GENETIC DIVERSITY, STRUCTURE, AND HYBRIDIZATION IN A HARVESTED GRAY WOLF (*Canis lupus*) POPULATION IN MINNESOTA

A THESIS
SUBMITTED TO THE FACULTY OF
UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Advisors, Dr. Jared L. Strasburg and Dr. Ronald A. Moen

December 2015

Acknowledgements

I would like to start by thanking my two advisors, Dr. Ron Moen and Dr. Jared Strasburg, for their invaluable guidance and support throughout my time at UMD and in working on this research. I would also like to thank my committee members, Dr. Briana Gross, Dr. John Pastor, and Dr. John Erb, for discussions and comments in the planning, execution, and write-up of this research. Additionally, I would like to thank Dr. John Erb and the Minnesota Department of Natural Resources for sharing the samples used in these studies. I am also thankful to the Department of Biology at UMD for providing support as a teaching assistant throughout my time at UMD. The Swenson College of Science and Engineering and Integrated Biosciences programs, in addition to The Wildlife Society, provided funding for me to travel to conferences and share my research with other researchers and professionals, opportunities for which I am extremely grateful.

This research would not have been possible without the in-laboratory support of Abby Schumann and several Lee Lab undergraduates, including Stephanie Grewenow, John Statz, Kara Werner, and Joe Burger. It also would not have been possible without the support and encouragement of my fellow Lee Lab graduate student Tessa Tjepkes, with whom I spent more time than I thought possible throughout our time at UMD.

Finally, I would like to thank the many and wonderful members of my Integrated Biosciences cohort (and those in cohorts above and below me) who became my close friends during our time at UMD. You are some of the kindest, most amazing, and most inspiring people that I have ever met, and I know that I would not have made it through without the mutual support, shared frustrations, and ever-present laughter.

Dedication

This thesis is dedicated to my amazing family, who supported me in every way and made it possible for me to complete this research. In particular, this work is dedicated to my grandpa, Bob Rick, who initially sparked my interest in the natural world, taught me the importance of a connection to nature, and continuously encouraged me to pursue my interests, and with whom I enjoyed many insightful discussions about my research.

Abstract

The demographic, social, and genetic effects of harvest-based management practices are not fully understood, especially in social carnivore species. Minnesota was one of several states that instituted a public hunting and trapping season to manage gray wolves (*Canis lupus*) following the delisting of wolves from the Endangered Species Act in 2012. Hunters and trappers harvested 413 wolves in Minnesota in 2012, about three times the average number of wolves removed annually under depredation control in previous years. Using tissue from wolves harvested during the 2012 and 2013 seasons in Minnesota, I assessed the population genetic consequences of this increase in anthropogenic mortality to determine if the harvest led to changes in population genetic structure and diversity in the first post-harvest year. I also sequenced a portion of the mitochondrial DNA (mtDNA) control region to assess the extent of gray wolf-eastern wolf (*C. lycaon)* and gray wolf-coyote (*C. latrans*) hybrid ancestry in Minnesota wolves. I found no significant difference in genetic diversity indices or mtDNA haplotype frequencies between years; however, population genetic structure and effective gene flow among the sampled wolves changed from 2012 to 2013. These analyses provide a baseline to determine variation in structure between years is normal for Minnesota wolves and how changes in genetic structure positively or negatively impact wolf populations. Baseline population genetic analysis at the beginning of managed harvest enabled my analysis of initial genetic responses to harvest, and will allow for comparisons with the population genetic structure of historical and future wolf populations in Minnesota.

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CHAPTER 1: THE POPULATION, PACK, AND INDIVIDUAL-LEVEL EFFECTS OF ANTHROPOGENIC HARVEST ON WOLVES

Introduction

As anthropogenic effects on the natural world increase, wildlife conservation becomes increasingly important. Several success stories exist where conservation efforts have led to achieving restoration goals. In the United States, one of these success stories is the gray wolf (*Canis lupus*). Throughout the country's history, the wolf was hunted and trapped, resulting in near extirpation of the species from the contiguous United States (Boitani 2003). In 1973, the Endangered Species Act (ESA) was signed into law; *C. lupus* became protected under this legislation in 1978. As a result of federal protections, gray wolf populations in the western Great Lakes region (Minnesota, Wisconsin, and Michigan) and the northern Rocky Mountain region (Wyoming, Idaho, and Montana) recovered to ESA-designated recovery population sizes and were delisted from the ESA in 2012 (Creel & Rotella 2010; Gude *et al.* 2012; Bruskotter *et al.* 2014; Erb & Sampson 2013). Management of recovered wolf populations was transferred from the federal government to individual state agencies[1]. Many of these agencies have introduced regulated annual wolf harvest seasons to manage wolf populations and reduce human-wolf conflicts.

The harvest of mammalian carnivores can have cascading effects on the ecosystems to which they belong. In certain cases, loss of these top predators can lead to loss of ecosystem resilience (Estes *et al.* 2011), invasion of non-native species (Wallach *et al.* 2010), or a decline in biodiversity, termed "ecological meltdown" in extreme cases (Terborgh *et al.* 2001). These ecosystem-wide effects have been relatively well-studied, especially with regard to wolves. Two well-known examples have come from the colonization of wolves on Isle Royale (MI), and the extirpation and subsequent recolonization of wolves in Yellowstone National Park, where the restored presence of gray wolves has led to greater ecosystem health (Smith *et al.* 2003; Ripple & Beschta 2012). In both of these parks, the ecosystems existed wolf-free for many years. The years

[1] In December 2014, the delisting decision was overturned for the Great Lakes distinct population segment, and management returned to the federal government.

without top predators led to these systems becoming dominated by large populations of ungulates, which in turn decimated native vegetation, affecting nutrient cycling throughout the ecosystems (McLaren & Peterson 1994; Smith *et al.* 2003). Although these effects occur when predator populations decline naturally, humans can play a role in disrupting the natural system if they raise or lower mortality rates beyond natural rates or by disrupting the stability of populations in other ways, such as causing habitat loss and fragmentation.

Harvest management usually focuses on numeric population levels. However, harvest has additional effects at higher and lower levels of biological organization, including the ecosystem, the pack, and the individual level. Though much energy has been put into research on the ecology, social structure, and dynamics of wolf populations (Fritts *et al.* 2003), little research has focused on the total effect of anthropogenic harvest on wolves. Wolf populations generally recover quickly from disturbance events unless wolves are extirpated from an area (Creel & Rotella 2010; Gude *et al.* 2012), indicating strong compensatory abilities. However, there are processes affecting wolves in harvested populations outside of the ability to return to pre-harvest population sizes. Wolves maintain a complex social structure and therefore measures of abundance do not capture all impacts of harvest or the interactions between effects at the population, pack, and individual levels (Mech & Boitani 2003). Recent research has shed light on demographic and behavioral responses to and consequences of hunting and trapping (Table 1.1), yet it remains unclear how these responses interact and whether interactions change at different harvest intensities.

With the recently established wolf harvest in some of the contiguous United States, it is necessary to understand the effects that hunting may have outside of temporarily reducing population sizes. In the sections that follow, I synthesize recent research comparing harvested to protected wolf populations, in an effort to understand demographic and behavioral responses and predict potential positive or negative effects of harvest on previously protected populations of wolves. I then highlight areas requiring further research, with a focus on how molecular genetic techniques can aid wolf management

and population monitoring efforts related to the indirect effects of anthropogenic harvest on these social carnivores.

Unharvested wolf populations

In gray wolves, a pack generally consists of an unrelated breeding pair and their offspring from multiple years (Mech & Boitani 2003). Packs function as dispersal pumps that produce offspring, care for pups until maturity (12-36 months), and then release them to find their own packs and territories (Mech & Boitani 2003). Wolf pups generally remain in their natal packs until intrapack competition for food resources forces them to disperse. Older wolves tend to be dominant over younger wolves, and subordinate wolves disperse first when food resources become limiting. This leaves only the two breeding individuals and a small number of offspring in years when prey is extremely scarce (Mech & Boitani 2003). On rare occasions, unrelated dispersers are accepted into packs; in unharvested populations in Minnesota and Denali National Park, 3 of 13 packs and 2 of 13 packs respectively had more than two unrelated individuals, though other studies suggest that this is high for protected populations (Lehman *et al.* 1992; Meier *et al.* 1995; Packard 2003). However, detection of adoptees[2] requires sampling of entire packs, which is uncommon in most studies.

Wolf dispersal is regulated via both positive and negative feedbacks. Dispersal in wolves is stimulated by competition for resources, including the absence of potential mates and the presence of established packs. Wolves disperse to find mates, either by meeting another dispersing individual or by being adopted into an unrelated pack. Several studies suggest that wolves dispersing shorter distances have a higher probability of successful territory establishment and reproduction than those that disperse further away (Caniglia *et al.* 2014; Leonard 2014). Long-distance dispersal is common during the early phases of colonization (Mech & Boitani 2003, and references therein), presumably due to low wolf

[2] "Adoption" is a term used in the literature for unrelated individuals (adoptees) that join a pack already containing a breeding pair, and does not include individuals joining a pack to replace a lost breeder (Mech & Boitani 2003).

density and to avoid inbreeding (due to a high probability of wolves in the same area being closely related to one another). Wolves then disperse shorter distances as pack densities increase, creating a positive feedback on pack density in the area. Regulatory feedbacks and environmental carrying capacity often appear to restrict wolf density to an average of 40 wolves per 1,000 km^2, though specific maximum densities vary widely by region (Fuller *et al.* 2003).

Packs are arranged in a mosaic of adjacent, non-overlapping territories that tend to be relatively stable (Mech & Boitani 2003). Pack social structure benefits individual wolves by increasing their likelihood of surviving aggressive encounters (Mech & Boitani 2003), increasing hunting success (Sand *et al.* 2006), increasing pup survival rate (Brainerd *et al.* 2008), and generally making resource use more efficient (Stahler *et al.* 2006). Wolves living in stable packs have lower stress levels (as measured by glucocorticoid hormone levels) than lone wolves, which also increases reproductive success (Sands & Creel 2004). Packs compete with neighboring packs for resources and habitat, resulting in dynamic territory boundaries (Fritts & Mech 1981). Despite stochastic fluctuations in territory boundaries, territories largely remain stable within the territorial mosaic. Territoriality reduces the amount of energy that an animal or group of animals expends in competing with conspecifics, both for food resources and for breeding opportunities (Verner 1977), and thus a stable territorial mosaic benefits the individuals living within it.

In forming a network of wolf territories across a landscape, environmental carrying capacity and wolf territoriality provide the two largest negative feedbacks. Wolf pack size is limited by prey abundance within the pack's territory (Fuller 1989; cf. Cariappa *et al.* 2011; McRoberts & Mech 2014). Areas with higher prey density support packs with smaller territory sizes, and territory sizes often scale with prey density (Kittle *et al.* 2015). During colonization, the prey to wolf ratio tends to be high, and new packs are likely to establish territories, reducing the size of already-established pack territories. However, once territories reach a certain minimum size, wolves become more territorial (Cubaynes *et al.* 2014). Territoriality is energetically costly but becomes increasingly beneficial as the area being defended decreases (Kittle *et al.* 2015). Established packs use

scent and vocalization to deter other wolves from intruding into their home range (Mech & Boitani 2003). Field observations by Fritts and Mech (1981) demonstrate territorial patterning, with inter-pack buffer zones and an increase in scent marking behavior along territory boundaries. Lewis and Murray (1993) modeled the formation of a territorial mosaic as a function of wolf movement and behavior in response to the presence of other packs and foreign scent marks, demonstrating that foreign scent marks acting as a negative feedback on wolf movement is consistent with the previously observed territory patterns.

Effects on the individual wolf

Hunting pressures pose different levels of risk to wolves of different age class and status. Dispersing wolves comprise most of the harvest in many hunted populations (Murray *et al.* 2010), although the loss of breeding individuals has a disproportionately large effect when compared to non-breeding individuals (Brainerd *et al.* 2008). Juveniles and dispersers are more at risk of harvest-related mortality than those remaining in their packs. Lone wolves have lower hunting success and increased mortality in general, and this is accentuated in harvested populations (Murray *et al.* 2010). Dispersers have little knowledge about the areas they are traveling through and often are forced to travel through unfavorable territory in the interstices of the territorial mosaic to avoid encountering packs in occupied territories (Mech 1994; Mladenoff *et al.* 1995). These areas may also have higher human and road densities and thus increased mortality risk.

Individual wolf dispersal may be affected by hunting pressures via decreases in wolf densities, reductions in pup survival, decreases in competition, and ultimately increases in prey availability. Webb *et al.* (2011) found low dispersal rates in a population of hunted wolves in Alberta, Canada, when compared to protected wolf populations nearby. Mortality of other wolves decreases wolf densities, thereby increasing food availability for each wolf and reducing the impetus to disperse (Benson *et al.* 2014). In a heavily exploited population in northwest Alaska, the average age of dispersal increased from around one year to around three years of age (Ballard *et al.* 1997). Juveniles are allowed

to remain in their natal packs longer due to low pup survival in hunted populations. Fewer offspring surviving in the current year results in a pack territory being able to support more offspring from previous years (Mech & Boitani 2003). Hunting-related mortality can reduce individual reproductive rates as a result of stress (Rutledge *et al.* 2010), increases pack instability, and may cause infanticide by dominant females (Packard 2003). Increased stress in reproductive females affects the fitness of offspring, resulting in lower pup survival rates as hunting pressure increases (Hayes & Harestad 2000).

Harvest-related mortality of breeding individuals can benefit other pack members and dispersers from other populations by increasing the probability of becoming a breeder in an established pack. In addition, harvest-related pack dissolution can mean more potential mates and more high-quality habitat available for establishing territories. This reorganization of packs and territories can then lead to more random mating within the population, increasing genetic homogeneity and distributing alleles throughout the population. It also can increase the proportion of breeding individuals in the population, thus increasing the effective population size. Though reduced effective population sizes are typical for other harvested species (Harris *et al.* 2002), gray wolf effective population sizes could theoretically increase with harvest.

If densities are reduced and there is not a sufficient number of wolves in an area, individuals may choose less fit mates, such as close relatives (Rutledge *et al.* 2010), or unfit mates from other sympatric species, such as coyotes (*Canis latrans*), golden jackals (*C. aureus*), or feral dogs (*C. lupus familiaris*) (Randi *et al.* 2000; Hailer & Leonard 2008; Benson *et al.* 2014; Moura *et al.* 2014). In the wild, viable hybrids can backcross with gray wolves and introduce new genetic material into the population's gene pool (Stronen & Paquet 2013). Introgression resulting from hybridization and subsequent backcrossing has been observed between canid species in North America and Europe (Hailer & Leonard 2008; Benson *et al.* 2014; Randi *et al.* 2000; Moura *et al.* 2014). However, the frequency of such interspecific mating is unknown.

Effects on pack structure and dynamics

When pack members are lost to harvest, pack size can be maintained by replacing lost individuals. Pack size is important to hunting success (Sand *et al.* 2006), pup survival (Harrington *et al.* 1983; Borg *et al.* 2014), and territory defense (Mech 1994; Kittle *et al.* 2015). Packs can recover from the loss of members by replacing lost breeders, increasing reproduction, decreasing dispersal, and/or adopting unrelated dispersers. If packs are unable to return to a sufficient pack size, then harvest-related mortalities may result in pack dissolution and territory turnover. The threshold for size-related pack stability is around five individuals left after harvest mortalities (Brainerd *et al.* 2008), though this number varies among populations.

One of the greatest impacts that human harvest has on wolf packs is the removal of breeding individuals from the population. The loss of breeders disproportionately affects packs due to the importance of breeders in pack social structure (Mech & Boitani 2003; Borg *et al.* 2014). Packs that lose one or both breeders often fragment or completely dissolve (Adams *et al.* 2008). This risk is higher when both breeders are lost. In a meta-analysis of studies, Brainerd *et al.* (2008) found that 56% of packs recovered and successfully produced pups the year following the loss of a single breeder, whereas packs remained together in only 9% of the cases when both breeders were lost. When a female breeder is lost, the replacement is most often a daughter promoted to breeder status from within the pack (Jędrzejewski *et al.* 2005; Borg *et al.* 2014). Replacements for male breeders, on the other hand, are usually unrelated dispersers from other packs (Fritts & Mech 1981; Jędrzejewski *et al.* 2005).

When breeders are not lost, packs compensate for harvested members through increased reproduction. Mean litter size is largely density-dependent such that decreased wolf densities in the population result in increased litter sizes (Fuller *et al.* 2003; Sidorovich *et al.* 2007). The number of litters produced in a pack and in the larger population also increases if anthropogenic mortality increases. In harvested populations, females other than the dominant female produce litters in a small proportion of packs (up to 7-10%;

Ballard *et al.* 1987), whereas extra-breeder reproductions generally do not occur in stable, unharvested populations (Hayes & Harestad 2000; cf. Vonholdt *et al.* 2008; Stenglein *et al.* 2011). This has been attributed to a combination of instability and an increase in per-capita prey availability (Woolpy 1968). These extra litters, however, tend to have lower survival rates due to increased pack instability and maternal stress (Bryan *et al*. 2014; Sparkman *et al.* 2011). Compensatory reproduction aids in balancing out harvest-related mortality, as demonstrated in models of pack responses to varying levels of hunting pressure (Gude *et al.* 2012).

Compensatory reproduction is not an immediate solution to increased mortality. To more rapidly compensate for lost pack members, packs sometimes allow unrelated lone wolves to join. Adoption of unrelated individuals seldom occurs in unharvested packs (Lehman *et al.* 1992; Vonholdt *et al.* 2008; Stenglein *et al.* 2011), and most adoptees are young male dispersers (Meier *et al.* 1995). Exploited populations tend to have more female adoptees, as well as a high proportion of packs with adoptees (7-11%; Grewal *et al.* 2004; Jędrzejewski *et al.* 2005). Adoption rates as high as 80% of packs were observed in a heavily harvested population in Ontario (Rutledge *et al.* 2010). This high adoption rate also results from the higher number of transient wolves of both sexes seeking packs to join following harvest-related pack dissolution (Jędrzejewski *et al.* 2005). While many of these transient wolves will establish packs of their own, others will be adopted into an existing pack to increase pack size following harvest. One significant result of increased adoption rates is decreased relatedness within these packs when compared to packs in protected areas (Rutledge *et al.* 2010). Adoption further reduces relatedness by increasing immigration rates into populations experiencing greater hunting pressures. Several studies have found unexpectedly high levels of genetic diversity in relatively small wolf packs experiencing harvest pressure (e.g. Pilot *et al.* 2006; Rutledge *et al.* 2010; Hindrikson *et al.* 2013; Stronen *et al.* 2013). The prevalence of adoption also depends on the pattern of wolf harvest in the population. If many breeding wolves are harvested, there will theoretically be high rates of pack dissolution and many transient wolves in the population. If harvest randomly removes individuals from different packs, most of the

established packs will remain stable and adoption will be more common. On the other hand, if whole packs of wolves are harvested, there will be more holes in the territorial mosaic to fill and more opportunities for transient wolves to establish packs of their own.

Although some wolves become transient as a result of pack dissolution, harvest pressure reduces natal dispersal in packs that remain intact. Decreased wolf densities due to hunting pressures increase per-capita prey availability and reduce the need for juveniles to disperse from their natal territories (Benson *et al.* 2014). For this reason, wolves in hunted areas are more likely to delay natal dispersal. Delayed dispersal is also beneficial to the breeding pair, which depends on these individuals to help raise the youngest pups (Harrington *et al.* 1983; Sand *et al.* 2006). With fewer wolves leaving the population, at least some of the harvest-related loss is offset via these alterations to dispersal patterns.

Hunting pressure has an additional effect on pack stability via its influence on territory size. Although many factors influence a pack's territory size and many of these influences are poorly understood (Mech & Boitani 2003), the intensity of hunting pressure had a direct correlation to territory size in a Montana population of wolves (Rich *et al.* 2012). In comparing management districts, Rich *et al.* (2012) found larger pack territory sizes in areas with large harvest quotas. Stronger hunting pressure results in fewer packs in the area, which allows for increased territory sizes. Rich *et al.* (2012) further suggest that areas of higher harvest correspond to areas with lower prey abundance due to a correlation between high deer harvest and high wolf harvest, thereby necessitating larger territories to support each wolf pack. Such shifts in territory sizes alter the dynamics within a wolf pack, as well as those among packs as they interact across the landscape.

Harvest and the wolf population

Just as pack social structure affects pack stability and fitness, the interactions between packs affect the stability and fitness of the larger population. It is often difficult to delineate distinct wolf populations due to high rates of dispersal, immigration, and emigration (Fuller *et al.* 2003). In stable wolf populations, pack territories are non-

overlapping and adjacent to one another. The population is then the wolves living in a collection of territories adjacent to one another. Populations of wolves are affected by hunting via immigration, emigration, and territory turnover rates. Wolves can disperse long distances (>800km; Fritts 1983) and there are few landscape features that serve as significant barriers to dispersal, so populations tend to remain connected to one another via immigration and emigration over time regardless of harvest pressures.

The balance between dispersal and emigration/immigration dictates kinship within and relatedness among packs within a population. In stable, protected wolf populations or those with low levels of harvest, packs tend to be genetically related to nearby packs. At low levels of harvest, local dispersers fill empty spots in nearby packs, allowing the packs to maintain local relatedness (Adams *et al.* 2008) and local patterns of isolation by distance (IBD), where genetic similarity decreases with increasing geographic distance. With more intense harvest, such as in a heavily controlled population in Inuvik, Canada, local inter-pack genetic relatedness and IBD decrease (Lehman *et al.* 1992). Based on these and other studies, it has been hypothesized that populations can withstand a certain level of harvest pressure before they become "sinks" for wolves emigrating from other populations. Source-sink dynamics (Pulliam 1988) have not been explicitly examined with regard to hunting pressures in wolves but were recently described in harvested populations of mountain lions in Nevada (Andreasen *et al.* 2012). Increased immigration augments local reproduction to maintain a viable population (Larivière *et al.* 2000), but immigration also increases local competition for resources. Sink populations have increased allelic diversity and lower inbreeding, which can increase the evolutionary viability of the population (Reed & Frankham 2003; Spielman *et al.* 2004). However, source-sink dynamics can theoretically lead to genetic swamping, where locally adapted phenotypes are lost as individuals immigrate into the sink populations from elsewhere (Allendorf *et al.* 2008). The adoption of unrelated individuals into packs, which is more common in harvested populations, reduces kinship within packs and thus can lead to social instability (Mech & Boitani 2003). The exact harvest rate allowing for a population to remain stable and self-sufficient varies between populations and with different harvest

practices, but many wolf biologists agree that this threshold is within 15-41% of total population size (e.g. Creel & Rotella 2010; Gude *et al.* 2012). For Minnesota wolves, the sustainable threshold for anthropogenic mortality has been estimated to be 28% of the total population (Fuller 1989).

Harvest rate has also been correlated with territory instability for wolf packs. Although pack territories continually shift in response to environmental, intrapack, and interpack factors, the overall territorial mosaic remains relatively stable once established in protected wolf populations (Fuller *et al.* 2003). Pack and territory turnover occur more frequently in areas with heavy hunting and poaching than in protected areas (Jędrzejewski *et al.* 2005; Sand *et al.* 2006; Sparkman *et al.* 2011). Harvest pressures can create new barriers on the landscape and thereby fragment populations (Rich *et al.* 2012) or create holes in the territorial mosaic. In a population experiencing low levels of harvest in which most wolves harvested were pre-reproductive, Webb *et al.* (2011) found home range stability to be generally unaffected. However, studies in areas where a higher proportion of breeders were harvested have found increased territory turnover rates under strong harvest pressures (Caniglia *et al.* 2014). Instabilities in pack territories can lead to a less stable territorial mosaic population-wide.

Harvest can affect the effective population size and genetic diversity of wolf populations. The effect of reduced census population sizes on effective population size is variable, and dependent on the age-class and status of the harvested wolves. For example, a population where mostly juveniles and dispersers are harvested may actually experience an increase in effective population size (Jędrzejewski *et al.* 2005) due to an increase in the proportion of breeders in the population (Sparkman *et al.* 2011). Genetic variability can increase with harvest following increased mixing of packs and an influx of immigrants (Harris *et al.* 2002), as has been shown in several harvested wolf populations (Jędrzejewski *et al.* 2005; Rutledge *et al.* 2010; Hindrikson *et al.* 2013).

Monitoring harvest at multiple levels of organization

It is difficult to determine exactly how wolf populations will respond to new hunting pressures and how these responses will affect the population genetics in the short and long term. Genetic variation is important to the evolutionary viability of populations and species, and loss of population genetic variability generally correlates to a decrease in measures of population fitness (Reed & Frankham 2003). Small population sizes increase the risk of genetic diversity loss due to genetic drift and inbreeding, as has happened to the small Isle Royale and Swedish wolf populations (Ellegren *et al.* 1996; Räikkönen *et al.* 2009). These genetic consequences can be avoided through appropriate management and monitoring. Population dynamics and genetic diversity are important to understand with regard to anthropogenic harvest, and population genetic analysis provides a relatively inexpensive, resource-efficient, and powerful monitoring system.

In wolf packs experiencing recent harvest pressure in the United States, we have a unique opportunity to study population variation before and after the onset of harvest. Monitoring populations from the beginning of harvest will allow for analysis of immediate responses to harvest, as well as long-term studies of how harvest may affect wolves. Characterizing these populations at the onset of harvest using genetic and geographic data, in addition to standardizing monitoring practices, will allow researchers to analyze how stable populations use compensation mechanisms to maintain stability, what the timescale for this rebound may be, and what temporal and geographic scales are important to conservation and management of these social carnivores. We can make predictions based on past observations, but the best strategy involves monitoring wolves affected by these management schemes.

In subsequent chapters, I explore the dynamics of a newly harvested wolf population using population genetic analyses. In Chapter 2, I use microsatellite genotyping to infer the genetic diversity and structure of the population in two sample years spanning an increase in anthropogenic mortality that corresponds to the recent onset of legal wolf harvest in Minnesota. This chapter uses population genetic approaches to focus on

immediate responses to harvest in the wolf system, providing a baseline for answering questions related to the genetic consequences of disturbances. The following chapter (Chapter 3) focuses more narrowly on hybridization, a known consequence of historical reductions in wolf densities in some areas of the United States and Canada. In this chapter, I use two uniparentally inherited genetic markers (one maternally inherited and one paternally inherited) to assess the signatures of wolf-coyote hybridization in Minnesota and to analyze how ancestry based on these markers compares to the population structure seen in Chapter 2. Through these genetic analyses, we can elucidate the genetic state of the wolf population in Minnesota and provide an important baseline for monitoring this population into the future.

Table 1.1 Compensatory responses to harvest pressures. Responses are categorized as demographic or social, and on whether they have a positive (+), negative (-), or neutral or variable (o) effect on the stability and abundance of the population. Stability is based on the maintenance of pack social structure and stable territories. Information is based upon studies specifically addressing the response of *Canis lupus* populations to human harvest. These social and demographic responses can result in changes in population genetic diversity and structuring within the affected wolf populations.

		Effect on Population		
Type of Response	**Mechanism**	**Stability**	**Abundance**	**Reference(s)**
Demographic	Increased litter size	+	+	[1,2,3,4]
Demographic	Decreased emigration	+	+	[5]
Demographic	Increased immigration	-	+	[5,6,7]
Demographic	Multiple litters	-	o	[3]
Demographic	Increased hybridization*	o	o	[8,9]
Demographic	Reduced natural mortality*	o	+	[4,10,11]
Social	Decreased territoriality	+	+	[12]
Social	Breeder replacement	+	o	[6,8,13]
Social	Smaller pack size*	-	+	[8]
Social	Increased territory turnover*	-	+	[4,8,14]
Social	Increased territory size*	-	o	[15]
Social	Increased adoption	-	+	[1,6,11]
Social	Pack dissolution	-	-	[8,13]

*Mechanisms that have been identified as absent in other populations than those in the cited literature, indicating continued debate about the relative influence of each of these factors.

[1] Gude *et al.*2012, *J. Wildl. Manage.* [2] Webb *et al.* 2011, *Can. J. Zool.* [3] Hayes & Harestad 2000, *Can. J. Zool.* [4] Sidorovich *et al.* 2007, *Can. J. Zool.* [5] Adams *et al.* 2008, *Wildl. Monogr.* [6] Jedrzejewski *et al.*2005, *Acta Theriol.* [7] Hindrikson *et al.* 2013, *PLoS One* [8] Benson *et al.* 2014, *Ecology* [9] Koblmuller *et al.* 2009, *Mol. Ecol.* [10] Murray *et al.* 2010, *Biol. Conserv.* [11] Rutledge *et al*. 2010, *Biol. Conserv.* [12] Fuller *et al.* 2003, in *Wolves: Behavior, Ecology, and Conservation* (Mech & Boitani, eds) [13] Brainerd *et al.* 2008, *J. Wildl. Manage.* [14] Caniglia *et al.* 2014, *J. Mammal.* [15] Rich *et al.* 2012, *J. Mammal.*

CHAPTER 2: TEMPORAL CHANGES IN POPULATION GENETIC STRUCTURE OF A NEWLY HARVESTED GRAY WOLF (*Canis lupus*) POPULATION

INTRODUCTION

Understanding the genetic effects of anthropogenic harvest is important if harvest is used as a tool for population management. Biologically-based harvest management is often effective at regulating population sizes and reducing human-wildlife conflicts, but mismanaged harvests leading to reductions in genetic diversity or changes in population structure can reduce population recovery rates and increase extinction risks in extreme cases (Allendorf *et al.* 2008). If this happens, human harvest can significantly reduce the ability of a species to respond to environmental change or anthropogenic disturbances (Coltman 2008). Reduced population size increases the potential for genetic drift, which accelerates the loss of genetic variation in the population (Reed & Frankham 2003; Frankham 2005). In addition, hunting can decrease the overall fitness of the population by removing the fittest individuals (Harris *et al.* 2002). Genetic effects may be more important in social animals due to kinship-based social structures. However, genetic diversity is generally high in socially-structured mammals (Parreira & Chikhi 2015) and thus reductions in genetic diversity may be less detrimental than in populations without social groups.

In many species, dispersal plays an essential role in maintaining genetic variation. Dispersal is critical to the ability of a population to recover from high mortality, but high levels of harvest can reduce the resilience of a population by altering these movements (Adams *et al.* 2008). In general, harvest reduces the emigration rate, dispersal distance, and survival of dispersers from intensely harvested areas (McCullough 1996; Newby *et al.* 2013), thereby decreasing gene flow between populations (Harris *et al.* 2002). In addition, harvested areas can experience increased immigration from unharvested populations due to lower levels of intraspecific competition caused by harvest (Adams *et al.* 2008; Webb *et al.* 2011). This creates a source-sink dynamic between populations with low and high harvest rates. Areas with more intense hunting become sink populations as individuals from lightly hunted populations disperse there (Andreasen *et al.* 2012; Newby *et al.* 2013). Increased gene flow increases genetic diversity through the

introduction of alleles from other populations, but it can also lead to genetic swamping and the loss of locally adapted phenotypes (Allendorf *et al.* 2008).

The gray wolf (*Canis lupus*) has a long history of human harvest in North America. Wolves live in family-based social groups; packs generally contain a dominant unrelated breeding pair and their offspring from multiple years (Mech 1999). These packs have high relatedness and low inbreeding due to one male and one female breeder usually being the only reproducing individuals. Juvenile wolves disperse from their natal packs at sexual maturity to establish their own packs and territories (Mech & Boitani 2003). Packs defend non-overlapping territories across a landscape surrounded by undefended buffer zones. Packs rarely compete for resources except in the buffer zones they share with other packs (Mech 1994). Pack and territory stability increase pack hunting success (Stahler *et al.* 2006; Sand *et al.* 2006) and pup survival rates (Schmidt *et al.* 2007; Brainerd *et al.* 2008). Stability also reduces interpack conflicts (Sands & Creel 2004) and hybridization with other species (Rutledge *et al.* 2012a).

Strong harvest pressure can affect individual wolf fitness through altering social dynamics and pack persistence. In states using public hunting and trapping for wolf management, harvest levels are regulated and thus generally do not reach this extreme. In heavily harvested populations, the proportion of packs producing multiple litters per year increases (Hayes & Harestad 2000), although total pup recruitment decreases due to increased mortality rates and increased maternal stress resulting from harvest (Sparkman *et al.* 2011; Bryan *et al.* 2014). Low wolf densities lead to wolves mating with less fit mates such as close relatives (Rutledge *et al.* 2010), or individuals from other canid species such as coyotes (*C. latrans*) in eastern North America (Hailer & Leonard 2008; Benson *et al.* 2014) and feral dogs (*C. lupus familiaris*) and golden jackals (*C. aureus*) in Europe (Randi *et al.* 2000; Moura *et al.* 2014). Harvest can increase territory turnover rates (Brainerd *et al.* 2008) and territory sizes (Rich *et al.* 2012) following disrupted pack social structure. At low harvest rates, packs and populations compensate for harvest-related mortality through changes in demographic rates and dispersal behavior. However,

continuously high harvest levels keep populations from recovering and can lead to long-term population decline (Murray *et al.* 2010; Creel & Rotella 2010; Gude *et al.* 2012).

Genetic structure related to the geographic distance between individuals or groups (isolation by distance, or IBD) is expected in protected populations due to pack-based social dynamics (Rutledge *et al.* 2010). Unharvested packs generally exhibit high intra-pack relatedness due to family-based composition and are closely related to nearby packs (Wayne & Vilà 2003). The balance between dispersal and immigration affects kinship and relatedness among packs within a population. Reductions in wolf densities exceeding natural mortality rates, as could occur from hunter harvest, cause increased immigration into and decreased emigration from an area (Adams *et al.* 2008). Packs that have lost pack members to harvest can replace harvested individuals by accepting unrelated juvenile dispersers from other packs (Lehman *et al.* 1992; Grewal *et al.* 2004; Jędrzejewski *et al.* 2005), reducing average relatedness and increasing genetic variability within packs. At high levels of harvest, pack dissolution and breeder turnover both increase, leading to territory turnover and greater genetic homogenization among wolves in the population (Brainerd *et al.* 2008; Rich *et al.* 2012; Caniglia *et al.* 2014). Population-wide genetic homogeneity and loss of genetic structure are predicted within heavily harvested populations as a result of this increased movement of individuals between packs (Jędrzejewski *et al.* 2005).

Population genetic structure analyses using microsatellite markers can provide information on the recent demographic history of populations and have previously been used to determine admixture and effective gene flow among populations of wolves. Several studies have examined genetic structure in hunted populations (*e.g.* Jędrzejewski *et al.* 2005; Rutledge *et al.* 2010; Hindrikson *et al.* 2013) and protected populations (Vonholdt *et al.* 2008; Rutledge *et al.* 2010), finding that the amount of genetic structure in hunted populations varies based on the intensity and length of harvest. However, no study to date has tracked genetic variability and structure in a population transitioning from protected to unprotected (harvested) status.

When wolves were protected under the ESA in the United States, illegal killing and removal of wolves following wolf depredation on livestock or pets were the only sources of anthropogenic mortality in the population. In Minnesota, legal depredation control resulted in an average of 121 wolves (5-8% of estimated population size) killed per year between 1988 and 2012 (Twin Cities Ecological Services Field Office 2014; J. Erb, *personal comm.*). In 2012, a record-high of 296 wolves were killed for depredation control. This increase occurred in the same year as the first hunter harvest season. These two sources of human-caused mortalities resulted in the highest recorded annual wolf take in Minnesota in recent history (approximately 24% of the adjusted population estimate; Figure 2.1).

In this study, we use two years of hunter-harvested wolf samples to analyze genetic diversity and population structure at the onset of harvest-based management of wolves in Minnesota. These samples were used to determine whether the three-fold increase in the anthropogenic mortality rate between 2012 and previous years significantly affected the population genetics of these wolves. We used samples from the 2012-2013 harvest as a "pre-harvest" sample, as the baseline level of human-related mortality prior to the first year of legal harvest was below rates found to affect wolves in other populations. We analyzed the changes in population genetic variation, effective population size, and spatial population genetic structure of wolves in Minnesota over two years to provide insight into the short-term effects of high anthropogenic mortality on a stable wolf population and to provide a baseline for later long-term studies in the population.

METHODS

Samples and study area

Muscle tissue samples were taken from legally harvested wolves during carcass inspection by the Minnesota Department of Natural Resources (MNDNR). The samples in this study were collected during the 2012-2013 (n=413) and 2013-2014 (n=238) wolf harvest seasons in the state. Hunters reported the county, township, range, and section of the kill site. MNDNR officials determined the age and reproductive history of female

wolves (Stark & Erb 2013). There were approximately equal numbers of males and females from each year; other statistics for 2012-2013 have been reported previously (Stark & Erb 2013). The number of wolves in the state was estimated at 2,211 (90% CI: 1,652-2,641) following the 2012-2013 harvest season and 2,423 (90% CI: 1,935 - 2,947) following the 2013-2014 harvest season (Figure 2.1; Erb & Sampson 2013; Erb *et al.* 2014), and has not increased or decreased significantly since population size appeared to level off around 2000. Most wolves in the state are juveniles (Mech 2006), and this is reflected in the age distribution of harvested wolves in both years (Stark & Erb 2013; J. Erb, *personal comm.*).

Harvest occurred across most of the known wolf range in Minnesota, and wolves were harvested throughout this range in both years (Figure 2.2). Primary wolf range exists throughout much of the forested portion of Minnesota. The most recent estimates describe this area as occupying about 70,000 km^2 in the northern half of the state with approximately 33% woody wetlands, 24% deciduous forest, 10% emergent herbaceous wetlands, 15% mixed and evergreen forest, 5% open water, and less than 5% each shrub/scrub, farmland, open space, grasslands, barren lands, and developed lands (Erb & Sampson 2013). Average pack territory size within wolf range in Minnesota was around 161 km^2 in 2012 and 150 km^2 in 2013, with an average density of 3.1 wolves per 100 km^2 in 2012 and 3.4 wolves per 100 km^2 in 2013 (Erb *et al.* 2014).

Laboratory methods

Muscle tissue was stored at -20ºC until DNA extraction. DNA was extracted using the GeneJET Genomic DNA Purification Kit (ThermoScientific, Waltham, MA, USA). Extractions followed kit protocol, with modifications to improve DNA concentration and purity, including: (1) overnight incubation for the lysis step, (2) an extra minute added to each of the highest speed centrifugation times, (3) an increase to 3 minutes of elution buffer incubation, and (4) a second elution step, resulting in a final elution volume of 400µl. Extracted DNA was quantified and diluted to 2ng/µl for downstream analyses. To confirm extraction results, one Y-chromosome locus (SRY; Meyers-Wallen *et al.* 1995)

and two X-chromosome loci (FH2548, FH2584; Richman *et al.* 2001) were used for sex determination. These three loci were amplified in a multiplex reaction with a total reaction volume of 13µl using 2ng of template genomic DNA, 1X GoTaq Flexi buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 0.8µM forward and reverse primer, 0.05µl GoTaq DNA polymerase (Promega, Madison, WI, USA), and sterile water. PCR products were visualized using 1% agarose gel electrophoresis and scored based on the presence or absence of the SRY band in the presence of the X-chromosome bands. A second extraction was performed for individuals that did not have positive sex-determination results.

We amplified twenty-two microsatellite loci characterized in *Canis lupus familiaris* and previously used in *C. lupus* studies, chosen based on the observed heterozygosity and polymorphic information content (PIC; Table 2.1). These included 14 autosomal loci with tetranucleotide repeats (FH2054, FH2422, Breen *et al.* 2001; FH3313, FH3725, FH3853, Guyon *et al.* 2003; FH2004, FH2611, FH2785, FH3965, Francisco *et al.* 1996; PEZ06, PEZ08, PEZ11, PEZ12, PEZ15, J. Halverson in Neff *et al.* 1999) and 6 autosomal dinucleotide loci (CPH3, Fredholm & Winterø 1996; C05.377, C10.213, Ostrander *et al.* 1993; Ren106I06, Ren169O18, Ren239K24, Breen *et al.* 2001). These 20 autosomal microsatellites represent 17 canine chromosomes across the *C. lupus* genome, with only three pairs of loci on the same chromosomes (C10.213 and FH2422 on CFA10; Ren169O18 and Ren239K24 on CFA29; PEZ15 and C05.377 on CFA5), and only PEZ15 and PEZ06 showing evidence of linkage in *C. lupus familiaris* (Neff *et al*. 1999). The final two loci were tetranucleotide microsatellites on the X-chromosome, FH2548 and FH2584 (Richman *et al.* 2001). Loci FH3965, PEZ15, and FH2054 were later removed from analysis due to low amplification success.

Microsatellites were amplified in a combination of single and multiplex reactions for each sample using M13-labeled forward primers (Table 2.1; Schuelke 2000). Loci were amplified in a total reaction volume of 13µl using 2ng of template genomic DNA, 1X GoTaq Flexi buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 0.5ng/µl bovine serum albumin (BSA), 0.08µM unlabeled forward primer, 0.8µM reverse primer, 0.8µM M13-labeled

fluorescent primer, GoTaq DNA polymerase (Promega, Madison, WI, USA), and sterile water. Loci were amplified under the following conditions: 2 min denaturation at 95ºC followed by 30 amplification cycles of 95ºC for 30 sec, 30 sec at the locus- or multiplex-specific annealing temperature, and 1 min at 72ºC, and with a final 10 min extension at 72ºC. Amplified products were combined into genotyping panels, diluted to 1:10 using ddH_2O, and analyzed on an ABI 3730xl capillary genetic analyzer at the University of Minnesota Biomedical Genomics Center (UMGC). Alleles were scored using GENEMARKER (v.2.6.0, SoftGenetics LLC). Uncertain allele calls were re-amplified and re-analyzed to confirm the genotype and reduce scoring errors. To check for accuracy in genotyping results, at least five randomly chosen individuals were re-amplified at each locus, and error rates were calculated based on these duplicated amplifications.

Error checking

We obtained microsatellite genotypes for all individuals included here at a minimum of 16 loci. Individuals with fewer than 16 loci genotyped were not included in analyses. The presence and frequency of microsatellite errors such as allelic dropout, false alleles, and null alleles were determined using the software MICROCHECKER (Van Oosterhout *et al.* 2004). The analysis suggested the presence of null alleles in loci Ren106I06, CXX.213, PEZ11, FH2004, and PEZ08, with an average null allele frequency of 0.06 (SE = 0.02). The frequencies of null alleles and polymorphic information content (PIC) were also estimated using CERVUS (Kalinowski *et al.* 2007).

Genetic diversity and inbreeding

We estimated observed (H_o) and expected (H_e) heterozygosities, the number of alleles (N_A), and the population mutation parameter Θ_H in ARLEQUIN (v3.5, Excoffier & Lischer 2010). Deviations from Hardy-Weinberg equilibrium at each locus and globally were tested using *p*-values produced from the Markov chain method (with 1,000,000 steps and 100,000 dememorization steps), implemented in ARLEQUIN. We used FSTAT (Goudet 1995) to calculate allelic richness (A_R) and Wright's inbreeding coefficient (F_{IS}) for each locus individually and overall for the sample set. We tested pairwise linkage

disequilibrium between loci using the log likelihood ratio statistic, implemented in GENEPOP (v.4.3, Rousset 2008) using 100 batches with 10,000 iterations per batch and 1000 dememorization steps. Effective population sizes for each sampling year were estimated using the linkage disequilibrium method in NEESTIMATOR (v2, Do *et al.* 2014) assuming monogamy and excluding alleles with a frequency less than 0.01.

Population structure

We used Bayesian genotype assignment in the program STRUCTURE (v2.3, Pritchard *et al.* 2000) to infer the most probable number of genetically distinct clusters within the samples and to estimate admixture proportions within each cluster. In STRUCTURE, genotypes are assigned a probability of membership (Q) in each of K genetic clusters, where K represents the *a priori* inferred number of genetic subpopulations. The admixture model of STRUCTURE was run assuming correlated allele frequencies at $K = 1$ to $K = 10$ three times each for 100,000 iterations following a burn-in period of 20,000 iterations. We determined the most probable number of clusters K based on the ΔK method (Evanno *et al.* 2005), implemented in STRUCTURE HARVESTER (Earl & vonHoldt 2012). We then ran STRUCTURE again at the most probable value of K for 10 runs, from which the individual assignments were averaged using CLUMPP (Jakobsson & Rosenberg 2007) to obtain final probabilities (Q) of individual cluster assignment and admixture. We categorized individuals with any $Q_i > 0.6$ as belonging to cluster i, and those with all $Q_i < 0.6$ as admixed. F_{ST} and admixture (α) values were used to characterize differentiation between the STRUCTURE-produced clusters, and FSTAT (Goudet 1995) was used to determine their significance via permutation.

The program STRUCTURE can overestimate genetic divisions in populations structured by genetic isolation by distance (IBD) if IBD is not accounted for (Frantz *et al.* 2009; Schwartz & McKelvey 2009). To test for structure related to IBD, we used Mantel tests with 999 permutations to assess correlations between Euclidean geographic distance and linear genetic distance between each pair of individuals, implemented in ARLEQUIN (v3.5, Excoffier & Lischer 2010) using default parameters, including 1000 permutations for

significance. Genetic distances (â) were calculated in GENEPOP (v4.3, Rousset 2008). This measure of genetic distance is most appropriate for individuals at a small scale in a continuously distributed population (Rousset 2000), and thus was chosen for this study. Geographic distances were log-transformed for the Mantel tests. Mantel tests were first conducted for all individuals in each sample year, and then among the individuals in each STRUCTURE-identified clusters in each year, resulting in four separate Mantel tests for each sample year.

Population genetic structure analyses can also be affected by family groups within the data set. The wolves in our sample set have unknown family relationships, and therefore we used COLONY to determine which pairs are likely to be half-sib or full-sib relationships. One individual was kept from each family cluster that had probability ≥0.5 and analyses were repeated in STRUCTURE using this trimmed data set under the admixture model at K=1 to K=10 three times each with 100,000 iterations following a 20,000 iteration burn-in period. This trimmed result was then analyzed using STRUCTURE HARVESTER to determine the most probable number of clusters when controlling for family structure.

Geographic distribution of clusters

The locations reported by hunters to the MNDNR upon wolf carcass inspection were converted to latitude and longitude by using the centroid of the reported section. These locations were then mapped in ARCMAP (v.10.2.2, ESRI). Core areas for STRUCTURE-defined clusters were visualized through computing the core hotspots for each cluster, based on the Q-values for that cluster, rather than based on the individuals assigned to that cluster. This was done using Getis-Ord G* hot spot analysis (Getis & Ord 1992) implemented in ARCMAP. Getis-Ord G* statistics identify areas containing clusters of points with values higher than expected by random chance for population i, based on the values of Q_i. Each sample was then assigned a z-score representing the statistical significance of how clustered it was with other samples of similar Q_i values. Minimum convex polygons (MCP) were then drawn around all of the sample points with 95%

significance (to delineate the 95% core area) and 99% significance (to delineate the 99% core area) for cluster *i*. The MCP technique was used because of its independence from frequency distributions when creating polygons from distributions of points.

To visualize spatial patterns of gene flow, we used the recently developed individual-based estimated effective migration surfaces (EEMS) program (Petkova *et al.* 2014). Cluster-based methods of determining gene flow have traditionally been used in genetic structure analyses, but these are not well suited for use in populations with continuous patterns of genetic variation, such as those exhibiting IBD (Rousset 2000). EEMS circumvents the problem of modeling clusters in a continuous landscape by explicitly producing a surface representative of gene flow across a landscape as a function of individual-based migration rates (Petkova *et al.* 2014). A grid of demes is laid across a defined study area, and each individual is assigned to the deme within which it exists. Migration rates between demes are then calculated using a stepping stone model (Kimura & Weiss 1964), and these are used to fit an "isolation by resistance" model to predict genetic dissimilarities and interpolate a migration surface. This visualization displays areas of higher than expected estimated migration rates (i.e. corridors for gene flow) and those with lower than expected estimated migration rates (i.e. barriers to gene flow). We use this individual-based migration surface to analyze whether the landscape is characterized solely by isolation-by-distance (and clusters are an artifact of this) or if there is actual separation between STRUCTURE-identified clusters. Migration and diversity parameters were adjusted for each year separately to produce 20-30% acceptance rates, as recommended by the authors (Petkova *et al.* 2014). We averaged three runs each with 50, 150, 200, 300, and 400 demes to produce the final EEMS surfaces. Different numbers of demes can change whether micro- or macro-processes are detected in the analysis, and thus averaging several different deme configurations is recommended. With 50 demes, each deme is approximately 1800 km^2, and a 400 deme grid has 170 km^2 demes. This range of demes was chosen because the 400 deme grid covers the average size of wolf pack territories in Minnesota reported in the literature (78-260km^2; Fuller 1989; Fuller *et al.* 1992; Erb *et al.* 2015), while the 50 deme grid can detect processes occurring on a

larger scale. Each run consisted of 1,000,000 burn-in steps followed by 3,000,000 MCMC iterations sampled every 1,000 steps.

In addition to Mantel tests to quantitatively determine whether genetic distances could be explained by isolation-by-distance, we used spatial structure analysis in GENALEX (v6.501, Peakall & Smouse 2012) at 50km distance categories, with 999 permutations and 9999 bootstraps. Spatial structure analysis computes the correlation coefficient between genetic distance and geographic distance for individuals within certain geographic distance bins, to determine whether the correlation changes as the distance between individuals changes (Smouse & Peakall 1999). We expected wolves within the distance of a pack territory to be more related to one another, with decreasing relatedness with distance (Mech & Boitani 2003).

Bottlenecks and migration

In populations that have undergone a recent bottleneck, allelic diversity is reduced faster than heterozygosity. Bottlenecked populations are thus expected to show higher values for observed heterozygosity (H_o) when compared to the expectation under mutation-drift equilibrium (Cornuet & Luikart 1996). Evidence for a bottleneck in the Minnesota wolf population was tested using the program BOTTLENECK (v1.2, Cornuet & Luikart 1996). A significant difference between these two values indicates a recent reduction in N_e ($H_o>H_e$), or either a recent expansion in N_e or an influx of rare alleles from genetically distinct immigrants ($H_o<H_e$; Cornuet & Luikart 1996). We used a one-tailed Wilcoxon signed-rank test to check for heterozygosity excess using 1000 replications each under the infinite alleles model (IAM, Maruyama & Fuerst 1985), the stepwise mutation model (SMM, Ohta and Kimura 1973; Kimura & Ohta 1978), and the two-phase mutation model of microsatellite evolution (TPM, Di Rienzo *et al.* 1994) with 90% probability of single step mutation and variance of 30.

We used MIGRATE (Beerli & Palczewski 2010) to estimate the historical migration rates between STRUCTURE-defined clusters in the Minnesota wolf population and BAYESASS (v3, Wilson & Rannala 2003) to estimate contemporary gene flow between the clusters.

In MIGRATE, the single step stepwise mutation model was chosen with a threshold of 100bp, with F_{ST} used as the start parameter for population mutation rate (Θ) and migration rate. A Bayesian Markov Chain Monte Carlo (MCMC) search strategy was used with 20,000 steps discarded before sampling 500,000 steps. In BAYESASS, we averaged three runs for each sample year. Each run consisted of 9,000,000 MCMC iterations following 1,000,000 iterations discarded as burn-in. MCMC steps were sampled every 2,000 runs, with mixing parameters adjusted to produce acceptance rates between 20% and 60% (for 2012, m=0.1, a=0.2, f=0.1; for 2013, m=0.1, a=0.2, f=0.2).

RESULTS

Microsatellite genotyping

A total of 637 samples (n=403 from 2012, n=234 from 2013) were successfully amplified at 16 or more microsatellite loci and were included in analyses. All 19 microsatellite loci were polymorphic in both sample groups, with values of H_e ranging from 0.59 to 0.92 and H_o ranging from 0.29 to 0.88 (Table 2.1). Overall, the combined set of samples had observed and expected heterozygosities that were not significantly different from one another (Table 2.1; $H_e = 0.82 \pm 0.08$, $H_o = 0.72 \pm 0.15$). However, this sample set deviated from Hardy-Weinberg equilibrium at individual loci: all loci had a significant deficiency in heterozygotes except Ren169O18, CPH3, and C05.377. The average allelic richness per locus was 13.8 and there was a positive inbreeding coefficient (F_{IS}=0.12, p=0.0024), indicating an excess of homozygotes. There was significant linkage disequilibrium at 50.9% of the 173 possible pairwise comparisons between loci genotyped (n=88 pairs at α=0.05).

Observed and expected heterozygosity and inbreeding coefficients were not significantly different between the 2012 and 2013 sample years at α=0.05 (Table 2.2). Both years had a positive inbreeding coefficient (Wright's F_{IS}; 2012: 0.124, p=0.0024; 2013: 0.117, p=0.0029). Allelic richness was slightly but not significantly reduced in the 2013 sampling year (A_R=12.2±0.95) when compared to individuals from 2012 (A_R=13.1±1.29; $p > 0.05$). The mean estimated effective population size using the linkage disequilibrium

method in NEESTIMATOR was also slightly lower in the 2013 samples than in the 2012 samples, though the two estimates are not significantly different (Table 2.2).

Population genetic structure

The most probable number of clusters *K* for both sampling years was 3 (Figure 2.3). In 2012, 23% of individuals assigned to cluster 1, 40% assigned to cluster 2, and 27% assigned to cluster 3. The final 10% did not have any clustering assignment $Q_i > 0.6$ and were considered admixed. At $K=3$, the admixture coefficient (α) was 0.25, the average F_{ST} was 0.025, and the mean number of migrants between clusters, calculated using the private alleles method, was 10.6 (Table 2.3). In 2013, 32% of individuals assigned to the first cluster, 35% to the second, and 15% to the third. Another 18% were admixed between the clusters. These clusters had an admixture coefficient of 0.11, a mean F_{ST} of 0.033, and a mean N_m of 3.9 individuals per generation. The median and distribution of the *Q* values did not differ between clusters between years (Figure 2.4).

In the combined 2012 and 2013 dataset (n=602), the most probable number of clusters was $K=4$. COLONY identified 200 different family groups, with a range of 1 to 8 individuals. STRUCTURE was run from $K=1$ to $K=10$ using a subset including one random individual from each of these family groups (n=200), resulting in $K=4$ as the most likely number of clusters. This is the same number of groups produced from running STRUCTURE from K=1 to K=10 with the whole combined data set, and thus it is unlikely that family structuring had a great effect on the results in this study. The majority of individuals (77%) assigned to the same family group were also assigned to the same cluster in a STRUCTURE run of the combined data set at the most probable *K* ($K=4$).

In both years, the Getis-Ord G* hot spot analysis of the locations produced core areas generally corresponding to the south-central (SC, cluster 1), northeast (NE, cluster 2), and northwest (NW) portions of wolf range in Minnesota. In 2012, the three 99% core areas are approximately the same size and are mutually exclusive (Figure 2.5c). The 95% core areas, on the other hand, differ greatly in size and overlap in the central part of wolf range. In 2013, the 99% core areas were similar to one another in size and smaller than

those in 2012 (Figure 2.5d). The 95% core areas did not overlap at all, supporting the F_{ST} and N_m results suggesting increased differentiation and decreased migration between clusters in 2013 when compared to 2012.

Geographic distribution of clusters

Mantel tests were used to determine whether there was a significant correlation between genetic distance ($\hat{a}$) and the log-transformed geographic distance between individuals. The 2012 and 2013 sample years were both significantly structured geographically, such that genetic distance could be explained at least in part by geographic distance (Table 2.5; $p < 0.001$). All three STRUCTURE-defined clusters in 2012 also had significant correlations between genetic distance and geographic distance ($p < 0.005$); however, only one of the clusters in 2013 had a significant association (SC, $p = 0.028$). Geographic and genetic distance in the other two clusters did not correlate significantly (NE, $p = 0.14$; NW, $p = 0.43$).

This difference in genetic structuring with distance was also evident in the spatial structure analyses for 2012 and 2013. In 2012, the sample set as a whole had a high correlation between genetic distance and geographic distance at small distances, and the correlation weakened with increasing distance and intercepted the x-axis at 98km, indicating IBD within this distance and a breakdown of IBD for samples greater than 98km apart (Figure 2.6a). The correlograms for spatial structure analysis were significantly different from $r = 0$ in the sample set as a whole ($p = 0.001$), as well as in the northeast ($p = 0.02$) and northwest ($p = 0.001$) populations, but not in the south central population ($p = 0.05$). In the 2013 sampling year, the sample set as a whole and all three STRUCTURE-defined clusters had correlograms that were significantly different from the null hypothesis of no correlation ($r = 0$; $p \leq 0.001$; Figure 2.6b), although the r values were smaller than those in 2012 and had larger confidence intervals, indicating weaker correlation between genetic and geographic distances. The 2013 sample set as a whole had an x-intercept of 91km, which was not significantly different from that in 2012.

From the individual-based effective gene flow estimates in EEMS, there is an area with high resistance to gene flow in the west-central portion of wolf range in Minnesota using the 2012 samples (Figure 2.7). In addition, there is a slightly resistive band running east to west across wolf range. There are corridors to gene flow in the northeast, south, and northwest parts of wolf range in the state, roughly corresponding to the core areas for each of the three STRUCTURE-defined clusters. In 2013, the effective migration surface has greater resistive areas, as evidenced in the greater proportion of area covered by the dark orange coloration and indicative of areas with reduced gene flow (Figure 2.7). As with the 2012 sampling year, the resistance surface for 2013 has pockets of enhanced gene flow in the northeast, south, and northwest parts of wolf range in the state.

Population bottlenecks and historical and contemporary gene flow

Allele frequencies had typical L-shaped distributions for the combined data set, indicating no detectable shift in distribution. The sign test and standardized differences tests in BOTTLENECK indicate that there is evidence for deviation from mutation-drift equilibrium in the population under both the infinite alleles and stepwise mutation model (IAM: $p < 0.0001$, SMM: $p < 0.0001$). The direction of deviation from the two models differed: 19 loci had heterozygosity excess under the IAM, while 18 of 19 loci had heterozygosity deficiency under the SMM. The data did not deviate from mutation-drift equilibrium under the two-phase mutation model using either the sign test (p=0.28) or the standardized differences test (p=0.09).

Historical maximum likelihood migration rates, estimated in MIGRATE using the 2012 samples, show net effective migration predominantly from the northeast cluster toward the northwest and south central clusters (Figure 2.8). Modal mutation-scaled migration rates were highest leaving the NE cluster ($M_{NE\text{-}SC}$ = 282, $M_{NE\text{-}NW}$ = 254), and lowest entering the NE cluster ($M_{SC\text{-}NE}$ = 86, $M_{NW\text{-}NE}$ = 145). Estimated migration rates between the NW and SC clusters were also relatively small compared to the emigration rates from the NE cluster ($M_{NW\text{-}SC}$ = 176, $M_{SC\text{-}NW}$ = 128). This directionality of historical migration shows wolves dispersing from the interior of the range toward the edge of the range.

BAYESASS was used to determine more recent migration rates between STRUCTURE-defined clusters in 2012 and 2013. All three runs for each year produced similar migration rates, indicating convergence in the MCMC. The magnitude of effective migration and the directionality of net migration between clusters changed from 2012 to 2013. However, the net migration rates and directions in both years were not significant at α=0.05 and 95% confidence intervals for all migration rates included 0 (Table 2.6).

DISCUSSION

In this study, we used two consecutive years of harvest samples from across wolf range in the state of Minnesota to determine whether the distribution of genotypes and gene flow across the state changed between the first and second year of legal wolf harvest. There was evidence for reduced isolation by distance, reduced gene flow, and increased differentiation between clusters in 2013 when compared to 2012, suggesting a contraction in the observed clusters in response to harvest pressures. Both sampling years had high genetic diversity and a slight deficiency in heterozygotes, as is common in wolves due to non-random breeding and social structure (Wayne & Vilà 2003).

A small portion of the differentiation in the 2012 samples could be explained by isolation by distance (IBD), but the 2013 clusters no longer had a significant association between genetic and geographic distances (Table 2.5). This change is also evident in the spatial structure analysis differences between 2012 and 2013. Though all four correlograms were significantly different from the null hypothesis of no correlation (r = 0) in 2013, the correlation coefficients were smaller than those in 2012 and had larger confidence intervals, presumably due to smaller sample size. Reductions in local genetic structuring could emerge from a disruption of the normal pack composition and territorial mosaic seen in stable wolf populations. More unrelated wolves joined harvested packs in harvested populations in Ontario (Grewal *et al.* 2004) and Poland (Jędrzejewski *et al.* 2005). Increased pack dissolution has also been associated with increased anthropogenic mortality in populations in several studies, based on a meta-analysis by Brainerd *et al.* (2008). Both pack dissolution and the incorporation of non-related wolves into packs

would result in a decrease in local spatial genetic structure, and the differences observed here between 2012 and 2013 suggest that wolves in Minnesota may have responded similarly.

In contrast to the decrease in local genetic structure, population-wide differentiation was higher between the three genetic clusters in 2013 compared to clusters in 2012. The number of migrants was larger in 2012, and the admixture coefficient was higher, supporting an increase in differentiation between clusters following the increase in anthropogenic mortality. This is also supported by the increased geographic separation between cluster core areas in 2013 when compared to 2012. Decreased dispersal and emigration rates in harvested areas have been observed in other populations in response to low or moderate levels of harvest (Adams *et al.* 2008). Here, the observed decreases in effective gene flow, increased spatial separation, and decreased admixture between clusters in the post-harvest sample year suggest that there were fewer migrants after the first hunt. It is unknown if this trend will continue in the years following harvest.

The private allele-based, individual-based, and cluster-based migration analyses produced conflicting results. The private alleles method implemented in GENEPOP indicated that the number of migrants decreased significantly in 2013 compared to 2012 (Table 2.3). Visual comparisons of the individual-based EEMS effective migration surfaces support this decrease in gene flow in some areas across Minnesota. In contrast, migration rates estimated using cluster-based migration analyses in BAYESASS increased in 2013 when compared to 2012. There are several possible explanations for these seemingly contradictory results. One explanation could be the differences in data used for these different estimations. The private alleles method of migration estimation uses only those alleles that differ between clusters for analysis (Slatkin 1981). In contrast, Bayesian inference in BAYESASS uses full genotypes for the maximum likelihood inference of recent immigration rates (Wilson & Rannala 2003). This difference in inputs for analysis may be significant enough to affect inferences in weakly differentiated populations, such as those in this study. BAYESASS can overestimate migration rates in many situations, including when population structure is weak (Meirmans 2014). In addition, likelihood

methods in general tend to not perform as well as F_{ST} and private allele analyses in simulations when few clusters are analyzed (Slatkin & Barton 1989), and thus may not perform well in our 3-cluster analysis.

Another surprising result was the lack of evidence for a past bottleneck event in the Minnesota wolf population. Wolves in Minnesota were nearly extirpated prior to ESA protection in the 1970s, and therefore we might expect to see evidence of this past bottleneck in our population genetic analyses. However, there was no significant evidence for a bottleneck in the population under the two-phase mutation model expected to be the best fit for microsatellite data, and furthermore, the infinite alleles model and stepwise mutation models produced conflicting trends of heterozygosity excess and deficiency, respectively. The conflict between these models indicates that caution should be taken in interpreting these results. Microsatellite evolution remains fairly poorly understood (Schlötterer 2000) and the SSM and IAM are considered the two extremes in the range of microsatellite evolution models (Cornuet & Luikart 1996). In addition, canine microsatellites have a high mutation rate ($1.1x10^{-2}$ to $4.7x10^{-3}$, Parra *et al.* 2009), which can result in frequent allele size homoplasy in large populations and thus reduced rates of heterozygosity dependent on the mutation model used (Estoup *et al.* 2002). High mutation rates over many generations may also result in the bottleneck signal being confounded by the subsequent expansion in the wolf population. Despite the apparent contradiction with known demographic history, the absence of a bottleneck in Minnesota supports bottleneck analyses previously conducted in Great Lakes wolves (Koblmüller *et al.* 2009). Koblmüller *et al.* (2009) suggested that the discrepancy could be attributed to high levels of introgression from other wolf populations and coyotes, or because the geographical extent of their analysis included individuals from both the US and Canada. The Canada wolves did not experience the same reduction as US populations, and therefore the signature may have been diluted by including Canadian wolves in the analysis. In this study, a similar explanation applies. The Minnesota wolf population is contiguous with the Canada population, which continues to be managed as a furbearer species. Wolves move freely across the border and therefore the meta-population size

remained large despite the range contraction and population reduction in Minnesota. Thus, the recovery of wolves in Minnesota could have been a range contraction and expansion for this widely distributed carnivore rather than a true population bottleneck across their contiguous range.

The observed strengthening of population genetic structure and reductions in dispersal may be consequences of high anthropogenic mortality in the Minnesota wolf population. However, the two sampling periods differ only by a year, and thus caution should be exercised in interpreting the results as solely the result of harvest pressures. Reduced dispersal suggests that more wolves are either remaining in their natal areas or not successfully dispersing (presumably a result of high disperser harvest). Gese & Mech (1991) observed changes in dispersal between declining, stable, and increasing wolf populations in Minnesota, such that fewer individuals dispersed in demographically stable years. The reduced dispersal observed here between 2012 and 2013 could be indicative of the recent plateau in the Minnesota wolf population estimate (Erb *et al.* 2014), rather than decreased dispersal resulting from harvest pressures. Prey density also regulates dispersal in wolf populations (Messier 1985, Ballard *et al.* 1987) and thus changes in prey availability between 2012 and 2013 would also have an effect on gene flow. In both years, there are areas of reduced gene flow in the western part of the state, where there are few resident wolf packs, less forested area (more farmland and prairie grasslands), and fewer state- or federally-protected lands. Moose numbers in the northwest portion of the state have declined in recent years (Delgiudice *et al.* 2002), which may also contribute to low prey availability in the area and few wolves immigrating to the area.

This study provides an important baseline enabling comparisons with population samples in the future, or retroactive comparisons to past years based on previously collected samples. Data from more years will be necessary to determine whether this affects year-to-year variation in the wolf population or if it is indeed correlated with the increased anthropogenic mortality. Monitoring the genetic diversity and structure of wild populations will be important to understanding how anthropogenic mortality may

positively or negatively affect these populations. In studying population genetic structure, we can determine whether effective gene flow rates are high enough to maintain connectivity in fragmented populations (Schwartz *et al.* 2007). The reductions in gene flow observed in this study do not preclude the possibility of further decreases in gene flow or an increase in immigration from other populations after more time has passed. Genetic monitoring allows for the estimation of several parameters from the same data, in addition to providing information indiscernible from traditional abundance, distribution, and vital rate monitoring. Genetic monitoring has been informative in many harvested species and will be important for the appropriate management of harvested wolves in the future (Schwartz *et al.* 2007). The Western Great Lakes distinct population segment was re-listed under the ESA in 2014 and currently is federally protected. However, a harvest-based management strategy is likely in the future and the baseline population genetics provided here will be important for long-term population monitoring.

Table 2.1 Characteristics of the 22 microsatellites chosen for genetic analysis. Microsatellites were PCR-amplified in the multiplexes shown using the designated M13 fluorescent primer, then combined in equal amounts into sequencing panels and diluted to a final volume of 10µl for genotyping. Locations refer to *Canis lupus familiaris* (CFA) chromosomes. Polymorphic information content (PIC) and null allele frequencies were estimated using CERVUS (Kalinowski *et al*. 2007).

Multiplex	Locus	Location	T_a (ºC)*	M13	Size range (bp)	N‡	PIC	Null Allele Frequency	H_o	H_e	A_R	Reference
Genotyping Panel 1												
1	PEZ06	CFA 27	48	FAM	183-219	645	0.829	0.022	0.824	0.839	13.7	[1]
	REN239K24	CFA 29	48	FAM	315-327	644	0.596	0.048	0.583	0.708	7.5	[2]
2	CXX.213	CFA 10	55	PET	160-182	501	0.711	0.469	0.273	0.682	10.4	[3]
	FH3313	CFA 19	55	PET	363-439	641	0.909	0.045	0.864	0.93	27.8	[7]
	PEZ15	CFA 5	55	PET	187-267	498	0.842	0.183	0.59	0.858	18.9	[1]
3	REN169O18	CFA 29	54	VIC	154-184	642	0.793	0.0099	0.793	0.81	9.0	[2]
	REN106I06	CFA 24	54	VIC	256-282	641	0.815	0.076	0.709	0.864	12.4	[2]
4	FH3853	CFA 22	54	NED	322-398	608	0.848	0.015	0.833	0.886	13.0	[7]
5	FH2548	CFA X	55	NED	176-216	631	0.847	0.078	0.744	0.865	13.0	[8]
	FH2584	CFA X	55	NED	308-351	638	0.868	0.098	0.707	0.855	14.0	[8]
Genotyping Panel 2												
6	PEZ11	CFA 9	55	FAM	133-169	611	0.771	0.220	0.492	0.844	14.5	[1]
	FH2004	CFA 11	55	FAM	252-340	643	0.798	0.091	0.671	0.842	9.7	[4]
7	CPH3	CFA 6	57	PET	175-199	589	0.782	-0.0014	0.803	0.794	10.5	[6]
	PEZ08	CFA 17	57	PET	228-276	604	0.887	0.169	0.623	0.871	16.6	[1]

	FH2785	CFA 28	57	PET	339-357	604	0.554	0.035	0.55	0.613	9.3	[4]
8	FH2611	CFA 36	57	VIC	200-232	613	0.821	0.022	0.783	0.818	12.8	[4]
	PEZ12	CFA 3	57	VIC	281-397	564	0.818	0.032	0.781	0.859	12.3	[1]
Genotyping Panel 3												
9	FH3725	CFA 14	50	FAM	145-243	553	0.805	0.032	0.776	0.802	21.3	[7]
10	FH2054	CFA 12	48	PET	165-197	562	0.740	0.030	0.724	0.767	20.7	[2]
11	FH2422	CFA 10	48	VIC	195-255	611	0.843	-0.0061	0.884	0.844	11.0	[2]
12	C05.377	CFA 5	55	NED	154-188	590	0.758	-0.0052	0.775	0.807	14.5	[3]
Average						602	0.792	0.079	0.704	0.817	13.9	

* T_a, optimal annealing temperature; ǂ N, number of individuals successfully genotyped, out of 646 individuals included in analysis.

References: [1] *J. Halverson in* Neff *et al.* 1999; [2] Breen *et al.* 2001; [3] Ostrander *et al.* 1993; [4] Francisco *et al.* 1996; [5] Neff *et al.* 1999; [6] Fredholm and Winterø 1995; [7] Guyon *et al.* 2003; [8] Richman *et al.* 2001

Table 2.2 Summary of population genetic diversity indices for Minnesota wolves harvested in the 2012-2013 and 2013-2014 seasons. Parenthetical values for N_e show 95% confidence intervals for the linkage disequilibrium method, implemented in NEESTIMATOR.

Statistic	**2012**	**2013**
Number of individuals	403	234
Mean Allelic Richness (A_R)	13.12	12.20
Frequency of Private Alleles	0.0087	0.024
Observed heterozygosity (H_o)	0.715 ± 0.149	0.717 ± 0.147
Expected heterozygosity (H_e)	0.816 ± 0.082	0.812 ± 0.078
Wright's F_{IS}	0.124 (*p*=0.0024)	0.117 (*p*=0.0029)
Θ_H	4.42	4.31
Effective population size (N_e)	642 (596-694)	573 (516-641)

Table 2.3 Summary of differentiation between STRUCTURE-identified clusters in each sampling year. Population differentiation (F_{ST}) and mean sample size-corrected number of migrants (N_m) per generation (using the private alleles method) were calculated in GENEPOP. The significance of differences between years is based on 1000 permutations in FSTAT.

	2012	**2013**	Significance
	n = 403	n = 234	
Number of Clusters (K)	3	3	
Admixture (α)	0.25	0.11	*
Mean F_{ST}	0.025	0.033	
Mean N_m	10.6	3.9	*

*Significant at α = 0.05

Table 2.4 Differentiation between STRUCTURE-defined clusters in 2012 and 2013 sample years. Clusters are named by their geographic location within wolf range in Minnesota (east-central, EC; northeast, NE; northwest, NW). F_{ST} values are below the diagonal; numbers above the diagonal are the number of migrants between clusters, calculated based on F_{ST} values. All F_{ST} values are significant with $p < 0.001$.

	2012			**2013**		
	SC	NE	NW	SC	NE	NW
SC		11.7	6.3		9.0	5.2
NE	0.021		11.8	0.027		6.5
NW	0.038	0.020		0.046	0.037	

Table 2.5 Mantel test results for samples from the 2012 and 2013 years testing 1000 permutations of linear genetic distances and the natural log of geographic distances between individual samples. The cluster assignments (SC, NE, NW) refer to STRUCTURE cluster assignments, with a cutoff of $Q > 0.6$. Significant isolation by distance at $\alpha = 0.05$ was found in the 2012 samples as a whole, as well as within the individuals assigned to each of the 2012 clusters. In 2013, the samples as a whole had significant isolation by distance (IBD), the south-central cluster had weakly significant IBD, and NE and NW clusters did not have significant IBD. All significant IBD relationships had small correlation coefficients, indicating weak association despite being significant.

	2012				**2013**			
	All	**SC**	**NE**	**NW**	**All**	**SC**	**NE**	**NW**
Number of Individuals	403	91	161	109	234	74	81	36
Mean Genetic Distance ($\hat{a}$)	0.13	0.12	0.11	0.12	0.14	0.13	0.11	0.12
Mean ln(GeogDist)	0.47	0.32	0.38	0.32	0.47	0.22	0.27	0.080
Regression coefficient (b)	0.015	0.021	0.007	0.021	0.014	0.013	0.001	0.009
Correlation coefficient (r)	0.12	0.190	0.063	0.19	0.11	0.087	0.008	0.072
Significance	***	***	***	***	***	*	NS	NS

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ^{NS} $p > 0.05$

Table 2.6 Estimated effective migration rates from BAYESASS for the 2012 and 2013 sampling years. Values show the proportion of individuals in the cluster listed in the column heading that are migrants from the row cluster per generation, with standard deviations in parentheses.

		Migration to		
	2012	SC	NE	NW
Migration from	SC	--	0.0069 (0.0058)	0.006 (0.0053)
	NE	0.0069 (0.0064)	--	0.0089 (0.0072)
	NW	0.0088 (0.0069)	0.0087 (0.0069)	--

		Migration to		
	2013	SC	NE	NW
Migration from	SC	--	0.011 (0.010)	0.030 (0.019)
	NE	0.0079 (0.0073)	--	0.027 (0.022)
	NW	0.0075 (0.0068)	0.012 (0.0094)	--

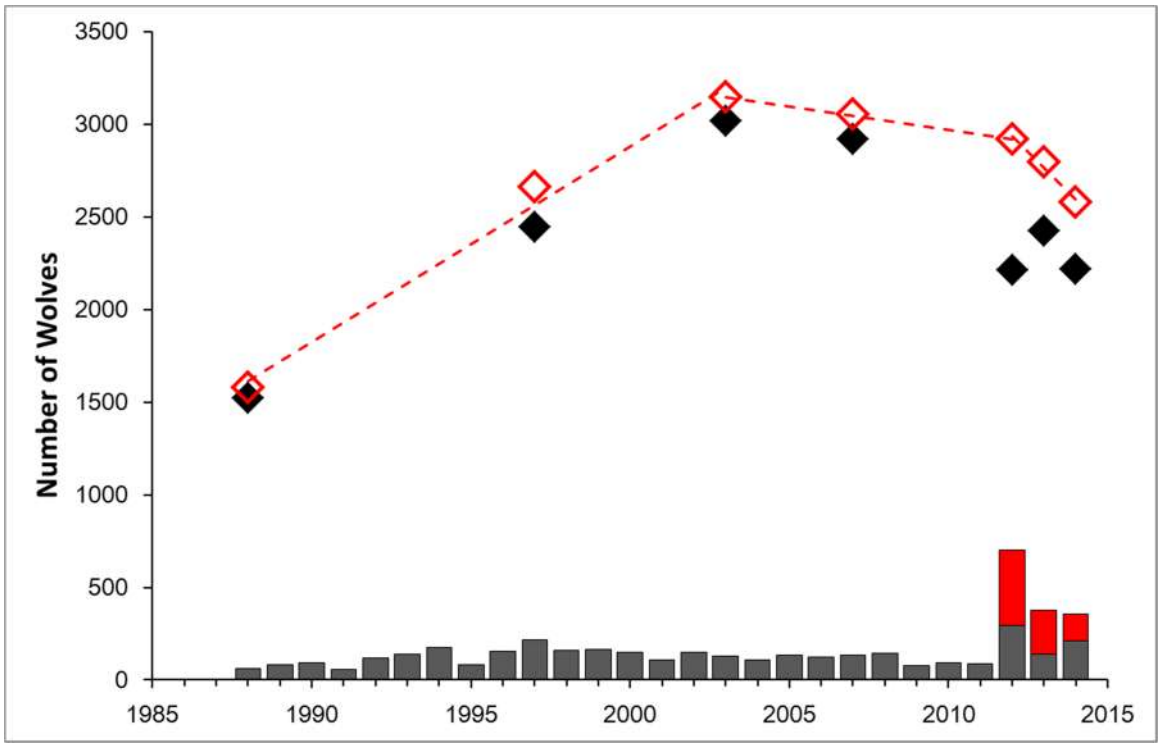

Figure 2.1 Population estimates and human-caused wolf mortalities in Minnesota. Data for mid-winter population estimates (solid black) come from Minnesota Department of Natural Resources population surveys (Erb *et al.* 2014). Adjusted population estimates (hollow red, with trendline) were calculated from adding known human-caused deaths from the previous year to the mid-winter population estimate. Gray bars represent animal damage control (ADC) mortalities recorded by the US Fish and Wildlife service and MNDNR. Red bars represent harvest mortalities in 2012- 2014, when legal harvest occurred in the state. Additional illegal mortality occurred each year at unknown rates.

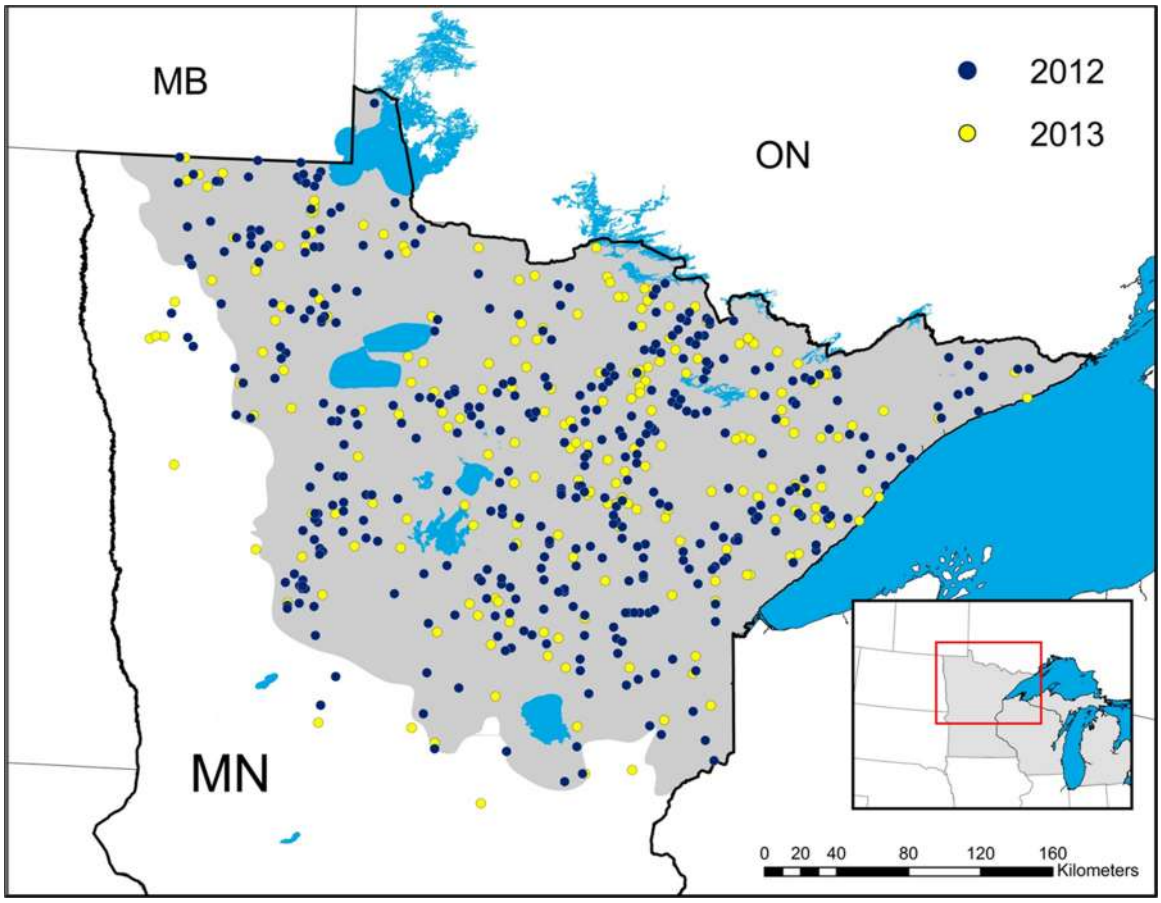

Figure 2.2 Sampling locations for 2012-2013 wolf harvest samples (blue, n = 413) and 2013-2014 harvest samples (yellow, n = 238) used in this study. The shaded area represents the current estimated occupied wolf range in Minnesota, adapted from Erb and Sampson (2013).

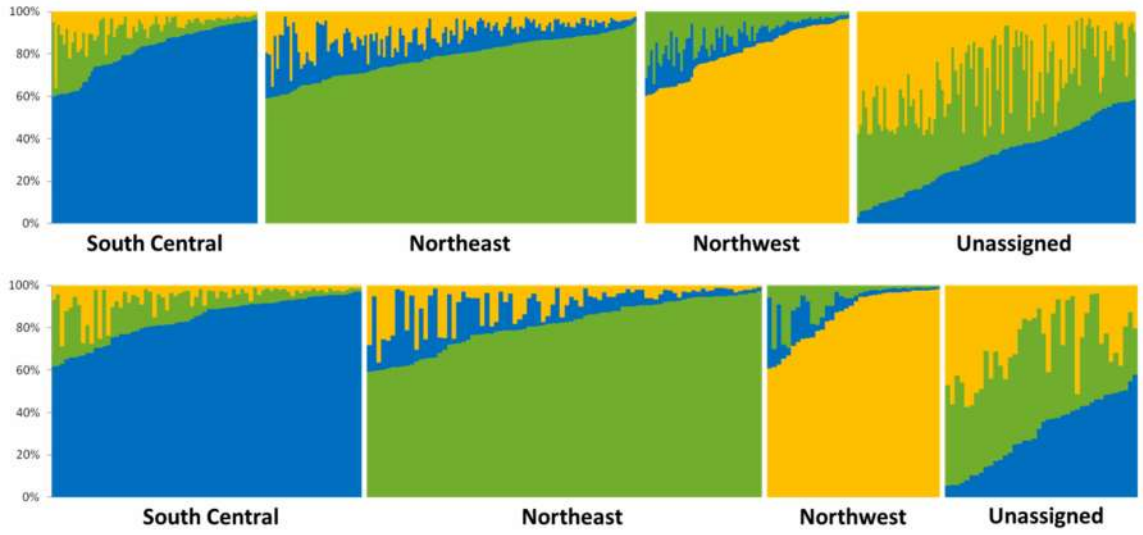

Figure 2.3 Bayesian clustering assignments at K=3 from STRUCTURE for wolves from the 2012-2013 harvest season and 2013-2014 harvest season. In both graphs, each vertical bar corresponds to an individual wolf, and the colors of each bar correspond proportionally to that individual's probability of cluster membership to the south-central (blue), northeast (green), and northwest (yellow) clusters. Samples are separated by cluster assignment, with unassigned (admixed) individuals as the final group. Individuals were assigned to clusters using a cutoff of $Q > 0.6$.

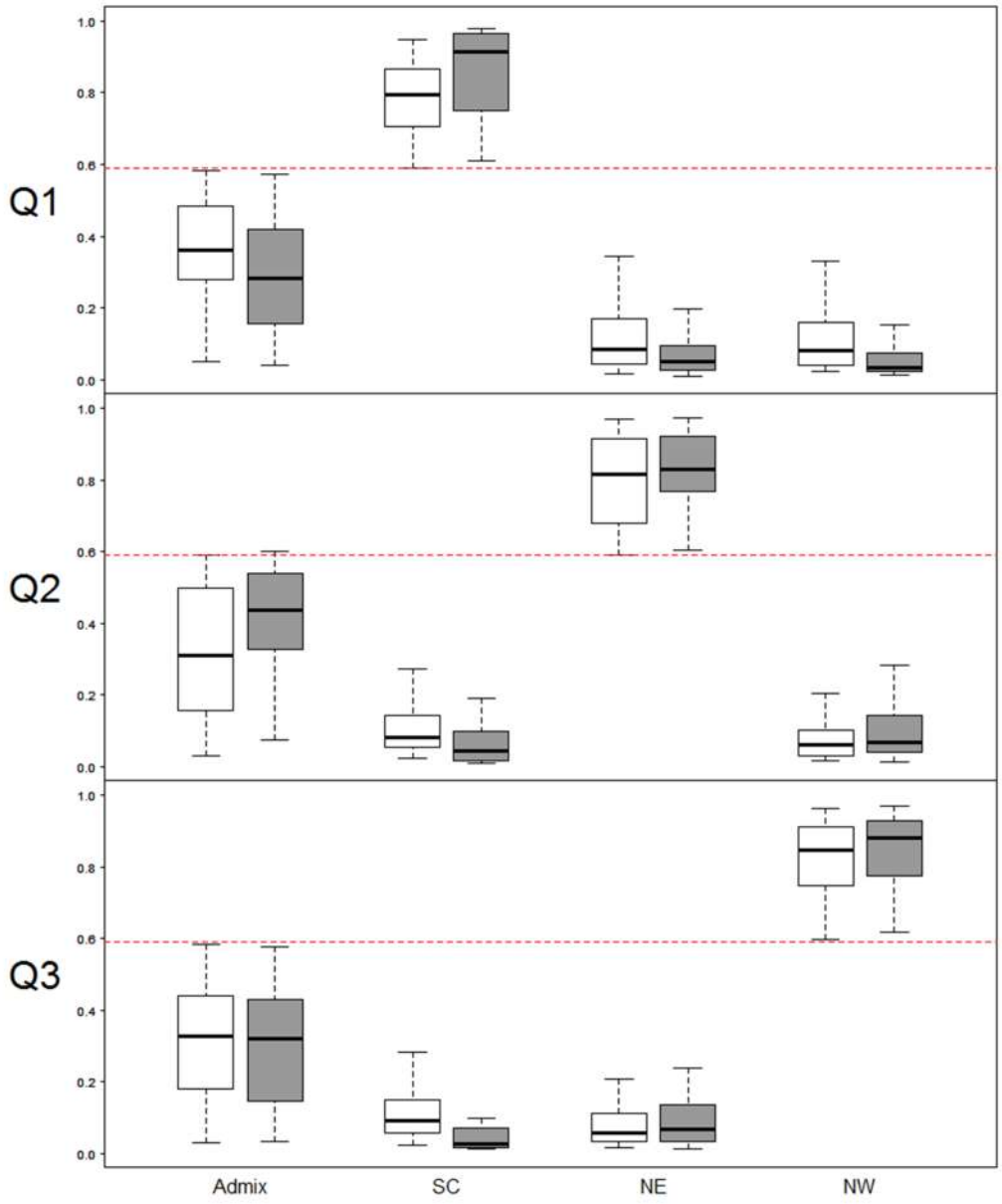

Figure 2.4 Boxplots of cluster assignment for the 2012 (open) and 2013 (shaded) sampling years. The proportion of cluster membership (*Q*) is shown along the vertical axis for each of the three clusters. Cluster assignment is shown on the horizontal axis, with admixed individuals grouped separately. Dashed lines show the $Q = 0.6$ cutoff used for cluster assignment.

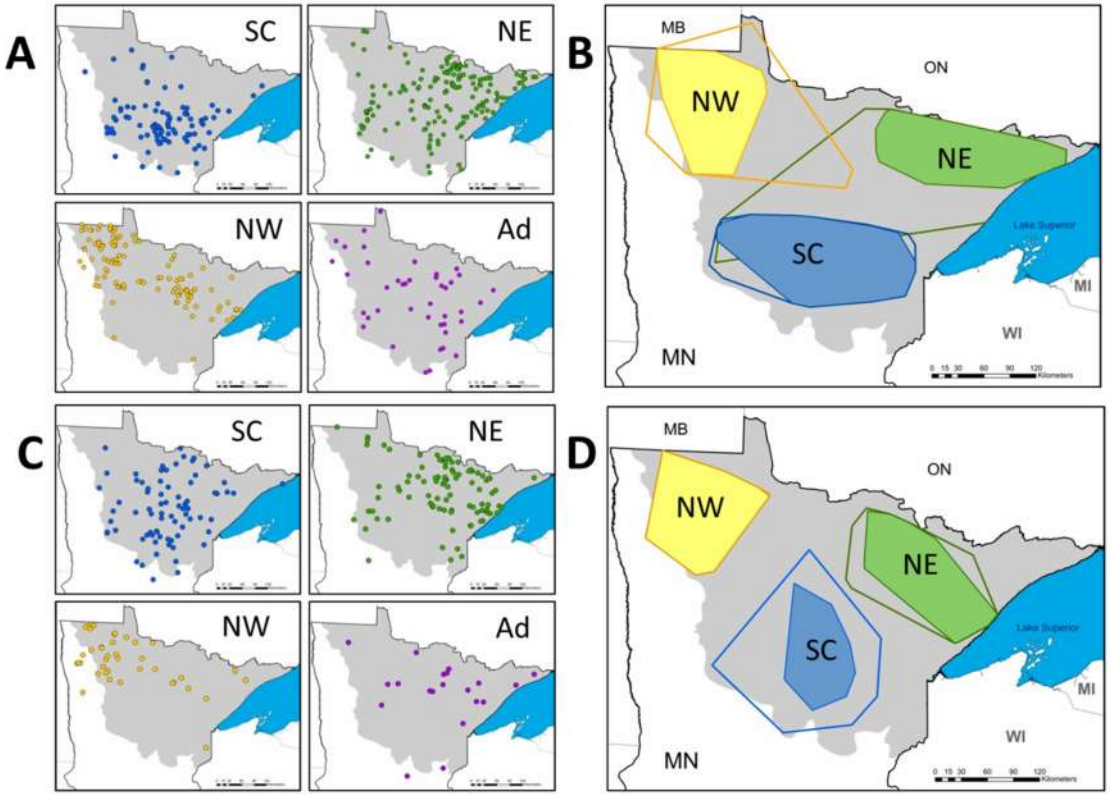

Figure 2.5 Spatial analysis of STRUCTURE-assigned Bayesian clustering results. (A) Maps of individuals assigned to each of the three clusters, and admixed individuals with no cluster assignment, from the 2012-2013 harvest season. (B) Locations for individuals assigned to each of the three clusters, and admixed individuals, for the 2013-2014 harvest season. (C) Getis-Ord G* Hotspot analysis core areas (solid: 99%, hollow: 95%) for the three populations in 2012, based on *Q*-values derived from STRUCTURE analyses. (D) Hotspot analysis-derived core areas for the 2013 sampling year.

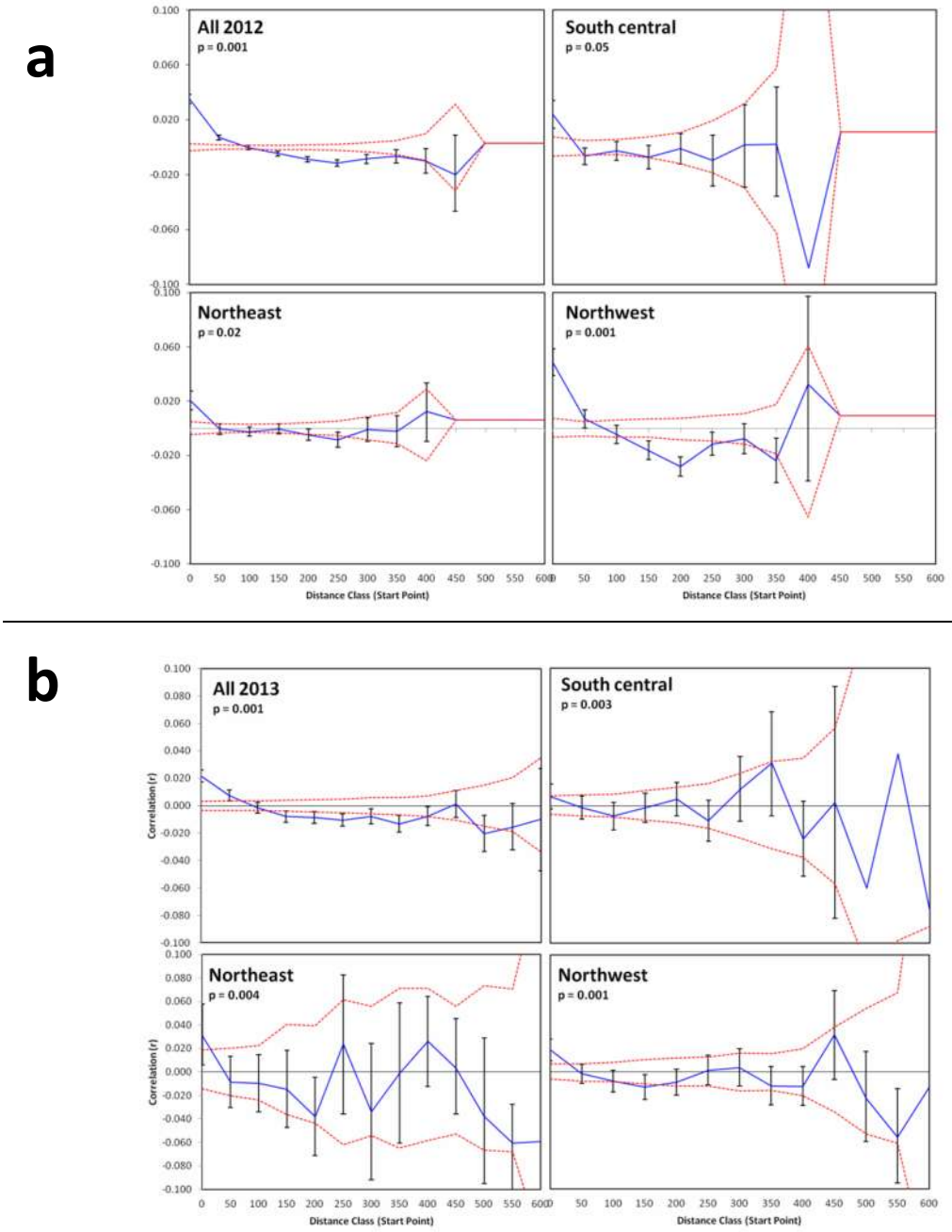

Figure 2.6 Results of spatial structure analyses in GENALEX for (a) 2012 and (b) 2013 sampling years for all individuals, individuals assigned to SC, individuals in NE, and individuals in NW. In each graph, the solid line shows the correlation coefficient (r), and the dashed lines and error bars show the upper and lower confidence intervals. Analyses were performed using 999 permutations and 10,000 bootstraps, with 50km distance classes (shown on the horizontal axis).

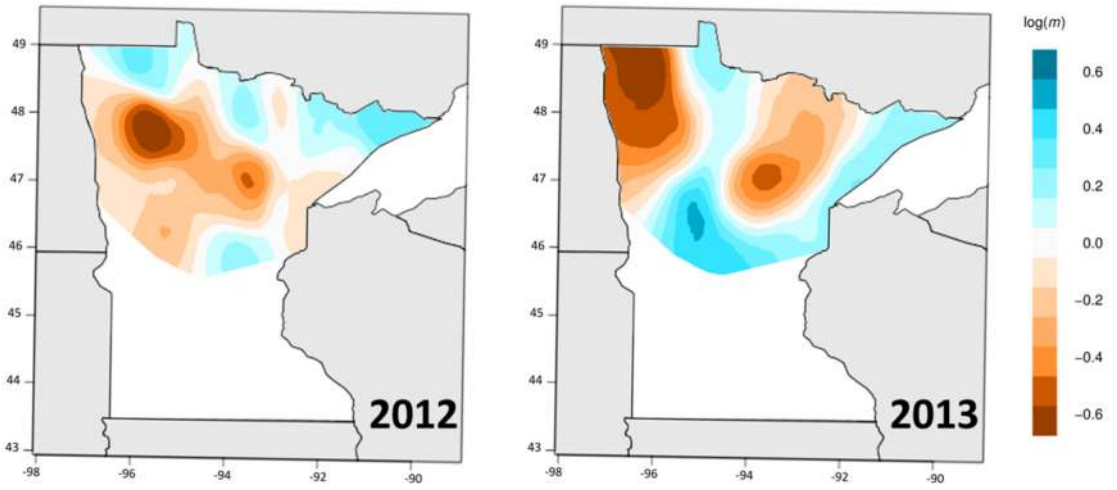

Figure 2.7 Individual-based EEMS analysis of effective migration rates (m) for the 2012 and 2013 sampling years. Colored regions indicate areas that deviate from isolation by distance (IBD). Darker (orange) areas have lower than expected effective gene flow, while lighter (blue) areas have higher than average gene flow, where genetic similarities decay faster with geographic distance than expected under IBD. In both years, there are areas of reduced gene flow in the western part of the state, where there are few resident wolf packs, less forested area (more farmland and prairie grasslands), and fewer state- or federally-protected lands. Migration surfaces are averages of 3 runs each with 50, 150, 200, 300, and 400 demes.

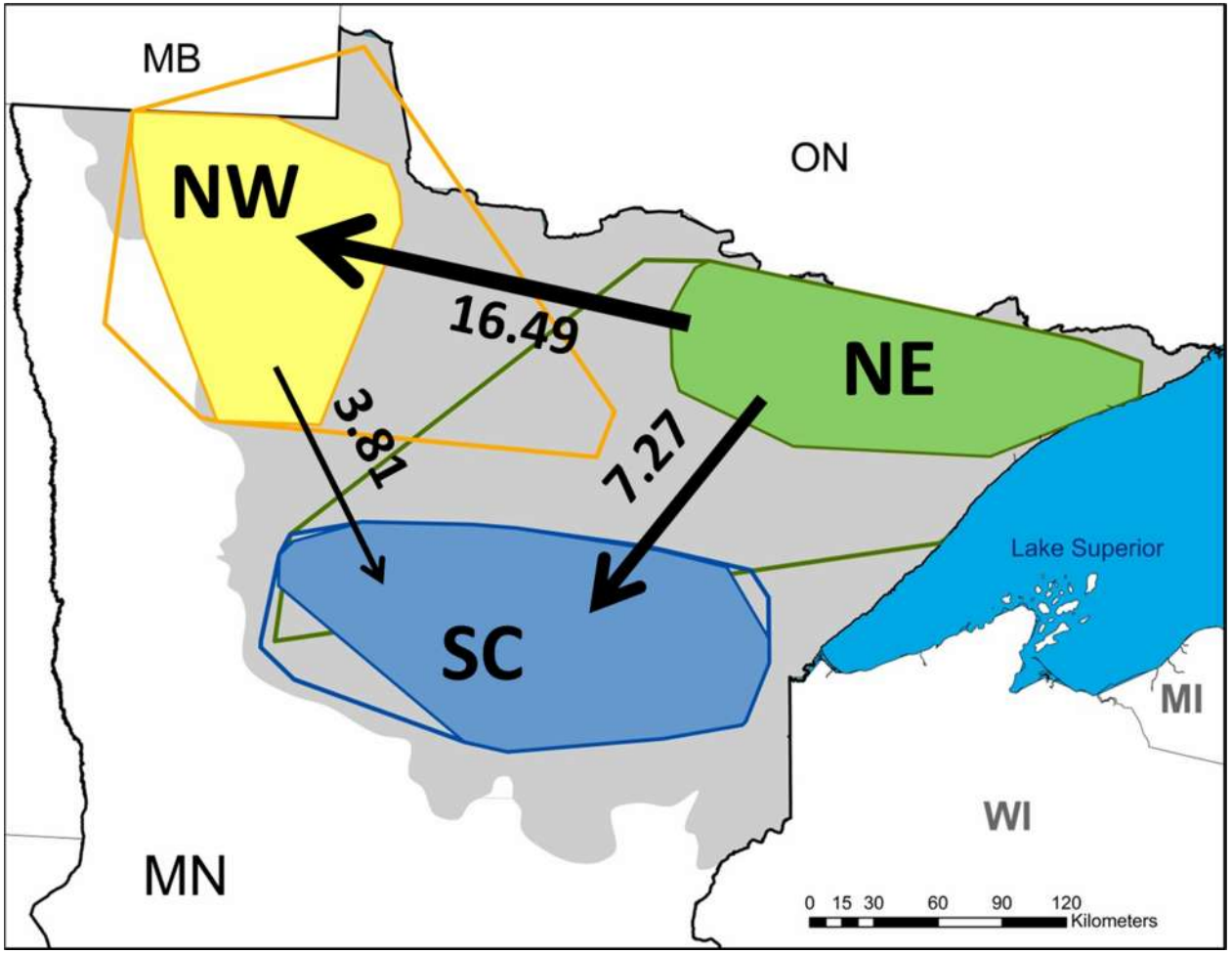

Figure 2.8 Historical net migration rates between STRUCTURE-defined clusters in Minnesota, as estimated using MIGRATE. Values are mutation-scaled net migration rates in numbers of migrants between clusters per generation, with directionality of net migration indicated by the arrows.

CHAPTER 3: HYBRIDIZATION AND INTROGRESSION IN MINNESOTA WOLVES (*Canis lupus*) USING A Y CHROMOSOME LOCUS AND MITOCHONDRIAL DNA

INTRODUCTION

Hybridization and subsequent introgression play a significant role in the adaptation of organisms across the globe (Hedrick 2013). Negative connotations associated with the term hybrid sometimes influence the perceived value of organisms with hybrid ancestry and affect the conservation of all taxa involved (Stronen & Paquet 2013). While hybridization has long been accepted by botanists as an important influence on evolution (Anderson & Stebbins Jr. 1954), its importance in animal evolution has only more recently been gaining scientific support (Dowling & Secor 1997; Abbott *et al.* 2013; Hedrick 2013). Hybridization provides the raw material for natural selection in the form of novel allelic combinations (Stronen & Paquet 2013) and can lead to the introgression of new genetic material at the population level. Hybrid zones can be maintained by a balance between dispersal and selection or by adaptation to different environments, even if hybrid offspring are less fit on average than their parents (Barton 2001; Seehausen 2004). An early classic example of adaptation via hybridization was demonstrated in the tephritid fly *Dacus tryon*, which exhibited expanded ecological tolerance and geographic range expansion attributed to hybridization with the closely related and sympatric *D. neohumeral* (Lewontin & Birch 1966). Adaptive introgression (Anderson & Hubricht 1938) is difficult to demonstrate, but recent studies have provided support for adaptive introgression in several plant (Martin *et al.* 2006; Whitney *et al.* 2010) and animal species (Payseur *et al.* 2004; Teeter *et al.* 2008; Fitzpatrick *et al.* 2009; Pardo-Diaz *et al.* 2012; Hedrick 2013) with varying levels of evidence. Novel methods have also been developed in recent years for measuring introgression at the genetic and genomic level (Payseur 2010). However, the frequency of natural hybridization and introgression, as well as which species are more prone to it, remain unknown.

Infertility or inviability of hybrids is often the limiting factor to successful hybridization in animals (Arnold & Martin 2009). Viable hybrids are more common between closely related animal species, as hybridization between such species may be frequent before reproductive isolation becomes complete (Koblmüller *et al.* 2009). Species that were

historically allopatric but have more recently become sympatric are also likely to lack pre-zygotic reproductive barriers and therefore could have higher rates of successful hybridization (Crispo *et al.* 2011; Stronen *et al.* 2012). Human influence, such as reductions in environmental heterogeneity and niche differentiation, can influence the relationship between species, and human-induced hybridization has been documented in several species of insects, birds, fish, mammals, and plants (Anderson 1948; Seehausen *et al.* 2008; Crispo *et al.* 2011; Brennan *et al.* 2014).

One species in particular in which hybridization is relatively common as a result of recent sympatry and a shared ancestry, and adaptive introgression has been hypothesized to occur, is the coyote *(Canis latrans*) in North America (Kays *et al.* 2010). The coyote is an opportunistic carnivore, able to adapt to a wide range of environments and predominantly associated with open, human-modified habitats (Kays *et al.* 2008). Prior to European settlement, coyotes were found in the Great Plains and Rocky Mountain regions of North America east to the Mississippi River (Young and Jackson 1951). As agricultural land expanded with increasing settlement of the eastern United States and Canada, coyote range expanded eastward and westward. As early as 1919, coyotes were sighted in southwestern Ontario (Hilton 1978), and they had reached west-central Quebec in the mid-1990s (Stronen *et al.* 2012).

As coyotes expanded their range, they increasingly came into contact with gray wolves (*Canis lupus*). Gray wolves had previously inhabited much of the lower 48 states, but were driven northward by habitat destruction and persecution by humans, and wolves were nearly extirpated from the contiguous United States by the mid-20th century (Boitani 2003). This extirpation from the southern parts of their range resulted in extensive range contraction for wolves (Leonard *et al.* 2005). In areas of historic sympatry in the western US, wolves and coyotes generally were mutually exclusive. Wolves are well-known to kill coyotes in these areas (Paquet 1992). However, in regions where sympatry has occurred more recently, wolves and coyotes frequently do not exhibit competitive exclusion.

The relative recent sympatry, along with high mobility and weak intraspecific differentiation, has led to extensive hybrid zones between *Canis* species in North America (Vonholdt *et al.* 2011). This hybridization has been the source of much recent discussion over the taxonomic and legal status of these species and their hybrids (e.g. Wilson *et al.* 2009; Fain *et al.* 2010; Wheeldon *et al.* 2010; Vonholdt *et al.* 2011). In the Great Lakes region of the northern United States and southern Canada, four wolf-like canid species can potentially interbreed: the gray wolf (*Canis lupus*), the red wolf (*C. rufus*), the eastern (or Great Lakes) wolf (*C. lycaon or C. lupus lycaon*), and the coyote (*C. latrans*). The gray wolf originated in the Old World and has existed throughout North America since the late Pleistocene (Nowak 1978). The eastern wolf and red wolf are both of uncertain New World ancestry, with support for hypotheses where they originated either from (a) admixture between coyotes and wolves (supported by mitochondrial analysis in Wayne *et al.* 1992, microsatellite analysis in Roy *et al.* 1994, SNP analysis in Von Holdt *et al.* 2011, and morphological analysis in Nowak 2002) or (b) from the parallel evolution of a wolf-like phenotype from a common coyote-like ancestor in the New World (Wilson *et al.* 2000; Kyle *et al.* 2006; Rutledge *et al.* 2015).

One region of recent sympatry between wolves and coyotes is northern Minnesota, where the range of gray wolves contracted through the mid-20th century before expanding as a result of population growth mediated by increases in prey populations and protection under the Endangered Species Act (Fuller *et al.* 1992). Though substantial genetic work has been completed on canids in the Great Lakes region, evolutionary history and taxonomy remain muddled by shared ancestry and introgressive hybridization. Several studies have found that the mitochondrial DNA of Minnesota wolves includes gray wolf (Old World) haplotypes along with coyote-like (New World) haplotypes (Lehman *et al.* 1991; Roy *et al.* 1994). Some recent studies have suggested that "New World" haplotypes are roughly 150,000-300,000 years divergent from coyotes and more likely belonged to eastern wolves than coyotes (Wilson *et al.* 2000). Y chromosome haplotypes support the scenario of New World genetic material coming to Great Lakes wolves through gene flow from eastern wolves, with Fain *et al.* (2010) finding that gray wolf and

coyote composite Y chromosome haplotypes at seven microsatellite loci were mutually exclusive. However, significant admixture between wolf and coyote DNA at autosomal microsatellite loci (Fain *et al.* 2010) and SNP markers (Vonholdt *et al.* 2011) indicate the presence of *C. lupus*-*C. lycaon* and *C. lupus*-*C. lycaon*-*C. latrans* hybrids in the region.

Despite genetic evidence for wolf-coyote hybridization in Minnesota and surrounding states and provinces, hybrids between wolves and coyotes have not been observed in the area (Wheeldon *et al.* 2010; Mech *et al.* 2014). However, Great Lakes wolf populations tend to genetically cluster halfway between populations of non-hybridizing wolves and populations of coyotes (Roy *et al.* 1994). Hybridizing coyote populations, on the other hand, cluster with non-hybridizing populations, suggesting that hybridization has a greater effect on the genetic content of wolf populations than those of coyotes, perhaps due to backcrossing being more common with wolves than coyotes. This is also evident in the presence of coyote-like (New World) mitochondrial haplotypes in wolves and the absence of wolf-like (Old World) haplotypes in coyote populations (Lehman *et al.* 1991) and suggests asymmetric gene flow between the two species. Despite this evidence for coyote alleles in wolf populations, it is unknown whether the introgression is of phenotypic or functional importance.

In this study, we sequenced regions of the uniparentally-inherited Y chromosome and mitochondrial control region to conduct a fine-scale assessment of introgression and phylogenetic history of western Great Lakes wolves residing in Minnesota. In addition, we tested whether mitochondrial control region haplotypes were strongly associated with characteristics of individual wolves, their location, or breeding status (for female wolves). We also compared wolves from two different sampling years spanning the beginning of recent legal harvest in Minnesota, to determine whether the distribution of haplotypes changed during this time.

METHODS

Samples and DNA extraction

Muscle tissue samples were obtained from wolves harvested during the 2012-2013 (n=192) and 2013-2014 (n=235) wolf harvest seasons in Minnesota during carcass inspection by the Minnesota Department of Natural Resources (MNDNR). There were approximately equal numbers of males and females from each year (Stark & Erb 2013). Harvest occurred across most of the known wolf range in Minnesota and sampled wolves were taken from throughout this range (Figure 3.1).

Muscle tissue was stored at -20ºC until DNA extraction. DNA was extracted using the GeneJET Genomic DNA Purification Kit (ThermoScientific, Waltham, MA, USA). Extractions followed kit protocol, with modifications to improve DNA concentration and purity, including: (1) overnight incubation for the lysis step, (2) an extra minute added to each of the highest speed centrifugation times, (3) an increase to 3 minutes of elution buffer incubation, and (4) a second elution step, resulting in a final elution volume of 400µl. Extracted DNA was quantified and diluted to 2ng/µl for downstream analyses. To confirm extraction results, one Y-chromosome locus (SRY; Meyers-Wallen *et al.* 1995) and one X-chromosome locus (FH2584; Richman *et al.* 2001) were used for sex determination. These loci were amplified in a multiplex reaction with a total reaction volume of 13µl using 2ng of template genomic DNA, 1X GoTaq Flexi buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 0.8µM each forward and reverse primer, GoTaq DNA polymerase (Promega, Madison, WI, USA), and sterile water. PCR products were visualized using 1% agarose gel electrophoresis and scored based on the presence or absence of the SRY band in the presence of the X-chromosome band. A second extraction was performed for individuals that did not have positive sex-determination results.

These samples have previously been genotyped at 19 microsatellite loci (Chapter 2). Using these microsatellite genotypes, individuals were assigned to one of three

genetically distinct clusters in Minnesota based on Bayesian clustering analysis in STRUCTURE (Pritchard *et al.* 2000). Core ranges of these clusters were located towards the northeast (NE), northwest (NW), and south-central (SC) parts of wolf range in the state.

Mitochondrial control region sequencing

An approximately 350bp region of the mitochondrial DNA control region was amplified using primers AB13279 (5'-GAA GCT CTT GCT CCA CCA TC-3'; Pilgrim *et al.* 1998) and AB13280 (5'-GGG CCC GGA GCG AGA AGA GGG AC-3'; Wilson *et al.* 2000). The control region was amplified in a total reaction volume of 13µl using 2ng of template genomic DNA, 1X GoTaq Flexi buffer, 2mM $MgCl_2$, 0.2mM dNTPs, 0.8µM forward and reverse primer, GoTaq DNA polymerase (Promega, Madison, WI, USA), and sterile water. This amplification involved the following PCR conditions: 5 min denaturation at 94ºC followed by 30 amplification cycles of 94ºC for 30s, 60ºC for 30s, and 72ºC for 1 min, with a final 10 min extension at 72ºC. PCR products were cleaned using Exosap-IT (Affymetrix, Santa Clara, CA) prior to performing the sequencing reactions. These products were then used as a template for sequencing in a total reaction volume of 12.0µl including 5X Tris-HCl sequencing buffer, BigDye, 10uM forward or reverse primer, 10ng cleaned PCR product, and sterile water, which was amplified using 25 cycles of 96ºC for 10s, 50ºC for 5s, and 60ºC for 4 min. Samples were then sequenced on an ABI 3730xl sequencer at the University of Minnesota Biomedical Genomics Center.

Y chromosome sequencing

For a subset of male samples (n = 63), we amplified a region of the Y chromosome unique to wolves (*Canis lupus*) and domestic dogs (*C. lupus familiaris)*, but absent in coyotes (*C. latrans*), jackals (*C. aureus*), and foxes (*Vulpes* spp*)*, using primers 650F (5'-GTCCTGGGTTCGGGTTAGTGTTAG-3') and 650R (5'-GTCCTGGGTTGAAGCCCTACATTG-3') from Olivier *et al.* (1999). This primer pair amplifies a 650bp region localized to the nonrecombining portion of the Y chromosome,

and most likely occurs on the short arm (Olivier *et al.* 1999). This region was amplified using the same reaction mixture as the mtDNA control region. Reaction conditions were as follows: 2 min of 94ºC denaturation; 10 cycles of 94ºC for 30s, 58ºC for 30s (decreasing 1ºC each cycle), 72ºC for 1 min; 20 cycles of 30s at 94ºC, 30s at 48ºC, 1 min at 72ºC; and finishing with 10 min elongation at 72ºC. PCR products were cleaned and sequenced as described for the mtDNA control region above.

Sequence and haplotype analysis

Chromatograms for sequencing results were visualized and base calls were manually checked using GENEIOUS version 8.0 (http://www.geneious.com, Kearse *et al.* 2012). Sequences for mtDNA and Y chromosome regions were checked manually and mtDNA sequences were aligned to known sequences obtained from GenBank. Y chromosome sequences were aligned manually, and ambiguous base calls were marked as such. Average sequence divergence between haplotypes was calculated using MEGA (v6, Tamura *et al.* 2013). Measures of nucleotide diversity (π, θ), as well as Tajima's D statistic (Tajima 1989), were calculated using DNASP (v5.10.1, Librado & Rozas 2009). Median joining haplotype networks (Bandelt *et al.* 1999) were visualized and statistics calculated using POPART (http://popart.otago.ac.nz). For the Y chromosome sequences, haplotype reconstruction was inferred in PHASE (v2.1, Stephens *et al.* 2001; Stephens & Scheet 2005), implemented in DNASP.

RESULTS

Mitochondrial control region haplotypes

In the wolves sampled, five haplotypes were identified at the 350bp mitochondrial control region locus (Table 3.1, Figure 3.2). These haplotypes (C3, C12, C22, C23, and C97) had all previously been identified in gray wolves and eastern wolves (Wheeldon *et al.* 2010). Haplotypes C3 and C13 are considered New World, while C22, C23, and C97 are classified as Old World, following Wheeldon *et al.* (2010). Old World haplotype C22 was the most common, found in 47% of the sampled wolves. New World haplotypes C3

(21.5%) and C13 (29.7%) together made up half of the sampled wolves, while Old World haplotypes C23 (1.2%) and C97 (0.2%) were uncommon. Among these five haplotypes, 25 segregating sites were identified. The nucleotide diversity among mitochondrial haplotypes (π) was 0.060, θ =0.054, and Tajima's D indicated that the population did not differ significantly from mutation-drift equilibrium (D=0.77, p>0.10).

To determine whether the distribution of haplotypes differed among specific groups of wolves, we used a series of Fisher's exact tests with the wolves categorized in several ways: by age class (pups, yearlings, and adults), by sex, by breeding status among females (breeders and non-breeders, >1 year of age), and by harvest year (Table 3.2). None of these classifications had significant differences in the distribution of haplotypes (age class, $p_{\text{pup-yearling}}$=0.97, $p_{\text{pup-adult}}$=0.90, $p_{\text{yearling-adult}}$=0.98, $p_{\text{all ages}}$=0.51; sex, p= 0.58; breeding status, p= 0.50; harvest year, p= 0.36). We then grouped the haplotypes into Old World (wolf-like) and New World (coyote-like) and tested the same categorizations, again with no significant associations (Fisher's exact test; all p > 0.10). We used linear regression to test whether the prevalence of coyote-like haplotypes correlated with geographic location in Minnesota, finding that neither latitude (β=-0.040, r^2=0.22, p=0.10) nor longitude (β=0.046, r^2=0.08, p=0.30) was significant as explanatory variables for the proportion of coyote-like haplotypes. Latitude was marginally significant, such that the proportion of coyote-like haplotypes increased slightly from east to west across the state.

When comparing mitochondrial control region haplotypes to microsatellite genotyping results, the distribution of haplotypes was similar among the three STRUCTURE-defined clusters in each sample year (Figure 3.3). Pairwise comparisons between south-central (SC), northeast (NE), and northwest (NW) clusters were not significant, except for comparisons between the NW and the other two clusters in 2012 (Fisher's Exact Test; 2012: $p_{\text{SC,NW}}$= 0.003, $p_{\text{NE,NW}}$= 0.016, all others p>>0.05). Only the comparison between SC and NW clusters was significant after Bonferroni correction for multiple tests (p_{critical}=0.008), such that the northwest cluster has significantly more wolves with haplotype C3 and fewer wolves with haplotype C13 compared to the other clusters

(Figure 3.3). The proportion of Old World and New World haplotypes also did not significantly differ between any clusters in 2012 or 2013 after correction for multiple comparisons (Fisher's Exact Test, all $p > 0.008$).

Y chromosome haplotypes

Seven ambiguous sites were identified at the Y chromosome locus sequenced in the male wolves in this study. Using PHASE for haplotype analysis, two consensus Y chromosome haplotypes were identified: 650A at a frequency of 0.477, and 650B at a frequency of 0.523 (Table 3.3). Both 650A and 650B were found across wolf range in Minnesota; there was no significant correlation between haplotype and latitude or longitude. There was also no significant association between haplotype and age class (Fisher's exact test; p=0.81). The nucleotide diversity among Y chromosome haplotypes was π=0.0057 and Tajima's D indicated no evidence for selection at this site (D= -1.87, $p > 0.05$).

Of the wolves with both mitochondrial control region and Y chromosome haplotypes (n=63), there were roughly equal numbers of each mtDNA haplotype group that had each Y chromosome haplotype (Table 3.4). There was no significant association of mtDNA categorization with Y chromosome haplotype assignment (Fisher's exact test, p=0.62) or mtDNA haplotype with Y chromosome haplotype (Fisher's exact test; p=0.41). There was also no significant association between Y chromosome haplotype and STRUCTURE-identified cluster assignment (Fisher's exact test, p=0.20).

DISCUSSION

In this study, we used mitochondrial and Y chromosome sequencing to study the ancestry of wolves in Minnesota, with implications for past hybridization with coyotes. We found five distinct mitochondrial haplotypes (C3, C13, C22, C23, and C97), two of which are associated with eastern wolves and hypothesized to be of coyote origin. In the 650bp Y chromosome region, we identified two haplotypes (650A and 650B), which occur in roughly equal frequencies in the sampled wolves.

Two gray wolf mitochondrial haplotypes found in Minnesota wolves were previously found in wolves from northern Ontario, northern Quebec, northern Manitoba, and the Northwest Territories (C22, C23; Wilson *et al.* 2000), supporting their Old World origin. Haplotype C22, the most common haplotype found in Minnesota, is common among wolves in Manitoba and was previously found to be the second most common haplotype in the Great Lakes region (Wheeldon *et al.* 2010). The two New World haplotypes, C3 and C13, had been found previously in Manitoba and northwestern Ontario but are more common in eastern wolves (Wilson *et al.* 2000). The C13 haplotype was previously found in wolves from 1899 and 1900 in Minnesota (Wheeldon & White 2009), indicating its presence in the region for more than 100 years. Coyote encroachment on wolf range in the state would have been around the turn of the 20th century, and therefore either slightly predated or co-occurred with these historical samples. Introgression takes several generations of backcrossing, and thus we would not expect to see the occurrence of C13 in historic wolves if its presence resulted from hybridization with coyotes.

Our findings here support previous conclusions that the prevalence of New World mtDNA haplotypes in western Great Lakes wolves indicates hybridization with the eastern wolf, rather than hybridization with coyotes. In a study where coyotes from Minnesota, Wisconsin, and Michigan were sequenced at the same portion of the mitochondrial control region as was sequenced here, none of the 92 coyotes had haplotypes identified in this study (Wheeldon *et al.* 2010). All of these western Great Lakes region coyotes also clustered separately from sympatric wolves in both STRUCTURE and factorial correspondence analyses based on autosomal microsatellites. This, along with evidence for reproductive isolation between western gray wolves and western coyotes (Mech *et al.* 2014), suggests that wolves and coyotes do not—and have not—hybridized in the western Great Lakes region. Though two of the haplotypes found in this study and the Wheeldon *et al.* (2010) study are assigned to a *C. latrans/C. lycaon* origin (C3, C13), our results suggest that these haplotypes are more common in eastern wolves than Minnesota coyotes, indicating that their presence originates from eastern wolves rather than gray wolf-coyote hybridization. The Wheeldon *et al.* (2010) study

sampled nearly half of their coyotes from one region west of Minneapolis, and thus it is possible that the C3 and C13 haplotypes were simply not present in the sampled coyotes but do occur in the state. More sampling from a greater geographical area in the state would be necessary to confirm the lack of these haplotypes in Minnesota coyotes.

Mitochondrial DNA haplotypes have previously been associated with habitat types in wolves. This results from dispersal in gray wolves tending to follow natal habitat types (Leonard 2014). This idea is based on microsatellite marker and mtDNA evidence for structuring related to vegetation and prey types in western North America (Muñoz-Fuentes *et al.* 2009), a continent-wide meta-analysis demonstrating climate and forest type associations (Geffen *et al.* 2004), and a demonstration of structuring with climate, habitat, and prey type despite an apparent lack of migration barriers in Eastern Europe (Pilot *et al.* 2006). In Minnesota, there is similarly a lack of obvious barriers to migration in gray wolves, as well as indication that dispersers are more successful in establishing a territory closer to their home territories than they are at greater distances (Gese & Mech 1991). We did not find a general pattern of mtDNA haplotype structuring associated with any of the variables that we analyzed in this study (Table 3.2, Figure 3.3). However, we did not attempt to correlate mtDNA haplotype with habitat type or prey availability, and this would be an interesting area of further research for this study. While wolf range in Minnesota lies almost exclusively in the Laurentian Mixed Forest biome, two main dominant prey types are available within different portions of the study region: moose and deer. These two prey items have different body sizes, habitat preferences, and climate tolerances, and thus it is possible that a habitat or prey-related association may exist in these Minnesota wolves. Though the statewide moose population is declining (Murray *et al.* 2006; DelGiudice 2015), the moose population in northwestern Minnesota is much smaller than that in the northeastern part of the state. Deer numbers are lower in the northern and northeastern parts of Minnesota, primarily due to winter severity and snow depth (Delgiudice *et al.* 2002). This difference in deer and moose availability is reflected in wolf diet comparisons among the northeastern, northwestern, and central portions of the state (Fritts & Mech 1981; Chenaux-Ibrahim 2015), and future studies should explore

whether this difference in diet composition drives dispersal and population-level mtDNA haplotype structuring.

Prey type has also been associated with morphology in *Canis* hybrid zones, such that wolves and wolf-coyote hybrids consuming larger prey (i.e. deer, moose, and beaver) are larger, while those preying on smaller animals are smaller and more coyote-like in body and skull morphology (Sears *et al.* 2003). Eastern wolves are smaller, have longer ears, and have a narrower rostrum than their western gray wolf counterparts, while hybrids have roughly intermediate characteristics (Mech 2010). Mech and Paul (2008) and Mech (2011) demonstrated a gradient in body and ear length measurements from east to west across Minnesota, with wolves in the northeastern portion of the state having smaller bodies and longer ears, a morphology commonly associated with *C. lycaon* and hybrids and more similar to that found in eastern Ontario. Wolves in northwestern Minnesota, in turn, were morphologically more similar to wolves in Manitoba. This suggests more *C. lycaon* ancestry in the northeastern portion of the state, and more western wolf ancestry in the northwestern portion, a trend that is consistent in both historic and present-day sampling. Thus, we would also expect to see a higher proportion of New World mtDNA haplotypes in the northeastern part of the state, while the gray wolf Old World haplotypes would be more prevalent in the northwestern wolves. However, this was not the case in this study, as there was no significant association between longitude and the proportion of New World haplotypes in an area. This may suggest that the hybrid zone between the populations has shifted, that the two subspecies have been in contact for long enough that the cline across the state has become less pronounced with regard to mtDNA haplotypes, or that there are common selective forces depending on prey size.

Based on morphological and genetic analyses of historic and current wolves, the population in Minnesota has been composed of hybrids between the eastern wolf and western wolf throughout known history (Mech 2010). Measurements of ear lengths, cranial morphology, body size, and genetic composition all indicate that wolves prior to the 1970s were more closely related to eastern wolves, with western wolf influence being increasingly detectable following ESA protections in the United States. However,

throughout this time, Minnesota has remained a hybrid zone between these *Canis lupus* subspecies. Our mtDNA results support this mixing, with Old World and New World haplotypes found in nearly equal proportions in the current Minnesota wolf population.

Though we can detect historic hybridization between *Canis* species with Old World and New World haplotypes, there are limits to interpretation based on mtDNA control region sequencing. It is interesting to find that nearly half of the Minnesota wolf population has traces of *C. lycaon*/*C. latrans* ancestry, but these haplotypes do not indicate whether introgression is present in other regions of the genome or whether the introgression is functionally important. The mtDNA is only one genetic marker and may not be representative of processes occurring in the nuclear genome. The survival of hybrids between *Canis* species and prevalence of mitochondrial capture suggest the persistence of hybrids (either through neutrality or a fitness advantage), but we cannot determine whether this is the case through control region haplotype data without functional support. Caution should be taken in interpreting one line of genetic data in isolation from other genetic data, and especially in isolation from known characteristics of the species such as its ecology, natural history, or fossil record, especially in a well-studied species where these are known (Rutledge *et al.* 2012b).

Though only two Y chromosome haplotypes were identified in the wolves sampled, these two haplotypes included 7 ambiguous sites. This is likely due to tandem duplication of the 650 region on the Y chromosome. The sequenced region was identified as a gray wolf-specific region and was used because it does not amplify in other wild canid species (Olivier *et al.* 1999). However, Olivier *et al.* (1999) also indicated that the segment may be duplicated on the Y chromosome due to complications when amplifying this region for microsatellite genotyping, despite not being able to locate a duplication using FISH or LR-PCR. We ran into the same difficulties in our study, suggesting that its use in sequencing and genotyping studies should be cautioned.

Here, we found no difference in autosomal genotypes related to specific mitochondrial haplotypes, and no association of specific Y chromosome haplotypes with Old World or

New World mtDNA. In other studies that have used both maternally-inherited and paternally-inherited markers, hybridization was detected bi-directionally with one coyote having gray wolf mtDNA and four male coyotes having wolf-like Y chromosome haplotypes (Wheeldon *et al.* 2010). In contrast, Lehman *et al.* (1991) found that coyote genotypes were found in high frequency in sampled wolves, but wolf genotypes were absent from sampled coyotes. Roy *et al.* (1994) supported this with microsatellite genotyping, finding a genetic asymmetry between hybridizing populations of wolves and hybridizing populations of coyotes—the hybridizing coyotes still clustered with coyote populations, while hybridizing wolves clustered together, separate from the other wolves. These two lines of evidence indicate that female coyotes mate more with male wolves than female wolves mate with male coyotes, and that backcrossing to coyotes is much less common than backcrossing to wolves (Roy *et al.* 1994). The 650 Y chromosome locus used in this study does not amplify in coyotes, and thus there may be evidence for male coyote-female wolf hybridization in the Minnesota wolves that we simply could not detect.

These large carnivores play an important role in their ecosystems as top predators, and coyotes, eastern wolves, and western Great Lakes wolves have continued in this role despite hybridization. Our findings support the existing literature suggesting that hybrid ancestry is common in Minnesota wolves, but that individuals with New World mtDNA haplotypes do not differ significantly from those with Old World mtDNA. However, more research is needed to determine the functional importance of mtDNA capture in wild canid species. Whether mitochondrial capture is an artifact of shared evolutionary history or more recent hybridization, these carnivores are important to their ecosystems and thus should be conserved independently of any traces of hybrid ancestry (Stronen & Paquet 2013).

Table 3.1 Mitochondrial control region haplotype frequencies in individuals (n=427) sampled in this study and western Great Lakes (WGL) wolves haplotyped in previous studies. Mitochondrial haplotype names and sequences follow Wheeldon *et al.* (2010).

mtDNA Haplotype	Frequency (this study)	Relative Frequency[1] (Previous WGL Studies)	Species Assignment	Genbank Accession
C3[2]	0.215	0.28	*C. latrans/C. lycaon*	AY267720
C13[3]	0.297	0.24	*C. latrans/C. lycaon*	AY267730
C22[4]	0.474	0.36	*C. lupus*	FJ687608
C23[5]	0.012	0.11	*C. lupus*	FJ687608
C97	0.002	0.011	*C. lupus*	FJ687612

[1] Haplotype frequency calculated from a total of 853 haplotypes reported for historic and contemporary Minnesota, Wisconsin, Michigan, and Ontario samples in Vilà *et al.* (1999); Wilson *et al.* (2000); Grewal *et al.* (2004); Leonard & Wayne (2008); Wheeldon & White (2009); Koblmüller *et al.* (2009); Wheeldon *et al.* (2010). Relative frequencies indicate the proportion of individuals with the given haplotype out of the total for all five haplotypes. Haplotypes not found in this study were excluded from frequency calculations.
[2] Named GL2, GL19, and FWSCly21 in other studies.
[3] Named GL10, GL17, GL18, and FWSCly12 in other studies.
[4] Named W22, W48, lu32, and FWSClu06 in other studies.
[5] Named W20, W55, lu28, and FWSClu07 in other studies.

Table 3.2 Mitochondrial control region haplotype frequencies in wolves from Minnesota. Categorized by age class, sex, and harvest year; none of these categorizations had a significant association with the distribution of haplotypes among categories (Fisher's exact test, $p>>0.10$).

		mtDNA Haplotype				
		C3*	C13*	C22	C97	C23
Age Class						
Pups (Age 0)	n=142	0.211	0.310	0.465	-	0.014
Yearlings (Age 1-2)	n=144	0.201	0.299	0.493	0.007	0.001
Adults (Age 3+)	n=128	0.227	0.273	0.477	-	0.023
Unknown Age	n=13	0.308	0.385	0.308	-	-
Sex						
Male	n=204	0.235	0.294	0.446	0.005	0.020
Female	n=223	0.197	0.300	0.498	-	0.004
Female Breeders	n=49	0.163	0.245	0.592	-	-
Harvest Year						
2012-2013	n=192	0.229	0.328	0.427	0.005	0.010
2013-2014	n=235	0.204	0.272	0.511	-	0.013
Total	n=427	92 (21.5%)	127 (29.7%)	202 (47.3%)	1 (0.2%)	5 (1.2%)

*New World (*C. lycaon/C. latrans*) haplotype.

Table 3.3 Y chromosome haplotypes at the 650 locus identified in this study and frequency among individuals (n=86). Seven sites were ambiguous due to tandem duplication on the Y chromosome; analysis of these was completed using PHASE (Stephens *et al.* 2001).

Haplotype	Base call at ambiguous sites	Frequency in Minnesota
A	CAGCTCT TGGCCCT	0.477
B	CGATCCG TGGCTAG	0.523

Table 3.4 Contingency table analysis of the association between mtDNA control region haplotype classification (Old World: C22, C23, C97; New World: C3, C13) in male wolves sequenced at both loci in this study; Fisher's exact test of independence: $p > 0.10$.

Haplotype	Old World mtDNA	New World mtDNA	*Total*
650-A	14	17	*31*
650-B	17	15	*32*
Total	*31*	*32*	*63*

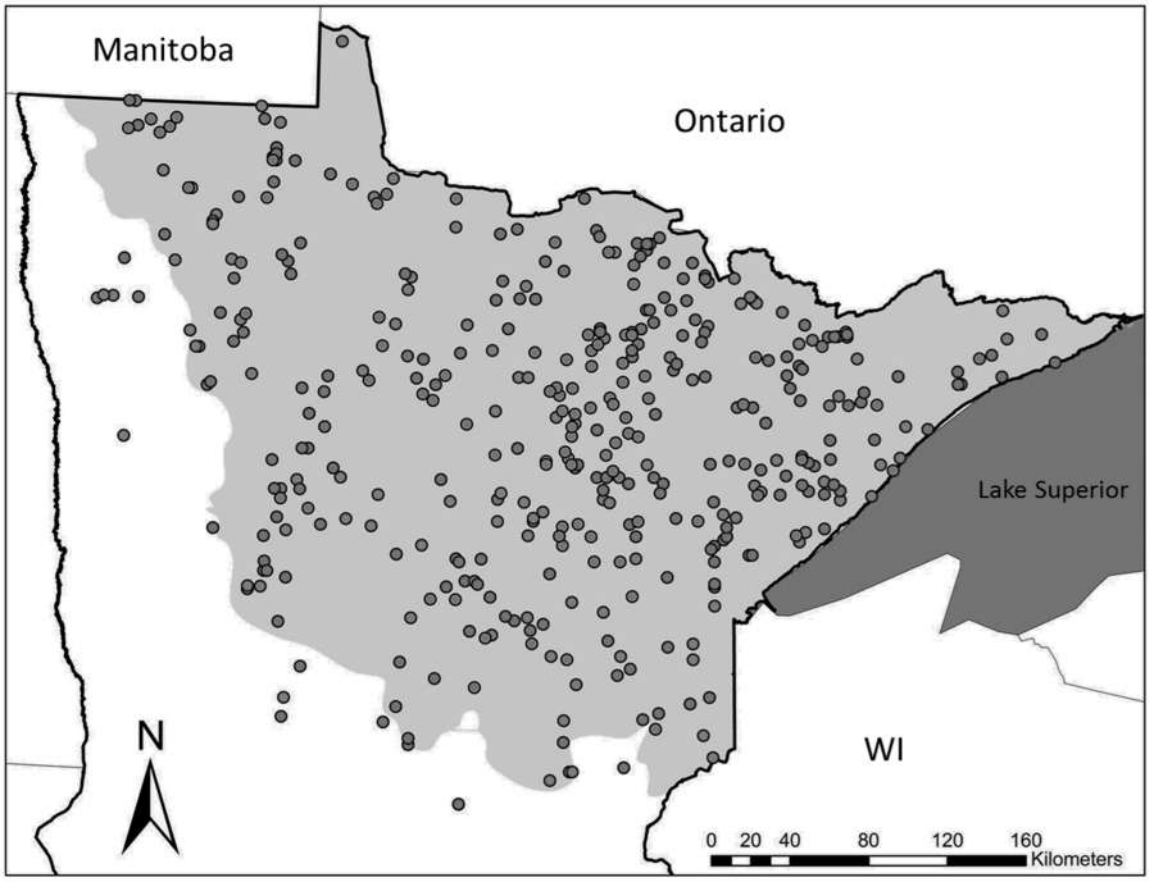

Figure 3.1 Reported collection locations for the wolves (n=427) used in this study. The light gray shaded region shows estimated core wolf range in Minnesota, adapted from MNDNR reports (Erb & Sampson 2013).

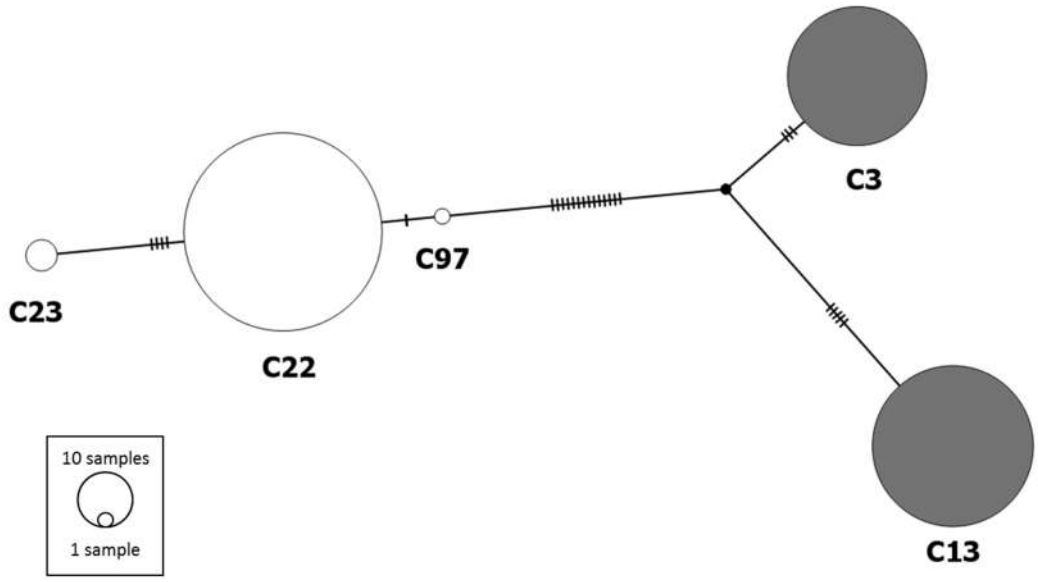

Figure 3.2 A median joining haplotype network of mtDNA control region haplotypes found in Minnesota wolves (n=428). The size of each circle is proportional to the haplotype frequency, and cross hatches indicate the number of mutations between haplotypes. Shaded haplotypes are New World in origin, while unshaded haplotypes are Old World. Nucleotide diversity within these haplotypes is $\pi = 0.0595$, with 25 segregating sites.

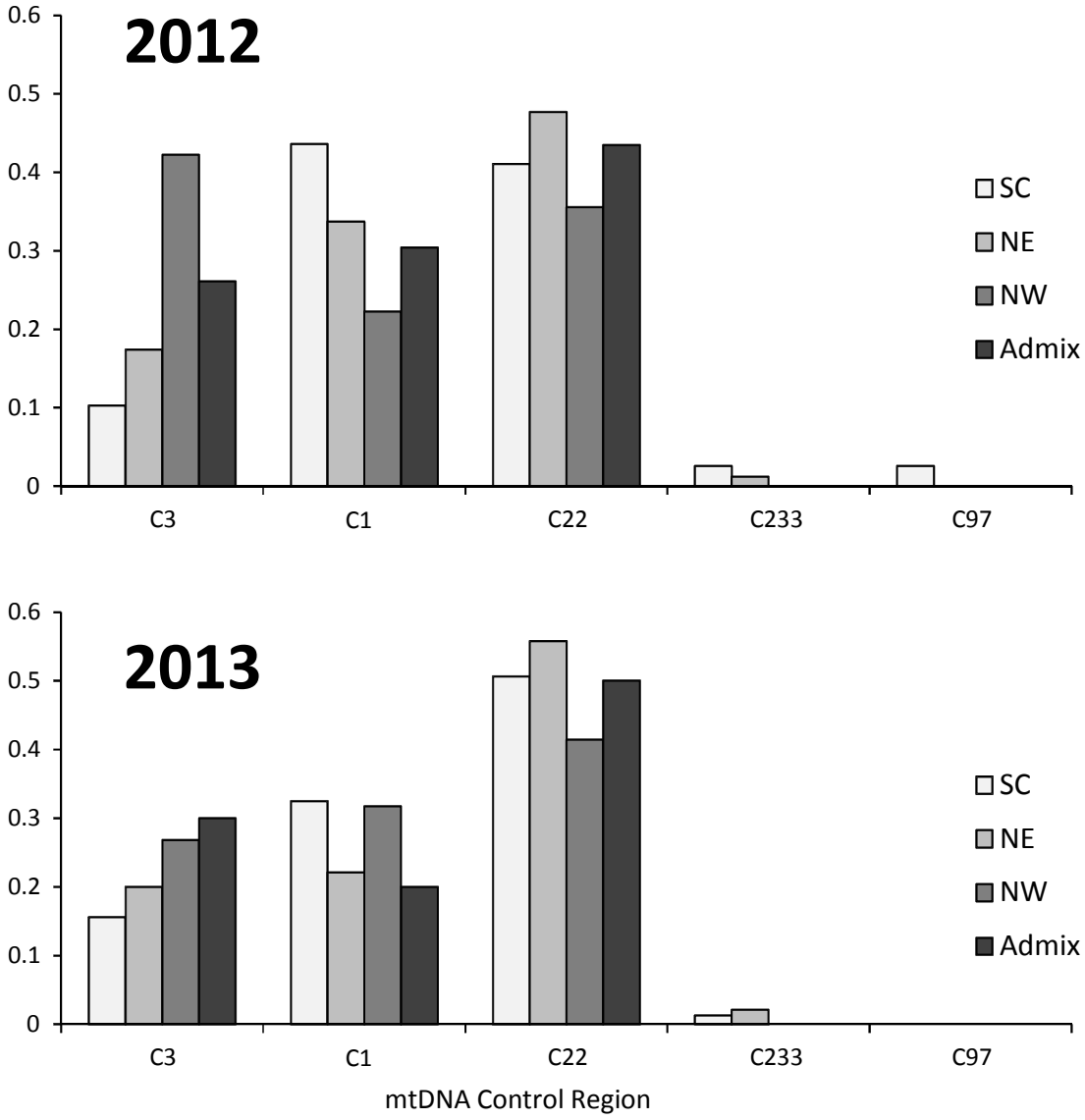

Figure 3.3 Haplotype proportions based on cluster assignment in the (A) 2012 and (B) 2013 sampling years. Cluster assignments are based on Bayesian clustering assignments using microsatellite genotypes, with K=3 clusters for each year (northeast, NE; northwest, NW; south central, SC). In the 2012 individuals, the distribution of haplotypes was significantly different between the SC and NW clusters, and between NE and NW clusters; no significant differences were found in comparisons between clusters of the proportions of individuals with each haplotype for 2013 (Fisher's Exact Test; 2012: $p_{SC,NE}$= 0.31, $p_{SC,NW}$= 0.003, $p_{SSC,Admix}$= 0.39, $p_{NE,NW}$= 0.016, $p_{NE,Admix}$= 0.72, $p_{NW,Admix}$= 0.47; 2013: $p_{SC,NE}$= 0.43, $p_{EC,NW}$= 0.47,$p_{EC,Admix}$= 0.44, $p_{NE,NW}$= 0.32, $p_{NE,Admix}$= 0.69, $p_{NW,Admix}$= 0.75).

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APPENDIX I: PROTOCOLS

Wolf Genomic DNA Extraction Procedure (Tissue Extraction)

Based on directions for the Thermo Scientific GeneJET Genomic DNA Purification Kit (#K0721, #K0722)

1. Turn water bath on to **56ºC** before beginning extractions.
2. Gather supplies:
    a. Forceps
    b. Razor blade
    c. Ethanol squeeze bottle
    d. Cutting board
    e. 12 samples
    f. 12 - 1.5mL microcentrifuge tubes
    g. Digestion Solution
    h. Proteinase K
3. Using forceps and a razor blade, cut and weigh out **20mg (0.020g)** of muscle tissue and place into the corresponding 1.5mL microcentrifuge tube. Repeat for remaining samples, making sure to wipe down forceps and razor blade with EtOH between samples.
4. Add 180µL of Digestion Solution and 20µL Proteinase K to each sample. Vortex thoroughly.
5. Incubate samples in 56º water bath until the tissue is completely lysed and no particles remain. For muscle tissue, this should be approximately **3-4 hours** (or overnight).
    a. During incubation, vortex the tubes occasionally.

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6. Add **20µL** of **RNase A** to each tube; mix by vortexing. Incubate for 10 minutes at room temperature.
7. While samples are incubating, gather the remaining necessary supplies:
    a. From GeneJET kit:
        i. 12 DNA purification columns
        ii. 12 extra collection tubes,
        iii. Lysis Solution
        iv. Wash Buffer I
        v. Wash Buffer II
        vi. Elution Buffer
    b. From lab:
        i. 12 - 1.5mL microcentrifuge tubes (label with sample IDs)
        ii. 50% EtOH
        iii. 50mL waste tube
8. Add **200 µL** of **Lysis Solution** to each tube. Mix thoroughly by vortexing for 15 s, until mixture is homogeneous.
9. Add **400 µL** of **50% EtOH** to each tube; mix by vortexing.
10. Transfer each lysate to the corresponding DNA Purification Column.
11. Centrifuge the columns for 1 minute at 6,000 rcf.

12. Discard the collection tubes containing the flow-through; keep the purification columns. Place each DNA Purification Column into a new 2mL collection tube. Add **500µL Wash Buffer I**.
13. Centrifuge columns for 1 min at 8,000 rcf.
14. Discard the flow-through into the waste tube using a pipette, and place purification column back into the collection tube. Add **500µL** of **Wash Buffer II**.
15. Centrifuge columns for 3 min at maximum speed (≥12,000 rcf).
16. Transfer the DNA Purification Columns to sterile, labeled 1.5mL microcentrifuge tubes. Discard collection tubes with the flow-through.
17. Add **200µL** of **Elution Buffer** to each DNA Purification Column membrane. Incubate for 3 min at room temperature.
18. Centrifuge for 2 min at 8,000 rcf.
19. Repeat elution step: add **200µL** of **Elution Buffer** to each DNA Purification Column. Incubate for 3 min at room temperature, then centrifuge for 2 min at 8,000 rcf.
20. Discard the purification columns.