008 unknown calves mentioned (field tag 86-68), the dam was confirmed but paternity was assigned to two potential sires (field tag 03-05, 44.82%; field tag 01-05, 45.03%). Each bull was recognized as having an equal chance of siring the calf. In Group B, matched parent pairs were identified for 12 of 13 calves (92.31%). For the last calf (field tag 9652), genetic mismatches between potential dams and calf led to matches of two potential dams (field tag 52-06, 2 mismatches; field tag 65-06, 3 mismatches), but the sire was unambiguously identified (field tag 58-06). The dam assignment could not be resolved but was decided based on behavioral interactions between one of the dams (field tag 52-06) and the calf. In the total BQFS herd, six of eight adult bulls (75%) accounted for 46 live progeny over the two year quarantine period. The bull that produced the most offspring sired 15 calves (32.61%). The average for the six bulls

siring offspring was 7.67 ± 5.19 offspring/adult male. Overall, the cows averaged 1.34 ± 0.59 offspring/adult female.

For Group A, four out of six bulls (66.67%) produced progeny in at least one breeding season. In 2008, the three bulls that produced offspring sired one, 5.5, and 9.5 calves (6.25%, 34.38%, and 59.38%, respectively). The following year, two males produced a single offspring (5.88%) and another bull produced 15 offspring (88.24%). Only two bulls (33.33%) produced at least one calf in sequential breeding seasons. There were seven (33.33%) cows that raised at least one calf and 13 (61.91%) that raised a calf both years. In Group B, there were only two bulls and both sired multiple calves. Notably, one bull dominated the siring by producing 10 calves (76.92%). Nearly every female (13 of 14 heifers; 92.86%) produced and raised a calf during the 2009 breeding season.

Discussion

Cattle DNA introgession into the bison genome is a common occurrence in North American bison subpopulations (Halbert 2003; Halbert *et al.* 2005; Freese *et al.* 2007; Halbert and Derr 2007). Markers identifying the presence of cattle DNA in bison have been used to determine the protective status and regulatory strategies for each bison herd in the US (Steklenev and Yasinetskaya 1982; Polziehn *et al.* 1995; Rhymer and Simberloff 1996; Ward *et al.* 1999; Halbert 2003; Halbert and Derr 2007; Sanderson *et al.* 2008; Hedrick 2009). Detecting hybridization of bison with cattle is an important factor in choosing animals for conservation herds and preserving purebred bison genetics. Assays using mitochondrial and nuclear DNA markers in this study population supported previous findings that cattle DNA has not introgressed into the YNP herd at a detectable level (Halbert 2003; Halbert *et al.* 2005; Freese *et*

al. 2007; Halbert and Derr 2007). Using both tests ensures the amount of cattle DNA introgression is not underestimated due to lack of detection (Halbert and Derr 2007). The results ensure that satellite herds originating from YNP contain genetically pure animals and future taxonomic status will not be threatened.

Genetic markers have been developed to compare genetic diversity and differentiation in bison herds from around North America and also to accommodate population control measures for each herd (Mommens *et al.* 1998; Wilson and Strobeck 1999; Schnabel *et al.* 2000; Boyd 2003; Halbert 2003; Halbert *et al.* 2004; Freese *et al.* 2007; Sanderson *et al.* 2008; Hedrick 2009). Levels of genetic variation in bison vary among North American herds. Among the herds with increased levels of genetic diversity compared to other herds, YNP has been highlighted as an important reservoir of bison genetic diversity (Halbert 2003; Halbert and Derr 2008; Hedrick 2009) in spite of its brucellosis disease status. Because of its potential to contribute to overall bison genetic diversity, research on the YNP herd has focused on conservation and decreasing the prevalence of brucellosis in the GYA. We found that heterozygosity in the BQFS population was comparable to that found in its parent YNP population despite having a slight decrease in allele preservation. The similar genetic composition between the BQFS satellite herd and its YNP parent herd effectively ensures YNP genetic diversity will continue to be present in the satellite herd. Validation of the YNP origin of individuals within the BQFS herd supports their utilization for satellite herd applications. In the future, another genetic inventory could be taken to determine whether supplemental breeding adults would be beneficial to the overall genetic composition of the herd.

Parentage analyses resulted in information about the breeding system and genetic contribution by males in the BQFS herd. This information is critical for monitoring potential

inbreeding and providing management advice based on minimizing the effects of inbreeding and genetic drift to conserve the diversity of the restored herds (Blouin *et al*. 1996; Frankham *et al.* 2002; Halbert *et al.* 2004). Data showed that nearly all adults were contributing to future generations. In the two quarantined breeding seasons, mating was polygynous as multiple males were confirmed to have sired at least one offspring. It appeared that these bison exhibited a hierarchical mating system where breeding was monopolized by a small number of highly successful males each year as seen in semi-natural bison populations and other wild species such as reindeer (*Rangifer tarandus*), red deer (*Cervus elaphus*), and African buffalo (*Syncerus caffer*; Wolff 1998; Taylor *et al.* 2000; Roden *et al.* 2005; Roden *et al.* 2011). It is promising to note that successful matings occurred for multiple bulls, ensuring genetic admixture for the offspring and promoting the potential to maximize the effective population size of the satellite herd which may subdue the effects of genetic drift for the initial generations.

Through parentage analysis and behavioral observations, we were also able to explain the maternal neglect and subsequent death of one calf (field tag 86-82) observed by researchers (Rebecca Frey, personal communication). Our analysis identified a mis-mothering event where two cows abandoned and did not care for their own calves (Aitken 2011). Because we identified one calf's (field tag 85-12) sire and dam (field tags 03-05 and 82-06, respectively) with high confidence based on our molecular analysis, the observed results suggested that one cow (field tag 12-05) abandoned her own calf (field tag 86-82) only to adopt an unrelated calf to raise. Consequently, the second calf (field tag 86-82) died due to neglect from both dams. This behavior has been recorded in other ruminant species including domestic cattle and sheep (Gonyou and Stookey 1985; Dwyer and Lawrence 2000; Aitken 2011). Due to the limited area in the quarantine study, the bison may not have had adequate area to separate or adequate bonding

time at the time of calving, thus increasing the chances of mis-mothering. Further, the dams had not previously calved which corroborates previous evidence that mis-mothering and offspring rejection appears to be more prevalent in primiparous or inexperienced females (Dwyer and Lawrence 2000).

The results of this study support the efforts of the BQFS conservation method for preserving the genetic diversity of YNP bison. However, limiting factors in animal selection from the parent YNP herd should be considered. As mentioned before, at least two genetically distinguishable breeding groups or subpopulations comprise the YNP herd (Halbert 2003; Halbert *et al.* 2012). The BQFS herd is believed to be represented by the northern YNP herd which has been shown to have slightly lower levels of genetic diversity (Halbert *et al.* 2012). However, it is not clear that these differences in genetic diversity are significant compared to the central herd. Therefore, it is recommended that the same genetic analyses should be conducted on future herds that are subjected to the quarantine protocol to warrant the animals as a viable satellite herd, that is, free of cattle DNA introgression and similar heterozygosity values compared to the parent YNP herd. Re-establishment of a satellite herd may benefit the overall bison genome by preserving some portion of high genetic diversity, but re-establishment also subjects the herd to environmental misfortunes such as natural disasters or unforeseen illness which may lead to local extinction and cause an irreversible allele loss. In addition, negative genetic effects such as genetic drift and inbreeding can be amplified in a herd with a small number of founding individuals. Future genetic analyses of satellite herds could attempt to quantify any loss of genetic diversity, but negative genetic effects may not be measurable for multiple generations.

Management Implications

Using DNA-based analyses to assess the BQFS satellite herd's genetic diversity provided important information for future management and conservation strategies. Together with identifying any domestic cattle DNA introgression, these genetic analyses provided a preliminary assessment of a satellite herd originating from YNP which might be used for conservation of the species and as a model for reintroducing a native species to areas of its historic range. By using genetic analyses when choosing animals to translocate from the founding population to a satellite herd, management teams increase the chances of retaining genetic diversity, minimizing inbreeding, and maximizing genetic variability in future generations. Preserving as much genetic variation as possible within satellite herds will prove vital to the preservation of the bison germplasm.

Satellite herds, like the BQFS herd, founded by animals originating in the YNP herd are subject to strict regulations in terms of translocation and handling due to the potential for *B. abortus* exposure. As such, it is important for managers of these satellite herds to be able to incorporate genetic, epidemiological, and ecological aspects into their conservation strategies. Our preliminary genetic assessment of this study herd supports its use as a satellite herd and using YNP bison as stocking animals for future satellite herds under the condition that the animals complete the quarantine protocol for being declared brucellosis-free, though the quarantine protocol may be cost prohibitive. Further genetic monitoring of these satellite herds could provide management teams with guidelines for translocating animals so as to protect stable genetic diversity and preserve the American bison for many years to come.

Figures

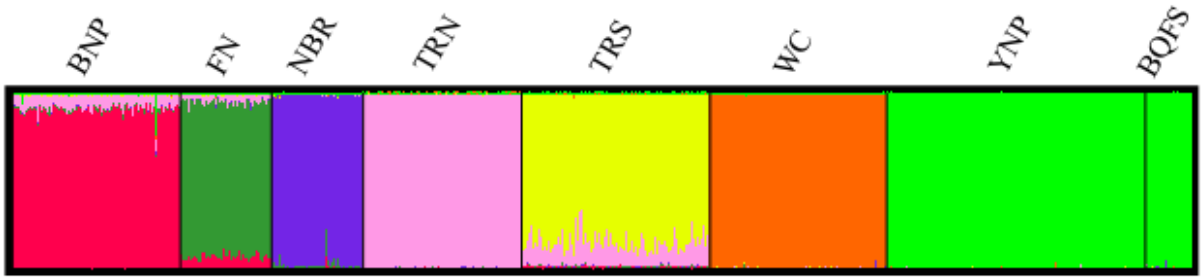

Figure 1. Results of STRUCTURE analysis comparing individual membership proportions of BQFS individuals to seven known bison populations. The colors represent the seven geographically defined populations of bison. Each vertical line represents one individual. The analysis demonstrates the genetic continuity of the BQFS individuals with their parent population (YNP) and also confirms the presence of seven populations of bison.

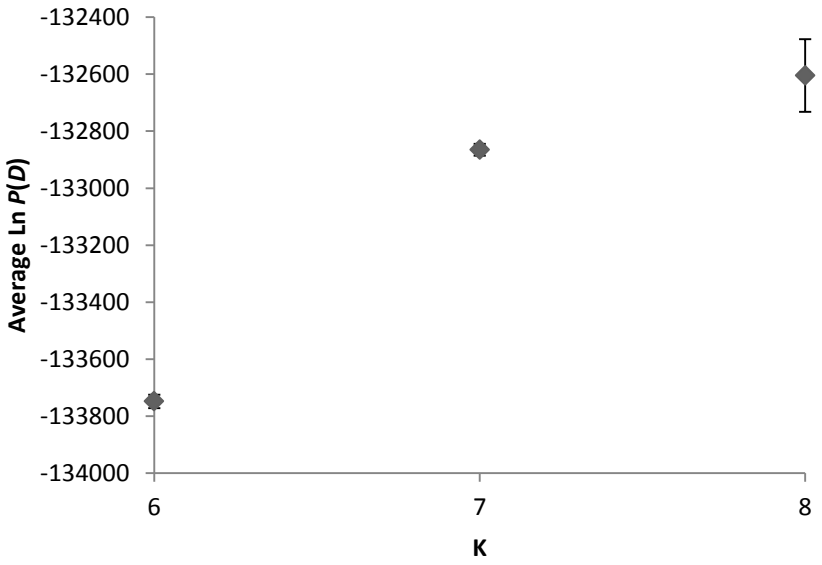

Figure 2. Assessment of STRUCTURE clustering for *K* values ranging from 6 to 8. Averages and standard deviations for Ln *P*(*D*) values based on six simulations for each value of *K* are shown. The model that best fits the data set includes seven genetically defined clusters based on the large average Ln *P*(*D*) for K_7 compared to smaller values of *K* and comparatively small standard deviation of Ln *P*(*D*) for K_7 compared with larger values of *K*.

Tables

Table 4. Sex distribution of live offspring born to Bison Quarantine Feasibility Study groups in 2008 and 2009. [a]

	2008		2009	
	Female	Male	Female	Male
Group A	11	5	10	7
Group B [b]	0	0	5	8
Total	11	5	15	15

[a] Stillbirths were not counted or used in the analyses.

[b] Group B included females that were not pregnant in 2007 and then divided into a separate pasture until palpable pregnancy occurred.

Table 5. Summary information for 28 nuclear microsatellite loci used in this study: range of alleles in base pairs (R_A), number of alleles observed (N_A), and observed heterozygosity (H_O) in Bison Quarantine Feasibility Study (BQFS) and Yellowstone National Park (YNP). Adapted from Halbert *et al.* 2004.

Marker	Chromosome (position)[c]	Label[a]	Multiplex[b]	R_A	$N_{A\text{-}BQFS}$	$N_{A\text{-}YNP}$	$H_{O\text{-}BQFS}$	$H_{O\text{-}YNP}$
BL1036	14 (78.7)	NED	85	177 – 193	4	4	0.73	0.60
BM1225	20 (8.0)	NED	PRTG 2	239 – 273	5	5	0.72	0.69
BM1706	16 (80.6)	6-FAM	PRTG 2	232 – 254	4	5	0.58	0.42
BM17132	19 (58.6)	6-FAM	PRTG 1	85 – 95	5	5	0.71	0.71
BM1862	17 (86.3)	6-FAM	80	201 – 215	5	5	0.74	0.71
BM1905	23 (64.3)	NED	PRTG 2	172 – 184	3	3	0.33	0.36
BM2113	2 (106.2)	6-FAM	PRTG 2	127 – 153	4	4	0.54	0.57
BM4107	20 (52.4)	HEX	85	159 – 185	5	5	0.69	0.63
BM4311	6 (89.7)	6-FAM	82	90 – 104	6	6	0.76	0.73
BM4440	2 (55.0)	NED	PRTG 2	123 – 143	4	5	0.62	0.61
BM47	23 (9.1)	6-FAM	85	103 – 111	2	3	0.20	0.20
BM6017	X (4.7)	HEX	82	104 – 122	5	5	0.44[d]	0.45[d]
BM711	8 (83.6)	6-FAM	82	161 – 177	4	4	0.51	0.54
BM720	13 (38.6)	VIC	PRTG 2	203 – 235	6	7	0.91	0.78
BMS1001	27 (5.1)	NED	80	107 – 115	5	5	0.65	0.64
BMS1074	4 (74.9)	NED	80	152 – 160	4	4	0.61	0.57
BMS1315	5 (31.8)	HEX	85	135 – 149	4	4	0.70	0.64
BMS1675	27 (64.1)	6-FAM	80	85 – 91	3	3	0.33	0.41
BMS1716	11 (47.7)	HEX	80	185 – 197	3	4	0.39	0.40
BMS1857	29 (0.9)	6-FAM	85	142 – 168	6	6	0.76	0.78
BMS410	12 (0.0)	NED	PRTG 1	83 – 97	4	4	0.65	0.63
BMS510	28 (22.1)	VIC	PRTG 1	91 – 95	4	4	0.79	0.68
BMS527	1 (55.9)	6-FAM	PRTG 1	159 – 177	5	6	0.70	0.68
HUJ246	3 (67.9)	NED	80	242 – 264	4	4	0.61	0.58
ILSTS102	25 (6.5)	NED	85	113 – 153	3	3	0.61	0.61
INRA189	Y	NED	82	96	1	1	—	—
RM372	8 (19.1)	VIC	PRTG 1	114 – 138	5	6	0.70	0.70
TGLA122	21 (67.3)	NED	82	136 – 150	4	6	0.66	0.79
Average					4.18	4.50	0.62	0.60
Standard deviation					1.16	1.26	0.16	0.14

[a] Flourescent label used with forward primer (Applied Biosystems).

[b] Thermocycler parameters: 96°C 3 min; 4 cycles of 96°C 20 s, 58°C 30 s (-1°C/cycle), 65°C 90 s; 26 cycles of 96°C 20 s, 54°C 30 s, 65°C 90 s; 1 cycle of 96°C 60 s, 54°C 60 s, 65°C 20 min.

[c] As reported in the USDA cattle gene mapping database.

[d] Calculated on female population only.

Table 6. Seven established North American bison populations used to compare genetic characteristics with the Bison Quarantine Feasibility Study (BQFS) herd. All herds were used to compare to genomes of BQFS individuals and establish relationships based on genetic clusters. Heterozygosity and alleles present in YNP samples were also used to compare with BQFS herd individuals. These samples are archived at Texas A&M University. [a]

Herd name	Abbreviation	Location	Sample size
Badlands National Park	BNP	South Dakota	328
Fort Niabrara National Wildlife Reserve	FN	Nebraska	178
National Bison Range	NBR	Montana	179
Theodore Roosevelt National Park - North Unit	TRN	North Dakota	309
Theodore Roosevelt National Park - South Unit	TRS	North Dakota	368
Wind Cave National Park	WC	South Dakota	345
Yellowstone National Park	YNP	Wyoming, Idaho, Montana	505

[a] Genetic data was used with permission (Natalie Halbert, personal communication).

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CHAPTER IV

MANAGEMENT IMPLICATIONS AND FUTURE CONSIDERATIONS

Managing wild populations and their diseases within the boundaries of national parks and other protected areas is challenging. Brucellosis is a prime example. Even though disease agent hosts such as bison or elk are restricted to YNP and the surrounding GYA, *Brucella abortus* bacterial agents are not limited by those boundaries. In some instances, such as brucellosis, human intervention may be justifiable in order to protect native populations, domestic animals, and humans from becoming infected with the pathogen. Significant management concerns are stimulated by wildlife diseases due to increasing interactions between wildlife and domestic animals (Aguirre and Starkey 1994).

Management practices for bison in YNP have long been debated between government officials and the public community. The YNP herd is subjected to management strategies that may negatively affect the retention of genetic diversity due to its brucellosis status. Controlling potentially brucellosis infected bison as they travel beyond YNP's borders remains a difficult task. Key considerations should include protecting the integrity of this historically and genetically important herd in conjunction with protecting uninfected animals and environments from the possibility of contracting infection through *B. abortus* transmission.

With such importance on preventing the transmission of *B. abortus* bacteria from infected animals residing in YNP and the GYA, it is understandable the number of regulatory approaches that have been attempted. Culling animals that venture outside the park's boundaries, vaccination protocols, or spatial separation between wildlife and livestock are just a few of the different practices. These methods have demonstrated an increased potential in retaining *B. abortus*

infection and subsequent brucellosis development to the GYA and may often involve the cooperation of many persons and agencies on the local, state, and national levels.

With a system such as the BQFS protocol used to determine YNP bison as free of brucellosis, it opens up the potential for using animals from this herd to stock conservation herds around the US. However, implementing the quarantine protocol for future satellite herds may be cost-prohibitive depending on available resources. Genetic analyses used in this study demonstrate a reliable method of quantifying the conservation herd's founder population genetics. High heterozygosity levels complemented the high genetic diversity levels and provide a positive outlook on the preservation of YNP genetics in smaller conservation herds. The analyses ensure the satellite herd as being genetically pure and without any detectable evidence of cattle DNA introgression which will aid in the herd's protective status in the future. The first group of BQFS animals has demonstrated a strong genetic foundation to initiate a conservation satellite herd in the future. If the quarantine procedures can continue to produce groups of animals declared free of brucellosis, further genetic analyses should be implemented in an attempt to introduce sustainable genetic diversity along with no cattle DNA introgression, in addition to the serological testing and removal of seropositive animals.

Genetic approaches used for identifying genetic natural resistance to brucellosis could be a valuable complement to the management of the disease in the GYA. Bison that are already being tested for brucellosis could also have a genetic screen completed to aid in culling decisions. In the present study, the bison PRNP genotype alone was not able to confidently determine susceptible or resistance genotypes to brucellosis in bison. However, it is possible that the bison PRNP may still be an integral part to an unknown genetic cascade determining natural resistance to brucellosis. A more comprehensive genetic analysis should be conducted across the

bison genome to resolve any discrepancies in the specific role of the bison PRNP in natural resistance. If a genetic cascade were found to confidently predict susceptibility or resistance to this disease, a long term study would be needed to note any physiological or genetic consequences in selecting for the resistant genes. Identifying genetic natural resistance has the potential to be a beneficial aide to YNP bison management decisions.

The BQFS herd provides unique potential to introducing brucellosis-free herds of YNP bison to areas of the species' historic range. As upcoming groups of animals pass through the quarantine stage, it is important to also monitor genetic aspects of the herd in hopes of releasing animals with high genetic diversity and no cattle DNA introgression. If this model can be followed in the future, the genetic and historic heritage of the YNP herd can be carried on for years to come without the threat of brucellosis which has afflicted the herd for nearly a century.

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APPENDIX I

Population dynamics of Bison Quarantine Feasibility Study animals

Field tag identification numbers of all BQFS samples are listed below in addition to sex, birth year, origin of animal, and parentage. Birth year was estimated for wild caught animals. Parentage was not known for wild caught animals but was determined through genetic analyses for animals born during the quarantine period.

Group	Field Tag	Sex	Birth year	Origin	Dam	Sire
A	01-05	M	2004	Wild caught	Unknown	Unknown
A	02-05	F	2004	Wild caught	Unknown	Unknown
A	03-05	M	2004	Wild caught	Unknown	Unknown
A	07-05	M	2004	Wild caught	Unknown	Unknown
A	11-05	F	2004	Wild caught	Unknown	Unknown
A	12-05	F	2004	Wild caught	Unknown	Unknown
A	14-05	F	2004	Wild caught	Unknown	Unknown
A	15-05	F	2004	Wild caught	Unknown	Unknown
A	16-05	M	2004	Wild caught	Unknown	Unknown
A	06-06	F	2005	Wild caught	Unknown	Unknown
A	13-06	F	2005	Wild caught	Unknown	Unknown
A	17-06	F	2005	Wild caught	Unknown	Unknown
A	19-06	F	2005	Wild caught	Unknown	Unknown
A	22-06	F	2005	Wild caught	Unknown	Unknown
A	23-06	M	2005	Wild caught	Unknown	Unknown
A	37-06	F	2005	Wild caught	Unknown	Unknown
A	39-06	F	2005	Wild caught	Unknown	Unknown
A	44-06	M	2005	Wild caught	Unknown	Unknown
A	46-06	F	2005	Wild caught	Unknown	Unknown
A	50-06	F	2005	Wild caught	Unknown	Unknown
A	55-06	F	2005	Wild caught	Unknown	Unknown
A	57-06	F	2005	Wild caught	Unknown	Unknown
A	64-06	F	2005	Wild caught	Unknown	Unknown
A	68-06	F	2005	Wild caught	Unknown	Unknown
A	73-06	F	2005	Wild caught	Unknown	Unknown
A	75-06	F	2005	Wild caught	Unknown	Unknown
A	82-06	F	2005	Wild caught	Unknown	Unknown

A	85-02	F	2008	Born in quarantine	02-05	01-05
A	85-12	F	2008	Born in quarantine	82-06	03-05
A	85-14	F	2008	Born in quarantine	14-05	01-05
A	85-15	M	2008	Born in quarantine	15-05	03-05
A	86-13	F	2008	Born in quarantine	13-06	01-05
A	86-17	F	2008	Born in quarantine	17-06	03-05
A	86-19	M	2008	Born in quarantine	19-06	01-05
A	86-22	F	2008	Born in quarantine	22-06	03-05
A	86-37	M	2008	Born in quarantine	37-06	03-05
A	8639	F	2008	Born in quarantine	39-06	16-05
A	86-46	F	2008	Born in quarantine	46-06	01-05
A	86-50	F	2008	Born in quarantine	50-06	01-05
A	86-55	M	2008	Born in quarantine	55-06	01-05
A	8657	M	2008	Born in quarantine	57-06	01-05
A	86-68	F	2008	Born in quarantine	68-06	Unknown [a]
A	86-73	F	2008	Born in quarantine	73-06	01-05
A	86-82 [b]	M	2008	Born in quarantine	Unknown	Unknown
A	01-09	F	2009	Born in quarantine	12-05	07-05
A	02-09	M	2009	Born in quarantine	15-05	07-05
A	03-09	M	2009	Born in quarantine	39-06	07-05
A	04-09	F	2009	Born in quarantine	22-06	03-05
A	05-09	M	2009	Born in quarantine	82-06	07-05
A	06-09	F	2009	Born in quarantine	11-05	07-05
A	07-09	F	2009	Born in quarantine	17-06	07-05
A	08-09	F	2009	Born in quarantine	50-06	07-05
A	09-09	M	2009	Born in quarantine	06-06	07-05
A	10-09	F	2009	Born in quarantine	02-05	01-05
A	11-09	M	2009	Born in quarantine	14-05	07-05
A	12-09	M	2009	Born in quarantine	55-06	07-05
A	13-09	F	2009	Born in quarantine	75-06	07-05
A	14-09	F	2009	Born in quarantine	68-06	07-05
A	15-09	M	2009	Born in quarantine	13-06	07-05
A	16-09	F	2009	Born in quarantine	37-06	07-05
A	17-09	F	2009	Born in quarantine	73-06	07-05
B	02-06	F	2005	Wild caught	Unknown	Unknown
B	04-06	F	2005	Wild caught	Unknown	Unknown
B	05-06	F	2005	Wild caught	Unknown	Unknown
B	08-06	F	2005	Wild caught	Unknown	Unknown
B	12-06	F	2005	Wild caught	Unknown	Unknown
B	20-06	F	2005	Wild caught	Unknown	Unknown
B	31-06	F	2005	Wild caught	Unknown	Unknown
B	33-06	F	2005	Wild caught	Unknown	Unknown
B	45-06	M	2005	Wild caught	Unknown	Unknown

B	52-06	F	2005	Wild caught	Unknown	Unknown
B	53-06	F	2005	Wild caught	Unknown	Unknown
B	58-06	M	2005	Wild caught	Unknown	Unknown
B	65-06	F	2005	Wild caught	Unknown	Unknown
B	66-06	F	2005	Wild caught	Unknown	Unknown
B	71-06	F	2005	Wild caught	Unknown	Unknown
B	81-06	F	2005	Wild caught	Unknown	Unknown
B	9602	F	2009	Born in quarantine	02-06	58-06
B	9604	F	2009	Born in quarantine	04-06	58-06
B	9605	F	2009	Born in quarantine	05-06	58-06
B	9608	M	2009	Born in quarantine	08-06	45-06
B	9612	M	2009	Born in quarantine	12-06	58-06
B	9620	M	2009	Born in quarantine	20-06	45-06
B	9631	M	2009	Born in quarantine	31-06	45-06
B	9633	F	2009	Born in quarantine	33-06	58-06
B	9652	M	2009	Born in quarantine	52-06 [c]	58-06
B	9653	F	2009	Born in quarantine	53-06	58-06
B	9666	M	2009	Born in quarantine	66-06	58-06
B	9671	M	2009	Born in quarantine	71-06	58-06
B	9681	M	2009	Born in quarantine	81-06	58-06

[a] Two potential sires were determined by the parentage analyses as having an equal chance of siring the calf (field tags 01-05 and 03-05)

[b] Calf died from neglect before a DNA sample could be collected. No genetic analyses were completed.
[c] Dam assignment could not be resolved due to genetic mismatches with the calf. Behavioral interactions decided dam-calf pairing.

LIST OF COMMON ABBREVIATIONS

Abbreviation	**Description**
APHIS	Animal and Plant Health Inspection Service
BNP	Badlands National Park
BQFS	Bison Quarantine Feasibility Study
FN	Fort Niabrara National Wildlife Reserve
GYIBC	Greater Yellowstone Interagency Brucellosis Committee
GYA	Greater Yellowstone Area
IBMP	Interagency Bison Management Plan
ID	Idaho, USA
MT	Montana, USA
NBR	National Bison Range
NRAMP1	Natural Resistance Associated Macrophage Protein Gene
NVSL	National Veterinary Services Laboratories
PRNP	Prion Protein Gene
SNP	Single Nucleotide Polymorphism
TRN	Theodore Roosevelt National Park – North Unit
TRS	Theodore Roosevelt National Park – South Unit
USDA	Unites States Department of Agriculture
WC	Wind Cave National Park
WY	Wyoming, USA
YNP	Yellowstone National Park