Phenotypic and genetic variation in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*)

by

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Abstract

Genetic variation is a ubiquitous feature of natural populations and underpins much phenotypic variation. Genetic variance can be partitioned and examined at various hierarchical levels of organization to address fundamental questions in ecology and evolution. Patterns of genetic variance among populations reveals population structure or potential local adaptation, whereas we need to examine genetic variation among individuals within populations to study the genetic architecture underlying phenotypic variation. My thesis examined genetic and phenotypic variance in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) across these scales of organization. I examined population level variation in bighorn sheep across the northern portion of their range in Alberta using microsatellite genetic markers and found that their spatial genetic structure was delineated by inter-river valley regions and showed a strong pattern of isolation-by-distance. Additionally, I identified patterns of spatial genetic structure which indicated gene flow occurred equally between the sexes up to 100km. I also identified declines in genetic diversity moving northwards, which suggests the post-glacial recolonization of the northern Rocky Mountains by bighorn sheep was sourced from the Southern refugium. Within the Ram Mountain population, I then examined the fitness consequences of phenotypic variation at the individual level. I found that longer horned females reproduced and successfully raised offspring earlier in life, and therefore produced more lambs over their lifetime. This highlighted how presumed vestiges of strong sexual selection on males can indicate individual fitness in females, similar to their male counterparts. To investigate the genetic basis of phenotypic variance, I developed a high-density species-specific SNP assay to genotype Rocky Mountain bighorn sheep at 50,000 loci. Using genotypes from this assay and phenotypic data from 305 individuals at the Ram Mountain population, I investigated the genetic basis of eight traits: male and female body mass,

male and female horn length, female age at primiparity, female age at first weaning success, female lifetime reproductive success, and female longevity. I identified 278 loci associated with these traits and further characterised eight focal loci. Of these eight loci, I identified one associated with male horn length that showed temporal patterns consistent with an evolutionary response to selection by trophy hunting in the Ram Mountain population.

Preface

This thesis is composed of original work produced by Samuel Deakin. However, much of this research would not have been possible without biological materials, data, and other resources provided by the Ram Mountain project, Banff and Jasper National Parks, the Alberta Fish and Wildlife forensics unit, and numerous collaborators. Therefore "we" is used throughout the data chapters of this thesis. This thesis used biological samples and phenotypic data from Rocky Mountain bighorn sheep, which were obtained under procedures approved by the Animal Care Committees of the University of Alberta, University of Calgary, and Université de Sherbrooke, all of which complied with guidelines for animal use as provided by the Canadian Council on Animal Care.

A version of Chapter 2 has been published as Deakin, S., Gorrell, J.C., Kneteman, J., Hik, D.S., Jobin, R.M. and Coltman, D.W., 2020. Spatial genetic structure of Rocky Mountain bighorn sheep (Ovis canadensis canadensis) at the northern limit of their native range. *Canadian Journal of Zoology*, *98*(5), pp.317-330. Myself and J.C.G. conceived the study, I conducted analyses, and drafted the original manuscript. J.C.G. and D.W.C. contributed to theoretical background, statistical analysis, and concept formation. D.W.C., D.S.H, J.C.K., R.M.J. provided tissue samples and genetic data used in all analyses. All authors provided input to the manuscript throughout its preparation.

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Chapter 1 - Introduction

1.1 Introduction

Genetic variation is a ubiquitous feature of natural populations and is the mechanism underpinning variation in many phenotypic traits. Selection acts upon this phenotypic variance and if this selection affects the genetic variance underpinning the phenotype evolution occurs. Genetic variance can be partitioned at varying scales of organisation. Genetic variance among individuals in a range results from processes influencing gene flow and evolution across the range, which become apparent when variance is partitioned. Within populations genetic variance between individuals is the basis underpinning much phenotypic variation, thus the genetic basis of phenotype can be investigated by examining the relationship between individual genetic variance and phenotypic variance. Molecular markers are the basis of contemporary conservation genetic and genomic studies and can be used to examine genetic variance at differing levels and make inferences about population structure (Forbes and Hogg, 1999) and the genetic mechanisms underlying phenotypes (Reid *et al.*, 2016; Salmón *et al.*, 2021).

Population structure is the pattern of genetic variation that result from the departure from panmixia in a population. Molecular markers can be used to examine the patterns of genetic variation across a range or within a species to identify and characterise population structure. Environmental or cryptic factors may result in population structure; distance (Wright, 1943) or physical barriers (Peres, Patton and Silvac, 1996; Hitchings and Beebee, 1997; Riley *et al.*, 2006), specialisation of individuals within a species to differing environmental niches (Morin *et al.*, 2010), and differing reproductive cycles within a species (Aspinwall, 1974), may all cease or reduce gene flow within or across an environment, and therefore result detectable differences and patterns of genetic variation.

Within populations, at the individual level, examining patterns of genetic and phenotypic variation using molecular markers can reveal the genetic mechanism underlying traits. By examining molecular markers alongside phenotypic data, correlated variance between phenotype and genotype can be identified, and therefore aid in identifying the genetic basis of phenotypes. However, though naturally occurring (Ritland, Newton and Marshall, 2001), genotype is rarely the sole determinant of phenotype. Instead it is more common for phenotype to be a result of complex

genotype-environment interactions (Bourret and Garant, 2015). Thus, to identify the genetic basis of phenotype, environmental data must be considered alongside genetic and phenotypic data.

Once identified and examined, molecular markers associated with phenotype can reveal evolutionary processes. Within a population, selection acts upon phenotypic variance among individuals. If phenotypic variance is underpinned by a genetic mechanism, selection on phenotype may lead to changes in the genetic basis of the trait and thus, evolution occurs. Therefore, examining changes at these markers across differing environments or along a temporal gradient can reveal how selection is acting upon the underlying genetic basis of the trait and if an evolutionary response is occurring.

The Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) inhabits highly heterogenous terrain (Festa-Bianchet, 1988; Loehr *et al.*, 2006), exhibits great variance in phenotype (Jorgenson *et al.*, 1993; Coltman *et al.*, 2002), and is under high selective pressure based on its phenotype (Coltman *et al.*, 2003; Pigeon *et al.*, 2016). Therefore, bighorn sheep provide an excellent species in which to examine patterns of genetic variation using molecular markers and investigate population genetic structure, the genetic basis of phenotype, and evolutionary responses to selection.

Rocky Mountain bighorn sheep inhabit rugged terrain along the Rocky Mountains of North America, thus it is likely they exhibit genetic structure and variance correlated with variation in the landscape, similar to other species inhabiting highly heterogenous habitats (Keyghobadi, Roland and Strobeck, 1999; Adams and Burg, 2015). Given their affinity for terrain found at high elevations (Festa-Bianchet, 1988; Loehr *et al.*, 2006), it is likely Rocky Mountain bighorn sheep population genetic structure is characterised by the geography of high-elevation habitat, similar to caribou (*Rangifer tarandus*) (Serrouya *et al.*, 2012). Furthermore, this high degree of population structure makes bighorn sheep well suited for examination of population level genetic variance, phenotypic variance, and adaptation, as there is likely little movement of individuals or gene flow between populations which would homogenize phenotypic or genetic variance.

In Rocky Mountain bighorn sheep there are large amounts of variation in phenotype for a variety of traits; including body mass (Jorgenson *et al.*, 1993; Festa-Bianchet, Gaillard and Jorgenson, 1998), horn morphology (Coltman *et al.*, 2002; Coltman *et al.*, 2003; Pigeon *et al.*, 2016), and female reproductive traits (Jorgenson *et al.*, 1993; Festa-Bianchet, Gaillard and Jorgenson, 1998; Poissant *et al.*, 2008). Thus, investigating if and how phenotypic variation in these traits correlates with genetic variation, sheds light on the evolutionary adaptations possessed by individuals within populations and how certain individuals may be better suited to their environment than others. Furthermore, Rocky Mountain bighorn sheep are a trophy hunted species, with harvest selectively targeting larger horned males, of typically higher fitness (Coltman *et al.*, 2003; Pigeon *et al.*, 2016); which has had effects which extend into female horn morphology (Pigeon *et al.*, 2016) . Thus, identifying the genetic basis of traits, particularly ones under selective harvest, allow for studies on the evolutionary responses of harvested sheep at quantitative trait loci.

1.2 Thesis objectives

For this thesis, my aim was to characterise patters of genetic and phenotypic variation within and among Rocky Mountain bighorn sheep populations in northern Alberta. First, I characterised the patterns of genetic variance in bighorn sheep across northern Alberta, to better understand patterns of diversity and variation in this region. I then examined the relationship between female phenotypic variance and female fitness. To examine population level variance at a higher resolution and to understand the genetic mechanisms underlying phenotypic traits I developed a high-density genotyping assay. I subsequently applied this assay to individuals with phenotypic data and examined the genetic basis of male and female body mass, male and female horn length, female reproductive traits, and female longevity.

In **Chapter 2** I examine the population level genetic variation of Rocky Mountain bighorn sheep in Alberta, Canada. To accomplish this I examined microsatellite and mitochondrial sequence data in 1,495 and 188 individuals, respectively. Using these markers, I examined broad-scale and fine-scale spatial genetic structure, sex-biased gene flow, and spatial patterns of genetic diversity. This chapter confirmed that Rocky Mountain bighorn sheep populations in this region are highly structured and that the Ram Mountain, Alberta population is a random mating population genetically distant from all other studied populations. Therefore, Ram Mountain is a suitable

population for examining selection and evolution in Chapters 3 and 5 as there is minimal phenotypic or genetic variance introduced into the population from elsewhere.

In **Chapter 3** I investigated how female phenotypic variation at Ram Mountain is associated with fitness, specifically, the associations between female morphometric and female reproductive traits. For this, I examined the life history and morphometric data of 217 females over ~45 years. I specifically examined how body mass and horn length were associated with female age at primiparity, age at first offspring weaned, fecundity, reproductive lifespan, and lifetime reproductive success (LRS). This chapter identified female body mass and horn length to be indicative of female fitness with heavier, longer horned females having lower ages at primiparity and first weaning, and thus greater LRS.

In **Chapter 4** I detail the design, implementation, and validation of a high-density species-specific genotyping assay for Rocky Mountain bighorn sheep, for high resolution analysis of population genetic structure (see final paragraph of this introduction) and investigating the genetic variance associated with phenotypic traits identified in Chapter 3 (Deakin *et al.*, 2022) and other studies to be associated with individual fitness (Coltman *et al.*, 2002; Coltman *et al.*, 2005; Poissant *et al.*, 2008). Specifically, the assay was designed to type 50,000 SNP loci using Tecan Genomics' (Redwood City, United States) Allegro Targeted Genotyping technology, a form of Single Primer Enrichment Technology (Scaglione *et al.*, 2019). The assay was validated on 40 Rocky Mountain bighorn sheep and 16 individuals from two other sub-species of bighorn sheep and two subspecies of thinhorn sheep.

In **Chapter 5** I investigate the genetic basis of traits in Rocky Mountain bighorn sheep. I used the high-density SNP assay designed in Chapter 4 to genotype 305 individuals from cohorts spanning ~40 years in the Ram Mountain population. Specifically, I performed genome wide association studies to identify the genetic basis of male body mass and horn length which are associated with male fitness (Coltman *et al.*, 2002), and female body mass, horn length, age at primiparity, age at first weaning, LRS, and longevity, which either directly or indirectly affect female fitness (Coltman *et al.*, 2005; Poissant *et al.*, 2008; Chapter 3 Deakin *et al.*, 2022). Furthermore, given that Ram Mountain was subject to trophy hunting until the mid-nineties, once I identified loci

associated with traits, I examined how allele frequencies changed over time with differing harvest regimes.

One of my original, ultimate goals was to use genetic data to examine if male Rocky Mountain bighorn sheep were leaving protected areas during the rut and subsequently being harvested. Chapter 2 (Deakin *et al.*, 2020) identified gene flow between bighorn sheep populations occurs over vast distances up to ~100km, and evidence from Pelletier *et al.* (2014) and Poisson, Festa-Bianchet and Pelletier (2020) suggests rams from protected populations may leave their home ranges in search of breeding opportunities, potentially venturing outside of protected areas. However, due to a temporal overlap between the rut and the hunting season, rams who leave refuge populations may subsequently be harvested. My aim was to identify rams which left protected regions and were subsequently harvested. Once I had identified harvested rams from protected regions, I would examine if these individuals were enriched for alleles know to be associated with horn length (from Chapter 5) when compared to rams from unprotected regions. To investigate this, I intensely sampled faeces from bighorn sheep within Banff and Jasper National Parks in the summer of 2021, obtained horn core samples from all rams harvested in Alberta in the fall of 2021, and subsequently genotyped all these samples on the high-density assay. My aim was to identify matches between these faecal and harvested samples, thus identifying rams who moved out of refuge areas in the rut and were subsequently harvested. Unfortunately, due to a machine malfunction and quality issues with faecal sourced DNA, I did not obtain any genotypes from the faecal sample portion of this project, thus the study could not be completed. I will, provide further details on this study, and what I hypothesize went wrong, in my concluding remarks.

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Chapter 2 - Spatial genetic structure of Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) at the northern limit of their native range

2.1 Chapter summary

The Canadian Rocky Mountains are one of the few places on Earth where the spatial genetic structure of wide-ranging species has been relatively unaffected by anthropogenic disturbance. We characterized the spatial genetic structure of Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) in the northern portion of their range. Using microsatellites from 1495 individuals and mitochondrial DNA sequences from 188 individuals, we examined both broad- and fine-scale spatial genetic structure, assessed sex-biased gene flow within the northern portion of the species range, and identified geographic patterns of genetic diversity. We found that broad-scale spatial genetic structure was consistent with barriers to movement created by major river valleys. The fine-scale spatial genetic structure was characterized by a strong isolation-by-distance pattern, and analysis of neighborhood size using spatial autocorrelation indicated gene flow frequently occurred over distances of up to 100 km. However, analysis of sex-specific spatial autocorrelation and analysis of mitochondrial haplotype distributions failed to detect any evidence of sex-biased gene flow. Finally, our analyses reveal decreasing genetic diversity with increasing latitude, consistent with patterns of post-glacial recolonization of the Rocky Mountains.

2.2 Introduction

In the absence of anthropogenic habitat fragmentation, the natural population genetic structure of species can be defined by natural geographic and ecological barriers (Slatkin, 1985, 1987), or the dispersal capabilities of the species (Wright, 1943; Kimura and Weiss, 1964). When natural fragmentation is present, population spatial genetic structure correlates with natural barriers to gene flow (Slatkin, 1985, 1987). When a habitat lacks natural barriers, spatial genetic structure may exhibit clinal differentiation if the population range exceeds the maximal dispersal distance of the species (Kimura and Weiss, 1964), referred to as isolation by distance (Wright, 1943). In the modern world, anthropogenic habitat fragmentation is common and increasing, and the extent of pristine habitats where natural phenomena can be studied is increasingly limited.

The Canadian Rocky Mountains are a vast, relatively undisturbed, mountainous habitat that are part of an important inter- national conservation initiative (Chester, 2015). Areas of high anthropogenic activity in the northern Rocky Mountains are avoided by certain species (Proctor and Paetkau, 2004; Rogala *et al.*, 2011); however, these areas are minor when considering the vastness of the region and the intensity of human activity in other regions. Prior studies in this region identified broad-scale spatial genetic structure of several species is correlated with natural landscape features that impede gene flow such as high elevation (Adams and Burg, 2015) and major valleys (Serrouya *et al.*, 2012), or subtle ecological factors such as landcover preference (Keyghobadi, Roland and Strobeck, 1999; Samarasekera *et al.*, 2012; Cullingham *et al.*, 2016). Finer scale spatial genetic structure in the northern Rocky Mountains is primarily influenced by isolation-by-distance (Forbes and Hogg, 1999; Keyghobadi, Roland and Strobeck, 1999; Samarasekera *et al.*, 2012; Weckworth *et al.*, 2013; Cullingham *et al.*, 2016). Therefore, this region offers a range minimally affected by anthropogenic activities, where the natural spatial genetic structure of a species can be observed.

Following the Wisconsin glaciation, many species of flora and fauna recolonized northwestern North American including the Rocky Mountains. The two main refugial sources for this postglacial recolonization were the Beringian refugium (in northeastern Siberia and northwestern North America) and the Southern refugium (comprised by most of the modern-day mainland United States) (Hewitt, 2004; Shafer *et al.*, 2010). Genetic signatures of recolonization, primarily resulting

from the founder effect (Frankham, 1997), are well documented for many contemporary populations of flora (Mitton, Kreiser and Latta, 2000; Coltman *et al.*, 2002; Anderson *et al.*, 2006; Godbout *et al.*, 2008; Beatty and Provan, 2010) and fauna (Dueck, 1998; Stone and Cook, 2000; Flagstad and Røed, 2003; Latch *et al.*, 2009; McDevitt *et al.*, 2009; Burns, 2010). By examining patterns of genetic diversity across the landscape, we can make inference about a species recolonization patterns in the northern Rocky Mountains (Shafer *et al.*, 2010).

The range of Rocky Mountain bighorn sheep (*Ovis canadensis canadenis*) extends along the Rocky Mountains from Arizona, USA to Alberta and British Columbia, Canada, to approximately 55°N. For the southern portion of the species range, studies into the movement (DeCesare and Pletscher, 2006) and genetic structure (Miller *et al.*, 2012; Driscoll *et al.*, 2015) of sheep populations are numerous. In the northern portion of their range, studies of movement patterns (Festa-Bianchet, 1986b; Poole *et al.*, 2016) and population genetic structure (Luikart and Allendorf, 1996; Forbes and Hogg, 1999) are more limited. Unlike the southern areas, the northern ranges are relatively unfragmented, unimpacted by translocations, and unaffected by population declines or disease-related die-offs. Notably, respiratory disease has never been reported north of the Bow River (Figure 2-1) (Government of Alberta, 2015). Thus, the northern Rocky Mountains are well suited to study the natural spatial genetic structure of Rocky Mountain bighorn sheep in a relatively intact landscape. Additionally, bighorn sheep in the northern Rocky Mountains are at the northern limit of the species range, and therefore should show signatures of the range edge effect and recent post-glacial recolonization.

Broad-scale spatial genetic structure of Rocky Mountain bighorn sheep is likely influenced by natural landscape features. Studies of desert bighorn sheep (*Ovis canadensis nelsoni*) indicate most fenced highways are barriers to gene flow (Epps *et al.*, 2005; Epps, Crowhurst and Nickerson, 2018). However, most highways in the northern Rocky Mountains lack fences. Instead, it is likely that natural features of the heterogenous landscape are barriers to the gene flow of these alpine ungulates. Alpine ungulates prefer to occupy high elevations; thus, their movement and distribution are primarily restricted by the availability of this habitat. Owing to this habitat preference, the population genetic structure of thinhorn sheep (*Ovis dalli*) and mountain goats (*Oreamnos americanus*) have been found to correlate with areas of high elevation (Worley *et al.*,

2004; Shafer, Côté and Coltman, 2011; Sim *et al.*, 2016). Similarly, Rocky Mountain bighorn sheep show preferences for higher elevation alpine meadows (Festa-Bianchet, 1988; Loehr *et al.*, 2006), situated close to steep cliffs that can be used to escape predators (Festa-Bianchet, 1988; Loehr *et al.*, 2006). Therefore, we expect broad-scale Rocky Mountain bighorn sheep spatial genetic structure to correlate with high elevations, owing to reduced gene flow across lower elevations.

At a finer scale, the adaptations of mountain sheep to alpine environments enable their movement across the rugged terrain of mountain ranges to be unimpeded. Marco Polo sheep (*Ovis ammon polii*) exhibit low levels of population genetic differentiation, indicating that high levels of gene flow persist, despite inhabiting mountainous terrain (Luikart *et al.*, 2011). For Rocky Mountain bighorn sheep, male seasonal movements of 33–48 km have been reported in Alberta, Canada and Montana, USA (Festa-Bianchet, 1986b; DeCesare and Pletscher, 2006), and analysis of microsatellite data (Forbes and Hogg, 1999) indicates moderate to strong patterns of isolation by distance across the entire range of the species. In the mountains, Rocky Mountain bighorn sheep are likely able to move relatively freely, and therefore their finer scale spatial genetic structure is likely to exhibit a pattern of isolation by distance.

Dispersal is well documented across many taxa, with resource competition, kin competition, and inbreeding avoidance commonly theorized as evolutionary drivers (Pusey, 1987; Lawson Handley and Perrin, 2007). Most mammalian species exhibit male-biased dispersal (Pusey, 1987); however, previous studies of mountain sheep found no evidence of sex-biased dispersal (Roffler *et al.*, 2014). Bighorn sheep social structure is highly segregated between the two sexes. Females and sexually immature males coexist until males become sexually mature, at which point they leave the herd to associate with exclusively male bachelor groups (Festa-Bianchet, 1991). Females exhibit high levels of natal philopatry and rarely emigrate from their home ranges (Festa-Bianchet, 1986b; Rubin *et al.*, 1998; Boyce *et al.*, 1999; DeCesare and Pletscher, 2006). However, instead of exhibiting permanent dispersal (Festa-Bianchet, 1991), males undertake seasonal breeding migrations (Hogg, 2000; Poole *et al.*, 2016), visiting other populations during the rut to maximize their reproductive success and returning to their home range following the rut (Hogg, 2000).

Owing to the rarity of female dispersal and the occurrence of male breeding migrations, we expect that the vast majority of gene flow across the landscape to be male mediated.

Two species of mountain sheep recolonized northwestern North America following the Wisconsin glaciation (Loehr *et al.*, 2006). Thinhorn sheep recolonized from the Beringian refugium and other minor northern refugia (Loehr *et al.*, 2006; Sim *et al.*, 2016). It is hypothesized that bighorn sheep recolonized northwestern North America from the southern refugium (Cowan, 1940; Geist, 1971). Luikart and Allendorf (1996) reported bighorn sheep from the northern portion of the range exhibit a reduction in genetic diversity compared with the southern portion, consistent with the hypothesis that the Southern refugium sourced the recolonization of northwestern North America. Given the linear distribution of bighorn sheep in the northern Rocky Mountains, the strength of the founder effect should increase with geographic distance from the Southern refugium, when examined on a finer scale. We therefore hypothesized that current populations of bighorn sheep in the northern Rocky Mountains will exhibit declining genetic diversity with increasing latitude.

We used nuclear microsatellite markers and mitochondrial DNA sequence data to characterize the population structure of the Rocky Mountain bighorn sheep in the northern portion of their range. Our main objectives were to (i) identify potential barriers to gene flow that cause broad-scale spatial genetic structure, (ii) examine fine-scale spatial genetic structure and the extent of contemporary gene flow between sampling locations, (iii) test for sex differences in population genetic structure and gene flow across the landscape of the northern Rocky Mountains, and (iv) characterize fine-scale spatial patterns of genetic diversity across the landscape that may have arisen as a result of post-glacial recolonization.

2.3 Materials and methods

2.3.1 Sampling

Biological material used for DNA analysis was collected over multiple years, using various techniques, by multiple personnel (Figure 2-1 and Table 2-1). The majority of our samples were collected as faeces during the winters from 2013 to 2015 by Government of Alberta staff. Additionally, blood, skin, and hair samples were collected from sheep at the Luscar-Greg mine site, Ram Mountain, Sheep River, Castle Yarrow, Narraway, and Radium by Government of

Alberta or Government of British Columbia staff, or university researchers under protocols approved by the University of Alberta Animal Use and Care Committee (certificate No. 610901) and the University of Calgary Animal Care Committee (protocol No. BI11R-14), following the guidelines set out by the Canadian Council on Animal Care. Finally, we obtained genetic data from hunter-submitted horn cores analysed by the Alberta Fish and Wildlife Forensics Unit (AFWFU). GPS coordinates were recorded for all sampling locations, with exception to hunter-submitted horn cores that were grouped by wildlife management unit. All sampling locations, except Ram Mountain, were entirely native populations. For Ram Mountain, we used a pedigree (Poirier *et al.*, 2019) to select only individuals of native ancestry for genotyping.

2.3.2 DNA isolation

We extracted DNA from biological material using protocols optimized for each material type. Skin and hair samples were digested and extracted using the DNeasy Blood and Tissue kit (Qiagen N.V., Venlo, Netherlands), following the manufacturer's recommended procedure. Blood was treated with ammonium–chloride– potassium (ACK) (Brown, Hu and Athanasiou, 2016) prior to undergoing the same procedure as skin and hair samples. For faecal samples, three pellets from each sample were soaked in 1X phosphate-buffered saline (PBS) for 20 min before being swabbed around the exterior with a cotton tip applicator, which in turn was washed with Aquastool (MoBiTec GmbH Goettingen, Germany); the material was digested and extracted following the Aquastool protocol.

2.3.3 Microsatellite genotyping

We amplified extracted DNA across 13 nuclear microsatellite loci and one sex-determining amelogenin marker (Table 2-2). Loci were amplified either individually or in one of three multiplexed groups (for further details regarding master mixes, thermocycler profiles, and co-loading see Appendix A1). We ran amplified products on an Applied Biosystems 3730 capillary sequencer and alleles were scored based on fragment size using GENEMAPPER software version 4.0 (Applied Biosystems, Foster City, California, USA). Faecal samples were amplified and scored in triplicate to ensure data quality and minimize allelic drop-out. A subset of skin samples was also genotyped by the AFWFU to ensure bin calibration prior to merging data sets.

We extracted and genotyped 1,252 faecal samples in triplicate; the software GIMLET version 1.3 (Valière, 2002) was used to produce consensus genotypes across the three replicates for each faecal sample. We accepted any allele that was present in at least two out of the three replicates, but then only retained samples that achieved >60% genotyping success across the 13 loci, which resulted in 957 samples (76% success rate). We used CERVUS version 3.0 (Kalinowski, Taper and Marshall, 2007) to identify identical genotypes as a result of repeatedly sampling the same individuals. Identical samples were pooled into consensus genotypes until only unique samples remained. In five instances where identical genotypes were found in two different sampling locations, each genotype was removed from one sampling location at random. When there was only one mismatch between samples in the same location, we scrutinized the electropherograms produced by GENEMAPPER version 4.0 a second time. Our final list contained 613 unique faecal genotypes (49% of the original starting samples). We also genotyped 309 sheep from blood, skin, and hair samples and pooled 573 horn cores from the AFWFU for a total sample size of 1,495 unique genotypes (922 samples with GPS coordinates).

2.3.4 Mitochondrial DNA D-loop sequencing

We selected 202 individuals from 18 georeferenced sampling locations spread across the length of the sampling area to be sequenced (Figure 2-1 and Table 2-1). We amplified a portion of the D-loop region using two different polymerase chain reaction (PCR) conditions depending on whether the template DNA was sourced from faeces or tissue and then performed clean-up using ExoSap (further details in Appendix A2). For each individual, two PCR amplifications were performed, which resulted in two technical replicates. We sequenced the D-loop portion using Applied Biosystems 3730 capillary sequencer. Both replicates were sequenced in both directions, resulting in two forward sequences and two reverse sequences for each individual. The four sequences were aligned in Geneious version 11.1.4 (https://www.geneious.com; Kearse *et al.*, 2012) to produce a consensus sequence. If two or more sequences were of poor quality and failed to align when producing a consensus sequence, then the individual was subject to resequencing. We used BLASTn to align the haplotypes discovered against other bighorn sheep accession numbers in the NCBI database.

2.3.5 Summary statistics

We used Microsatellite analyser version 4.05 (Dieringer and Schlötterer, 2003) to calculate the global expected heterozygosity (H_{exp}), observed heterozygosity (H_{obs}), and number of alleles, which we used to calculate Wright's inbreeding coefficient (f) based on the formula $f = (H_{exp} - H_{obs})/H_{exp}$. We repeated these calculations for each sampling location, with the addition of rarefied allelic richness to account for uneven sample size between sampling locations. We also used Microsatellite analyser version 4.05 to calculate Nei's (1972) standard genetic distance corrected for small sample sizes and Fst (Weir and Cockerham, 1984) among sampling locations. Significance of the F_{ST} distances were tested with 10,000 permutations.

2.3.6 Broad-scale spatial genetic structure

To investigate subtle population structure that might not be apparent when using pairwise distance estimates, we performed a principal coordinate analysis in the R package *adegenet* version 2.1.1 (Jombart, 2008). Additionally, we used the Bayesian approach developed in STRUCTURE version 2.3 (Pritchard, Stephens and Donnelly, 2000). The admixture analysis included correlated allele frequencies and was run for 500,000 Markov chain Monte Carlo iterations after a burn-in period of 50,000 and was repeated across 10 independent runs for values of K1–K8. This analysis was done for the entire 1495 individual data set and no prior sampling location information was included. Following the initial runs on the entire data set, individuals occurring between the Bow and the Athabasca rivers, as well as individuals north of the Athabasca River, were subjected to separate follow-up analyses, each with 10 independent runs of K1–K4 to investigate substructure as recommended by Janes *et al.* (2017). The entire admixture analysis was also repeated using prior location data. We used CLUMPAK (Kopelman *et al.*, 2015) to combine the 10 independent runs for each value of K and to produce admixture plots. We produced likelihood plots using the Evanno method (Evanno, Regnaut and Goudet, 2005) in the R package *POPHELPER* version 2.2.7 (Francis, 2017).

To further test if population genetic structure was influenced by the presence of major river valleys, we used a hierarchical analysis of molecular variance (AMOVA) in GENALEX version 6.5 (Peakall and Smouse, 2006, 2012). The 1495 individuals were grouped by the 49 sampling locations, which were in turn grouped a priori into five regions separated by the Bow River, the

North Saskatchewan River, the Athabasca River, and the Smoky River (Figure 2-1). Pairwise genetic distance was measured with F_{ST} and significance was tested using 9,999 permutations.

To examine the effect that each river valley had on genetic distance individually, we performed a series of partial Mantel tests using subsets of georeferenced samples from locations separated by major rivers. To test the Athabasca River, we created a subset of individuals from sampling locations between the North Saskatchewan and the Smoky rivers. For the Bow River, we created a subset of individuals from sampling locations south of the North Saskatchewan River. We used individuals from sampling locations between the Bow and the Athabasca rivers to test the North Saskatchewan River. Finally, for the Smoky River, we used a subset of individuals from sampling locations north of the Athabasca River. For each of these subsets, the R package *adegenet* was used to calculate Nei's (1972) standard genetic distances and Euclidean geographic distance matrices. Partial Mantel tests were performed between the matrix of Nei's distances and a binary matrix denoting whether a pair was on the same (0) or opposite (1) sides of the river, while conditioning on Euclidean geographic distance using the software *ZT* version 1.1 (Bonnet and Peer, 2002). Each partial Mantel test was performed for 1,000,000 permutations.

2.3.7 Fine-scale spatial genetic structure

To examine the effect of Euclidean geographic distances on genetic distances, we tested for the common effect of isolation by distance. To avoid problems caused by small sample sizes, some sampling locations were either pooled or removed. Any sampling locations with fewer than 10 individuals were pooled with the closest sampling location within 15 km, unless they were separated by one of the major rivers (Figure 2-1). Sampling locations that were pooled were assigned the GPS coordinates of the sampling location with the most samples prior to pooling. Any sampling locations with less than 10 individuals that were farther than 15 km or separated by a major river from another sampling location were removed from the data set. This resulted in a data set of 918 individuals from 33 georeferenced sampling locations. Nei's (1972) standard genetic distances and Euclidean geographic distances were calculated among sampling locations with the R package *adegenet*. We then performed a Mantel test in *adegenet* to compare Nei's distance with Euclidean distance among all pairs of locations and plotted this relationship with ggplot version 3.1.0 (Wickham, 2016). To address the potential issues that hierarchical population

structure can cause for analysis of isolation by distance (Meirmans, 2012), we performed a partial Mantel test between Nei's genetic distances and Euclidean distances while conditioning on a binary matrix denoting whether a pair belonged to the same (0) or different (1) genetic clusters identified by admixture analysis, using the software ZT version 1.1. Mantel and partial Mantel tests were performed for 1,000,000 permutations.

We performed a spatial autocorrelation to investigate spatial genetic structure and infer gene flow across the landscape. We calculated five relatedness coefficients between the 922 georeferenced individuals using SPAGeDi version 1.5 (Hardy and Vekemans, 2002). These were Moran's I (1950), Queller and Goodnight's (1989) estimate of relatedness, Lynch and Ritland's (1999) estimate of relatedness, Wang's (2002) relatedness estimator, and the measure of similarity developed by Li, Weeks and Chakravarti (1993). All of the metrics showed highly similar patterns; therefore, we used Queller and Goodnight's estimator of pairwise relatedness for subsequent analysis. We divided Queller and Goodnight's estimates of pairwise relatedness into 17 distance classes based upon the Euclidean geographic distance between the pair of individuals tested. The 17 distance classes ranged from 0 to 300 km split by 20 km intervals, with one class consisting of all individuals >300 km apart. The mean values for each class were then subjected to a two-tailed test to determine if the mean relatedness of the distance class exhibited a significantly positive or negative difference from zero.

2.3.8 Geographic patterns associated with mitochondrial variation and spatial patterns of sex-specific genetic variance

To assess differences in population genetic structure between the sexes and to make inferences about sex-biased gene flow, we examined the distribution and differentiation of maternally inherited haplotypes. We ran a hierarchical AMOVA in Arlequin version 3.5.2.1 (Excoffier and Lischer, 2010) for 99,999 permutations, using all sequenced individuals from 18 sampling locations that were in turn grouped a priori into five regions separated by the Bow River, the North Saskatchewan River, the Athabasca River, and the Smoky River (Figure 2-1). A haplotype network was produced in the R version 3.4.4 package adegenet. We calculated Nei's standard genetic distance and F_{ST} in the R package *adegenet* and *Arlequin*, respectively. Using nuclear microsatellite data and mitochondrial F_{ST} values, we performed a calculation used by Roffler *et*

al.,(2014) to quantify sex-biased dispersal. If gene flow rates are equal between the sexes, then mitochondrial F_{ST} values should be two to four times higher than nuclear F_{ST} values (Birky, Maruyama and Fuerst, 1983; Larsson *et al.*, 2008; Allendorf, Luikart and Aitken, 2013). Additionally, we performed the previously described spatial autocorrelation analysis for each sex separately.

<u>2.3.9 Geographic patterns of genetic diversity</u>

To investigate the effect of latitude on genetic diversity, we calculated the expected heterozygosity and rarefied allelic richness of georeferenced sampling locations. We used the same modified data set used to investigate the effect of isolation by distance. Allelic richness was rarefied to the number of samples in the smallest of the pooled sampling locations. The relationships between expected heterozygosity and latitude, as well as between rarefied allelic richness and latitude, were tested using a Pearson's correlation in R version 3.4.4 (R Core Team, 2013) and plotted with ggplot.

2.4 Results

<u>2.4.1 Genotyping success and quality control</u>

We genotyped 1,495 individuals from the 49 sampling locations across the northern Rocky Mountains, of which 587 were female and 908 were male (Table 2-1). The mean genotyping success across all samples and loci was 92.88%. Mitochondrial DNA was successfully sequenced in 188 of 202 individuals and aligned at a 220 bp segment of the mitochondrial DNA D-loop region, comprising six unique haplotypes. When aligned to the NCBI records for bighorn sheep, our haplotypes were a 99% match with the bighorn sheep mitochondrial genome (accession No. MH094035.1). The six haplotypes were uploaded to the NCBI GenBank, with the accession nos. MK660218, MK660219, MK660220, MK660221, MK660222, and MK660223.

<u>2.4.2 Summary statistics</u>

The total number alleles per locus ranged from four (BMC1222) to 15 (BM4505), with BMC1222 also having the lowest observed and expected heterozygosity. We found all loci deviated from Hardy–Weinberg equilibrium when samples were pooled as a single population (Table 2-2). Across sampling locations, the mean number of alleles per locus ranged from 2.92 to 7.85, and

when rarefied to the sample size of the location with the fewest individuals, allelic richness ranged from 2.45 to 3.37. Observed heterozygosity ranged from 0.47 to 0.78, whereas expected heterozygosity ranged from 0.51 to 0.73 across sampling locations. Wright's inbreeding coefficient within each sampling location ranged from –0.186 to 0.094 (full results in Table 2-1). The mean pairwise F_{ST} distances and Nei's standard genetic distances between all 49 sampling locations were 0.09 and 0.187, respectively (table of genetic distances available in Appendix A Table A1).

2.4.3 Broad-scale spatial genetic structure

Two clusters of individuals were apparent in a plot of the first two principal components (Appendix A Figure A1). One cluster contained all samples south of the Bow River including Fernie, Elko, and Radium (FR), Castle Yarrow (CY), South of Bow (SO), Sheep River (SR), and Radium (RD), whereas the other cluster contained all sampling locations north of the Bow River with geographically close sampling locations grouped together. Principal coordinates one and two explained 5.42% and 3.35% of the variance, respectively. The Bayesian clustering analysis indicated between four and six genetic clusters in the entire data set (Figure 2-2 and Appendix A Figures A2 and A3); K = 4 was indicated as the most probable K in the likelihood plots (Appendix A Figure A4). North of the Athabasca River, two genetic clusters were apparent (Figure 2-2 and Appendix A Figures A2 and A3) and K = 2 was most supported by the likelihood plots (Appendix A Figure A4). For the region between the Bow and the Athabasca rivers, two genetic clusters were apparent (Figure 2-2 and Appendix A Figures A2 and A3) and K = 2 was the most supported by the likelihood plots (Appendix A Figure A4). From the Bayesian clustering analysis, we concluded that there are five genetic clusters in the entire data set defined by major rivers. The clusters (as seen in Figure 2-2) correspond to regions south of the Bow River (dark orange, K4 plot), between the Bow and the North Saskatchewan rivers (light green, K2* plot), between the North Saskatchewan and the Athabasca rivers (dark blue, K2* plot), between the Athabasca and the Smoky rivers (dark green, K2* plot), and north of the Smoky River (red, K2* plot). The geographic distribution of genetic clusters is shown in Figure 2-1. Our AMOVA of 1495 individuals was significant (p < 0.01) at all hierarchical levels, with major river valleys accounting for 5% of the total genetic variance. Differences among the 49 sampling locations explained 8% of the total genetic variance, while differences among individuals within locations explained another 8% and

differences within individuals explained the remaining 79%. Sampling locations separated by the Athabasca, North Saskatchewan, and Smoky rivers were more genetically distant than expected for their geographic proximity, and sampling locations not separated by these rivers were less genetically distant than expected (Figure 2-3). Sampling locations either side of the Bow River appeared to only show a trend for isolation by geographic distance (Figure 2-3). Results of partial Mantel tests for each river confirm the Bow River ($r = 0.183$, $p = 0.234$) did not significantly influence genetic distance when controlling for Euclidean geographic distance. However, the North Saskatchewan ($r = 0.551$, $p < 0.001$), Athabasca ($r = 0.823$, $p < 0.001$), and Smoky ($r = 0.792$, $p < 0.001$) rivers had a significant influence on genetic distance when controlling for Euclidean geographic distance.

2.4.4 Fine-scale spatial genetic structure

A strong isolation-by-distance pattern was apparent between Nei's standard genetic distance and Euclidean geographic distance among the 33 georeferenced sampling locations (Figure 2-4). This pattern was statistically significant ($r = 0.84$, $p < 0.001$), with geographic distance explaining 84% of the variance in genetic distance. When controlling for genetic hierarchical population genetic structure, isolation by distance was still statistically significant ($r = 0.82$, $p < 0.001$), with geographic distance explaining 82% of the variance in genetic distance. We also observed a negative relationship between pairwise relatedness and increasing geographic distance across the study area. Positive mean relatedness values were observed out to 100 km (commonly referred to as neighborhood size) and negative relatedness values were observed past 200 km (Figure 2-5), a trend that was consistent across all relatedness estimators.

2.4.5 Geographic patterns associated with mitochondrial variation and spatial patterns of sex-specific genetic variance

We identified six haplotypes in the mitochondrial DNA D-loop region. The majority of individuals (151) shared the M haplotype, which was found throughout the sampling area. We observed no pattern in the distribution of the 201C haplotype; however, haplotypes 131A, 144G, and 186T spanned across different rivers, whereas haplotype 239C was restricted to a single sampling location (Figure 2-6). Grouping locations *a priori* into regions by major rivers accounted for 5.98% of the total genetic variance, whereas grouping sampling locations within regions accounted for

34.49% and grouping within sampling locations accounted for 59.53% (p < 0.001). The haplotype network (Figure 2-6) indicated the 131A, 144G, 186T, 201C, and 239C haplotypes all differed from the M haplotype by only one nucleotide each. Our mitochondrial F_{ST} values (Appendix A Table A2)1 had a mean of 0.224, which was 2.46 times higher than our mean microsatellite F_{ST} value of 0.091. Both our sex-specific spatial autocorrelation and comparison of mitochondrial and microsatellite F_{ST} values indicated the absence of sex-biased gene flow. When we performed spatial autocorrelation upon each sex independently, positive mean relatedness values were observed out to 100 km for both males and females, and negative mean relatedness values were observed past 200 km for males and past 300 km for females (Figure 2-5). Both our comparison of mitochondrial and microsatellite F_{ST} values and sex-specific spatial autocorrelation indicated the absence of sex-biased gene flow.

<u>2.4.6 Spatial patterns of genetic diversity</u>

We observed a reduction in genetic diversity with increasing latitude, indicated by the negative correlations between expected heterozygosity of the 33 georeferenced sampling locations and increasing latitude (Pearson r = –0.645, df = 31, p < 0.001; Figure 2-7), and rarefied allelic richness (Pearson r = –0.579, df = 31, p < 0.001; Figure 2-7) of the 33 georeferenced sampling locations and increasing latitude.

2.5 Discussion

Our analyses of bighorn sheep population genetic structure provide clear inferences about patterns of gene flow and historical recolonization in the northern Rocky Mountains region. We observed spatial genetic structure consistent with the hypothesis that major river valleys function as a barrier to gene flow. Additionally, we observed a significant isolation-by-distance pattern within our study area, with Euclidean geographic distances accounting for the majority of genetic distance between sampling locations, suggesting geographically limited gene flow. Observed patterns of individual pairwise relatedness indicate Rocky Mountain bighorn sheep in this region exhibit positive mean relatedness (neighbourhood size) up to 100 km. From our analyses of mitochondrial markers and sex-specific pairwise relatedness, we did not observe any indication of differing population genetic structure between the two sexes, thus suggesting the absence of sex-biased gene flow. Finally, the

distribution of genetic diversity that we observed indicates the post-glacial recolonization of the region proceeded from the Southern refugium northwards in a stepping-stone pattern.

We found all loci exhibited excess homozygosity when samples were pooled as a single population and a mean inbreeding coefficient of 0.094, possibly resulting from the Wahlund effect (Wahlund, 1928). When the data set was separated by sampling location, no substantial deviation from Hardy–Weinberg equilibrium was detected and the mean inbreeding coefficient was reduced to –0.012, thus indicating the initial deviations from Hardy–Weinberg equilibrium were due to population substructure. Sampling locations exhibited genetic differentiation, with a mean F_{ST} of 0.092 being found between all sampling locations.

2.5.1 Broad-scale spatial genetic structure

We observed spatial genetic structure defined by major river valleys, which functioned as barriers to gene flow. The AMOVA indicated that 5% of genetic variance within the data set was among the five regions separated by four major rivers (the Bow River, the North Saskatchewan River, the Athabasca River, and the Smoky River). From our principal component analysis, we observed that sampling locations south of the Bow River were genetically distinct from sampling locations north of the Bow River, and sampling locations north of the Bow River grouped together with other sampling locations based upon geographic proximity (Figure 2-2). Bayesian clustering analysis performed on the whole data set indicated the presence of five genetic clusters (Figures 1 and 2 and Appendix A Figures A2, A3, and A4). The five clusters were defined by major rivers; a single genetic cluster was identified for each of the following regions: south of the Bow River, between the Bow and the North Saskatchewan rivers, between the Athabasca and the Smoky rivers, and north of the Smoky River. This overall pattern indicated population genetic structure correlated with the inter-river regions, and these major rivers functioned as barriers to gene flow.

When further examined by region-specific isolation-by-distance plots and partial Mantel tests, we observed the North Saskatchewan, Athabasca, and Smoky rivers to have an effect on genetic distance (Figure 2-3). The Bow River did not appear to have an effect on genetic distance despite individuals from south of the Bow River appearing highly dissimilar from individuals from the main range (Figure 2-3). Gene flow between sampling locations either side of the Bow River is

already rare due to the geographic distances between sampling locations, thus we did not detect any effect of isolation caused by the Bow River. Instead, we observed genetic distances between sampling locations that were consistent with an isolation-by- distance pattern (Figure 2-3). The presence of major rivers appears to be a barrier to the gene flow of bighorn sheep populations, when populations are located within close geographic proximity to major rivers.

Although our analyses identify major rivers as barriers to gene flow, we cannot infer whether river valleys are a physical barrier to gene flow or represent lowland, heavily vegetated habitats far from escape terrain, which are avoided by bighorn sheep. The genetic structure of bighorn sheep is consequently correlated with areas of high elevation similar to thinhorn sheep and mountain goats (Loehr *et al.*, 2006; Shafer, Côté and Coltman, 2011; Sim *et al.*, 2016). We cannot infer whether it is solely rivers, the lowland habitats that rivers represent, or a combination of both which fragments the landscape for bighorn sheep. We conclude that the primary barriers to the gene flow of Rocky Mountain bighorn sheep are river valleys, as a result of the natural landscape features associated with them.

2.5.2 Fine-scale spatial genetic structure

Across the 680 km mostly linear sampling area, we observed a strong isolation-by-distance pattern characterized by a high correlation between Euclidean geographic distance and genetic distance (Figure 2-4). Euclidean distance accounted for 82% of genetic distance when controlling for genetic hierarchical spatial genetic structure. Thus, beneath the hierarchical population genetic structure caused by major rivers, geographic distance is a major factor determining the genetic variation of Rocky Mountain bighorn sheep in the northern Rocky Mountains. When compared with other large mammals from the region, the isolation-by-distance pattern observed in our data has a stronger correlation than reported in brown bears (*Ursus arctos*) (Forbes and Hogg 1999), woodland caribou (*Rangifer tarandus caribou*) (Weckworth *et al.*, 2013) and grey wolves (*Canis lupus*) (Forbes and Hogg, 1999; Cullingham *et al.*, 2016). It is unsurprising that bighorn sheep from this region show such a strong isolation-by-distance pattern. Bighorn sheep are habitat specialists; thus, their dispersal is more limited than more generalist species (Forbes and Hogg, 1999). Our data indicate a stronger correlation between Euclidean geographic distance and genetic

distance than previously observed in Rocky Mountain bighorn sheep (Forbes and Hogg, 1999); however, this is likely a result of our more intense sampling regime concentrated in a smaller area.

The strong isolation-by-distance pattern is indicative of a stepping-stone model of population structure, with gene flow occurring frequently among geographically close subpopulations; however, as subpopulations become separated by more distance, gene flow declines (Kimura and Weiss, 1964). Previous studies document male breeding migrations in which males leave their home range to reproduce with other nearby populations (Hogg, 2000). This behaviour fits the stepping-stone model (Kimura and Weiss, 1964). Breeding migrations result in localized gene flow; thus, genetic distance between subpopulations increases with geographic separation. This clinal genetic differentiation due to geographic separation is typical for any species occupying a region of greater size than its maximal dispersal capability (Kimura and Weiss, 1964).

We found bighorn sheep exhibit significant spatial genetic structure across the northern Rocky Mountains. The results of our spatial autocorrelation analysis indicated mean positive relatedness up to 100 km between pairs of individuals (Figure 2-5), commonly known as the neighbourhood size. The neighbourhood size indicates the geographical extent to which individuals are more related than expected, thus inferring the range at which gene flow occurs frequently. Although individual movement cannot be quantified from gene flow, gene flow cannot occur in the absence of movement. Our finding corroborates the telemetry data from DeCesare and Pletscher (2006) and observational data from (Festa-Bianchet, 1986b) indicating that bighorn sheep are capable of movements over significant distances.

2.5.3 Sex-biased gene flow

To assess the presence or absence of sex-biased gene flow, we examined mitochondrial DNA sequence data from a subset of individuals and performed sex-specific spatial autocorrelation across all individuals. A lack of variation in the mitochondrial DNA D-loop region that we sequenced restricted the extent to which we could assess female population genetic structure. We identified six haplotypes, the most common haplotype (M haplotype) occurred in 80% of individuals and was distributed across the entire region. Most other haplotypes appeared to be geographically localized (Figure 2-6). Similar to the pattern observed in nuclear markers, the

AMOVA found the presence of major rivers to account for 5.98% of genetic variance across our sampling range, thus indicating the gene flow of maternally inherited markers was equally restricted by the presence of major rivers. Our comparisons of mitochondrial marker and nuclear marker F_{ST} distances indicated no sex-biased gene flow. Mitochondrial F_{ST} distance values had a mean 2.46 times higher than the mean of the microsatellite F_{ST} distance values. In the absence of sex-biased gene flow, mitochondrial marker distances are expected to be two to four times greater than the microsatellite marker F_{ST} distances, indicating the absence of sex-biased gene flow, which is similar to the situation described for thinhorn sheep (Roffler *et al.*, 2014). We also did not observe sex differences in spatial autocorrelation based on nuclear markers. Thus, our analyses of mitochondrial and nuclear markers exhibited no evidence of sex-biased gene flow.

We conclude that each sex contributes to gene flow equally; however, previous literature on female philopatry (Festa-Bianchet, 1986a), male movement (Festa-Bianchet, 1986b), and male migrations (Hogg, 2000) suggest that male-biased gene flow is biologically probable. Our lack of evidence for male-biased dispersal possibly resulted from a combination of sampling methods and male behaviour. Field observations have shown that males perform seasonal breeding migrations but return to their home range after the rut (Hogg, 2000). We obtained the majority of our samples during the winter, after males would have already returned from their breeding migrations. Therefore, males may have been sampled in close proximity to their location of birth, inhibiting our ability to detect any difference between male and female neighbourhood size. Although our data did not support the hypothesis of male-biased gene flow, we cannot rule out the possibility that the sex-specific pattern of spatial genetic structure may change at different times throughout the year.

2.5.4 Post-glacial recolonization

We observed significant evidence that bighorn sheep recolonized the northern Rocky Mountains from the Southern refugium following the retreat of the Wisconsin glaciation. The reduction in the expected heterozygosity and rarefied allelic richness of sampling locations with increasing latitude (Figure 2-7), as well as the strong isolation-by-distance pattern observed, indicates the recolonization of the northern Rocky Mountains northwards in a stepping-stone (Kimura and Weiss, 1964) fashion from the Southern refugium. Our finding aligns with previous theories

(Cowan, 1940; Geist, 1971), genetic studies of bighorn sheep across the species range (Luikart and Allendorf, 1996), and studies of other taxa that recolonized this region from the Southern refugium (Rueness *et al.*, 2003; Runck and Cook, 2005; Godbout *et al.*, 2008; Latch *et al.*, 2009; McDevitt *et al.*, 2009; Samarasekera *et al.*, 2012). Based upon both our findings and the prior literature, we confidently conclude that the Southern refugium sourced the recolonization of the northern Rocky Mountains by bighorn sheep.

2.5.5 Conclusion

In summary, in the northern Rocky Mountains, Rocky Mountain bighorn sheep population genetic structure correlates with inter-river regions and isolation by distance explains genetic distance within and across the broad-scale population genetic structure. Additionally, spatial genetic structure indicates gene flow up to 100 km, with no evidence of sex-biased dispersal (but this is likely related to limitations of our sampling methods). Finally, patterns of genetic diversity indicate the post-glacial recolonization of the northern Rocky Mountains by bighorn sheep was sourced from the Southern refugium. Our results also have wider implications beyond just mountain sheep. Our study characterizes the population genetic structure of a habitat specialist across a vast area relatively unfragmented by anthropogenic disturbance. Second, our findings highlight the aversion that habitat specialists exhibit toward non-optimal habitats, as well as how the avoidance of non-optimal habitat can function to fragment populations and restrict gene flow across the landscape. Third, our inability to detect differential population genetic structure between the sexes highlights the difficulty of studying sex-bias gene flow in species that do not exhibit typical permanent dispersal. Finally, patterns of bighorn sheep post-glacial recolonization in the Rocky Mountains add further support to the growing body of knowledge suggesting that many vertebrates moved northwards to recolonize northwestern North America from Southern refugia.

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Table 2-1 Description of the 49 sampling locations, including site abbreviation (Abrv), latitude (Lat), longitude (Long), number of females typed at microsatellite loci (♀), number of males typed at microsatellite loci (♂), total number of individuals typed at microsatellite loci (Total), number of individuals with mtDNA sequence (mtDNA seq), number of alleles (A), allelic richness corrected for small sample size (Ar), observed heterozygosity (H_{obs}), expected heterozygosity (H_{exp}), and Wright's inbreeding coefficient (f). An asterisk (*) indicates samples from this location were submitted by hunters.

Sampling location	Abrv	Lat	Long	♂	♀	Total	mtDNA seq	A	Ar	H_{obs}	H_{exp}	f
Castle Yarrow	CY	49.27	-114.20	0	27	27	7	5.62	2.94	0.69	0.67	-0.033
Radium	RD	50.64	-116.05	2	2	4		2.92	2.57	0.56	0.56	0.000
Fernie, Elko, Radium*	FR			107	5	112		7.85	3.37	0.66	0.72	0.091
South of Bow*	SO			72	10	82		6.77	3.25	0.70	0.72	0.028
Sheep River	SR	50.64	-114.66	18	12	30	10	5.38	3.30	0.72	0.71	-0.011
Forbidden Creek Trail	FC	51.82	-115.93	9	13	22	10	4.62	2.95	0.68	0.67	-0.022
Malloch Creek-Banff Park	MB	51.88	-115.95	21	2	23		5.23	2.97	0.73	0.67	-0.101
Lost Guide	LO	51.94	-116.00	0	6	6		3.85	3.23	0.68	0.75	0.094
South Ram River Headquarters	RH	51.95	-116.16	7	11	18		5.00	2.99	0.66	0.66	0.009
Ram Range	RG	52.07	-116.15	10	14	24	9	5.00	3.10	0.70	0.69	-0.009

Cline River	CR	52.20	-116.62	6	6	12	10	4.85	3.02	0.74	0.68	-0.091
Abraham Lake	AL	52.22	-116.26	9	5	14		7.38	3.33	0.70	0.73	0.048
Ram River*	RR			154	0	154		5.30	3.06	0.67	0.66	-0.003
Ram Mountain	RM	52.36	-115.79	32	34	66	12	4.77	3.20	0.74	0.72	-0.037
Job Creek	JC	52.37	-116.72	6	5	11	10	4.62	3.21	0.78	0.72	-0.084
Bighorn River	BR	52.42	-116.51	9	0	9		4.62	3.07	0.71	0.69	-0.035
Opabin B	OB	52.43	-116.58	8	4	12		5.15	3.12	0.69	0.67	-0.025
Aztec Lake	AZ	52.46	-116.99	0	12	12	10	5.00	3.16	0.73	0.70	-0.039
Opabin A	OA	52.54	-116.72	3	5	8		3.91	3.07	0.61	0.64	0.046
Blackstone	BS	52.55	-116.53	4	9	13		3.77	2.76	0.61	0.64	0.046
Isaac Creek	IC	52.55	-117.01	8	11	19		5.00	3.05	0.70	0.68	-0.022
Cairn River	CA	52.69	-117.12	8	4	12	10	4.85	3.12	0.70	0.70	-0.004
Russel Creek	RC	52.82	-117.15	2	26	28		5.31	3.05	0.75	0.67	-0.119
Grave Flats	GF	52.90	-117.01	8	0	8		4.23	3.02	0.64	0.66	0.030
Drummond	DR	52.95	-117.38	8	0	8		4.23	3.06	0.71	0.67	-0.052
Cadomin Mountain	CM	52.97	-117.20	6	10	16	16	4.92	2.96	0.68	0.65	-0.050
Cadomin*	CD			69	5	74		6.54	3.15	0.66	0.69	0.032
Luscar-Gregg Mine	LG	53.06	-117.42	31	143	174	20	6.77	3.19	0.69	0.69	-0.006
Mystery Lake	ML	53.13	-117.67	19	22	41	10	5.77	3.04	0.66	0.68	0.019
Makwa Ridge	MR	53.05	-117.79	10	15	25		5.23	3.03	0.66	0.68	0.036

Name	Code	Lat	Lon									
Morro Peak	MP	53.04	-118.08	4	3	7		3.46	2.78	0.65	0.64	-0.026
Etna Knoll	EK	53.07	-118.02	6	3	9		4.08	3.11	0.69	0.65	-0.071
Disaster Point	DP	53.18	-117.96	3	6	9		4.38	3.13	0.66	0.66	0.008
Folding Mountain	FM	53.21	-117.69	16	5	21		5.08	2.93	0.67	0.64	-0.036
Windy Point	WP	53.21	-117.99	2	6	8		3.46	2.79	0.60	0.64	0.057
Brule Tunnel	BT	53.23	-117.87	1	20	21	10	4.54	2.78	0.64	0.62	-0.031
Stornoway Mountain	ST	53.29	-118.39	7	9	16	10	4.00	2.54	0.54	0.56	0.026
Munn Creek	MC	53.52	-118.32	8	11	19	8	4.77	2.93	0.65	0.63	-0.032
Monaghan	MO	53.61	-119.05	3	11	14		3.85	2.77	0.68	0.59	-0.138
Rocky Pass	RP	53.63	-118.83	4	17	21		4.15	2.75	0.59	0.60	0.019
North Berland	NB	53.67	-118.76	13	8	21	12	4.31	2.84	0.65	0.63	-0.032
Wilmore*	WM			72	3	75		5.15	2.85	0.61	0.62	0.019
Mount Mawdsley	MM	53.80	-119.12	4	10	14		3.77	2.72	0.66	0.60	-0.097
Stearn A	SA	53.91	-119.29	11	26	37	10	4.69	2.80	0.58	0.61	0.048
Horn Creek	HC	53.97	-119.53	4	9	13		3.77	2.61	0.58	0.57	-0.023
Grande Cache Coal Mine	GC	54.03	-119.20	13	22	35		4.23	2.56	0.50	0.53	0.062
Smoky River*	SM			76	0	76		4.62	2.52	0.51	0.53	0.047

Gorman Mountain	GM	54.18	-120.01	1	6	7		3.08	2.45	0.47	0.51	0.073
Narraway/Mount Torrens	NW	54.27	-119.91	2	6	8	10	3.62	2.67	0.67	0.57	-0.186

Table 2-2 Descriptive statistics for 13 microsatellite loci and one sex-determining marker used to quantify genetic diversity in bighorn sheep, including: locus name, number of genotypes, genotyping success rate, number of unique alleles (k), allele size range (bp),

observed heterozygosity (H_{obs}), expected heterozygosity (H_{exp}), and Wright's inbreeding coefficient (*f*).

Locus	Source	Genotypes	Success (%)	k	Size range (bp)	H_{obs}	H_{exp}	*f*
BM1225	Bishop et al., 1994	1407	94.11	11	237-261	0.653	0.735	0.112
BM4505	Bishop et al., 1994	1371	91.71	15	263-301	0.661	0.716	0.077
BMC1222	Bishop et al., 1994	1437	96.12	4	286-292	0.317	0.340	0.068
FCB266	Buchanan & Crawford, 1993	1434	95.92	6	88-102	0.715	0.770	0.071
MAF209	Buchanan & Crawford, 1992	1475	98.66	9	107-123	0.717	0.751	0.045
MAF36	Swarbrick, Buchanan & Crawford, 1991a	1477	98.79	7	90-108	0.629	0.693	0.092
MAF64	Swarbrick, Buchanan & Crawford, 1991b	1374	91.91	8	109-123	0.726	0.788	0.079
MAF65	Buchanan, Swarbick & Crawford, 1992	1349	90.23	12	101-135	0.681	0.794	0.142
OarAE16	Plenty et al. 1993	1453	97.19	11	82-106	0.775	0.854	0.093
OarCP26	Ede, Pierson & Crawford, 1995	1408	94.18	12	131-159	0.632	0.724	0.127
Rt9	Wilson et al., 1997	1159	77.53	11	119-147	0.734	0.797	0.079
TGLA122	Georges and Massey, 1992	1194	79.87	10	129-146	0.624	0.718	0.131
TGLA387	Georges and Massey, 1992	1406	94.05	13	132-155	0.701	0.783	0.104
SheepAML	Gokulakrishnan et al., 2013	1495	100	2	121-168	-	-	-
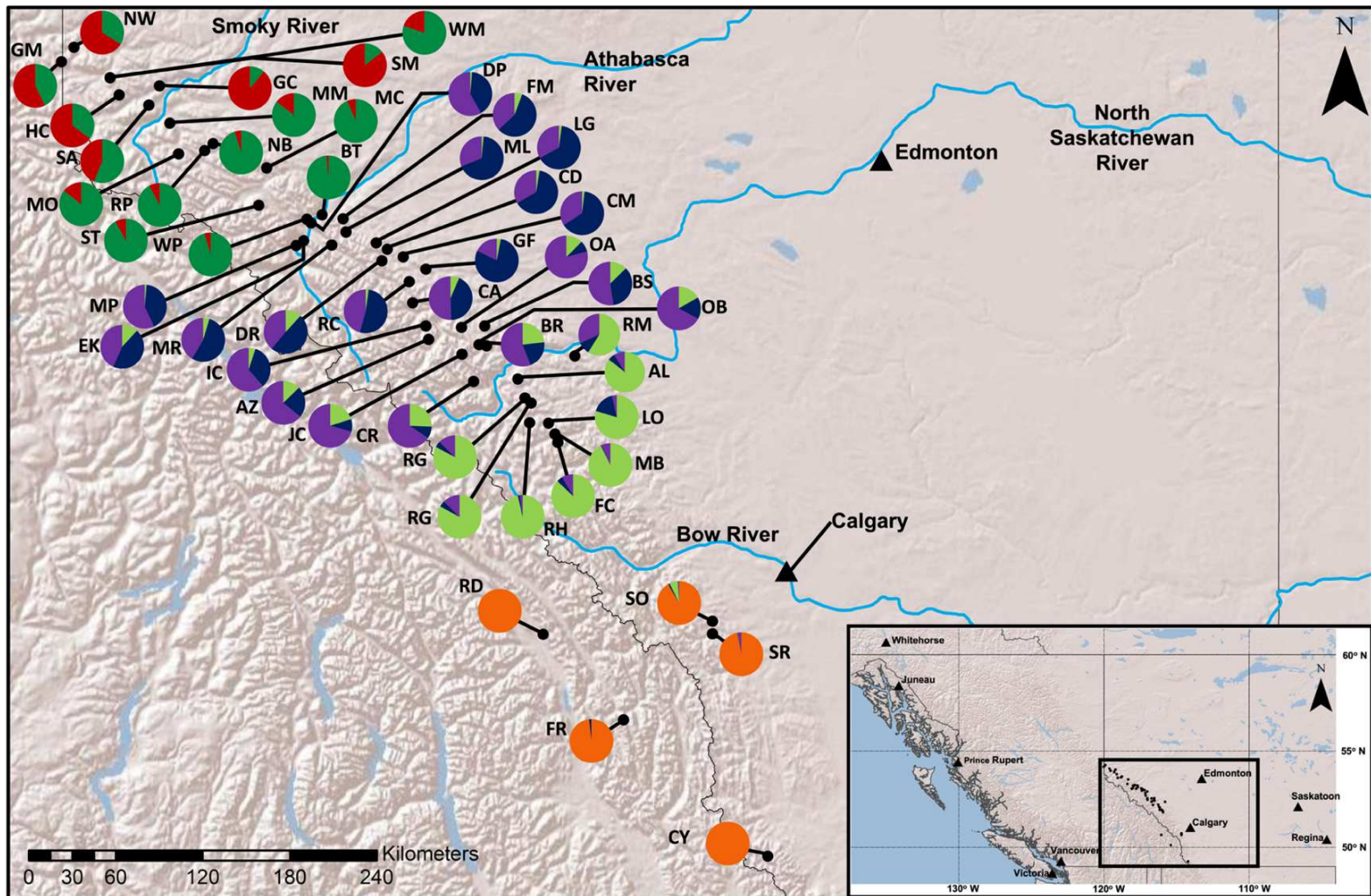
Figure 2-1 Sampling locations of Rocky Mountain bighorn sheep and their admixture of each of the six genetic clusters identified, with major river valleys highlighted in blue. See Table 2-1 for sampling location names and other details. *Due to the resolution of this figure the location of EK (Etna Knoll) and MP (Morro Peak) may appear ambiguous, we confirm both sampling locations are located south of the Athabasca River.
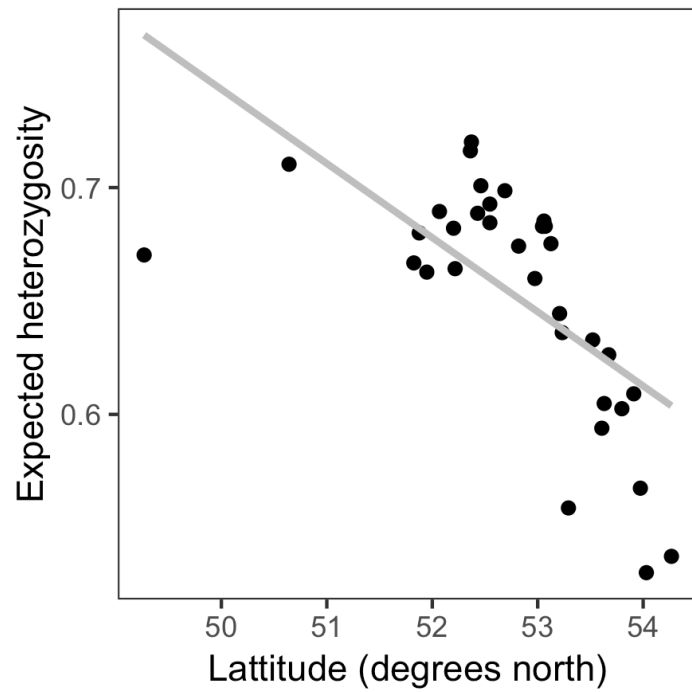
Figure 2-2 Correlation between the expected heterozygosity of
and latitude for 33 georeferenced sampling locations of Rocky
Mountain bighorn sheep (Pearson r=-0.634, p<0.001, df=31;
Equation of line y=-0.033+2.378).
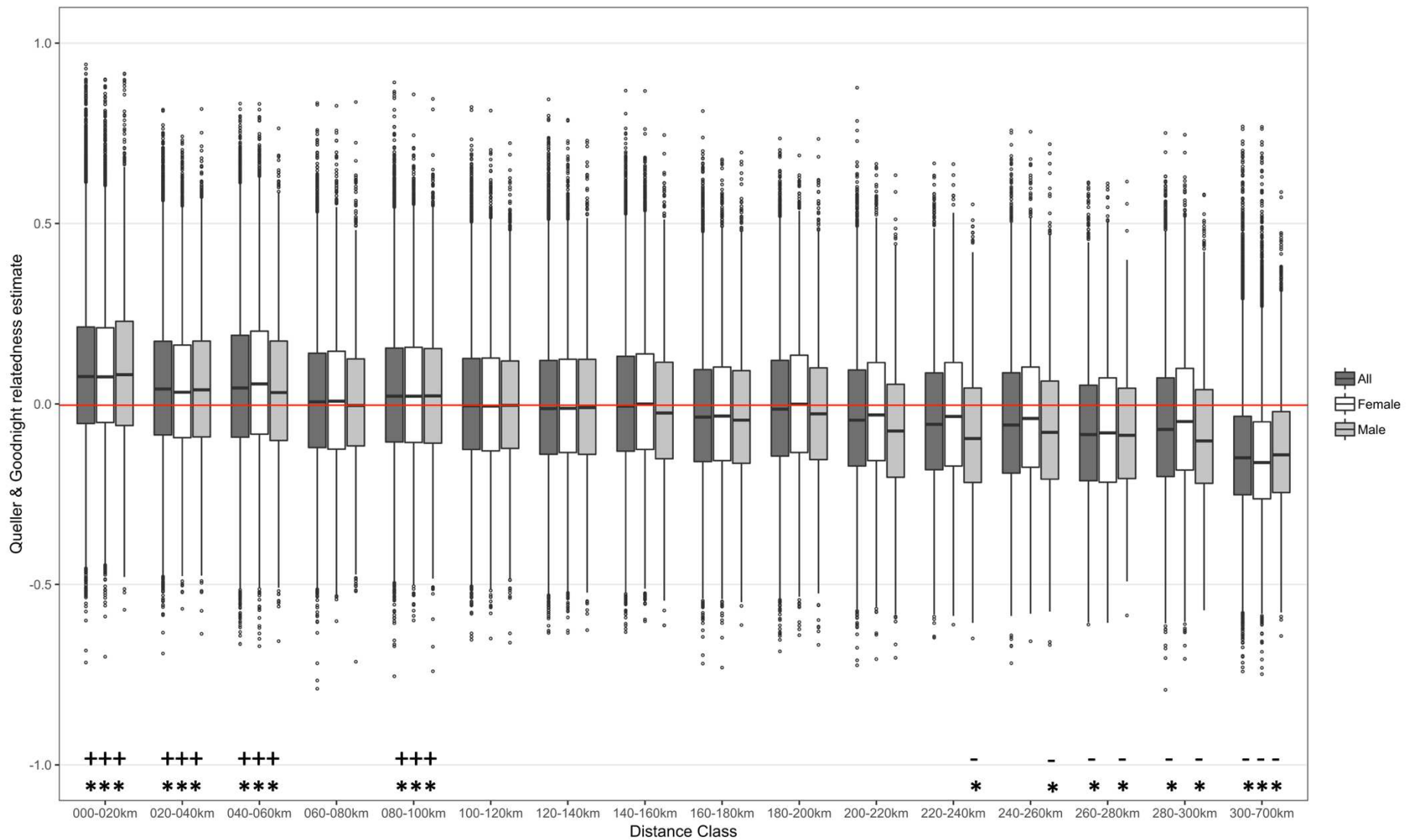
Figure 2-3 Average pairwise relatedness estimated by the Queller and Goodnight (1989) by distance class for Rocky Mountain bighorn sheep. The lower and upper limit of the box are the 25th and 75th percentile respectively, the middle bar represents the median, and the whiskers extend to the furthest data point which is within 1.5 times the inter-quartile range. An asterisk below + or – indicates a distance class mean pairwise relatedness significantly more or less than zero. Mean relatedness of the total population shown in red with a value of -0.0014.
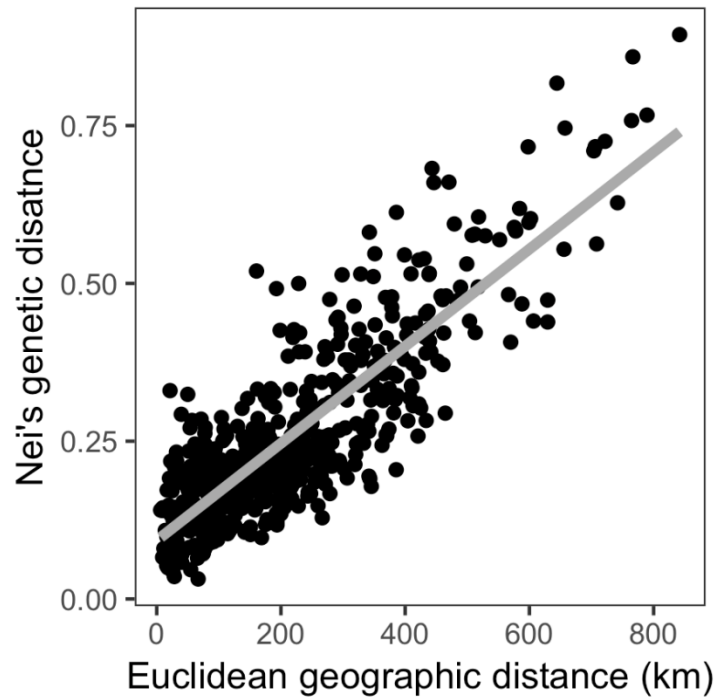
Figure 2-4 Regression of Nei's (1972) standard genetic distance on Euclidean geographic distance between 33 sampling locations of bighorn sheep in the northern portion of the species range (r^2= 0.846, p<0.001; equation of the line y=0.0008, intercept =0.0927).

Figure 2-5 Principal coordinate analysis of individuals with individuals from the 49 sampling locations, axis 1 and 2 explain 5.42% and 3.25% of variance respectively. Legend contains sampling location colour and abbreviations, see Table 2-1 for sampling location full names.
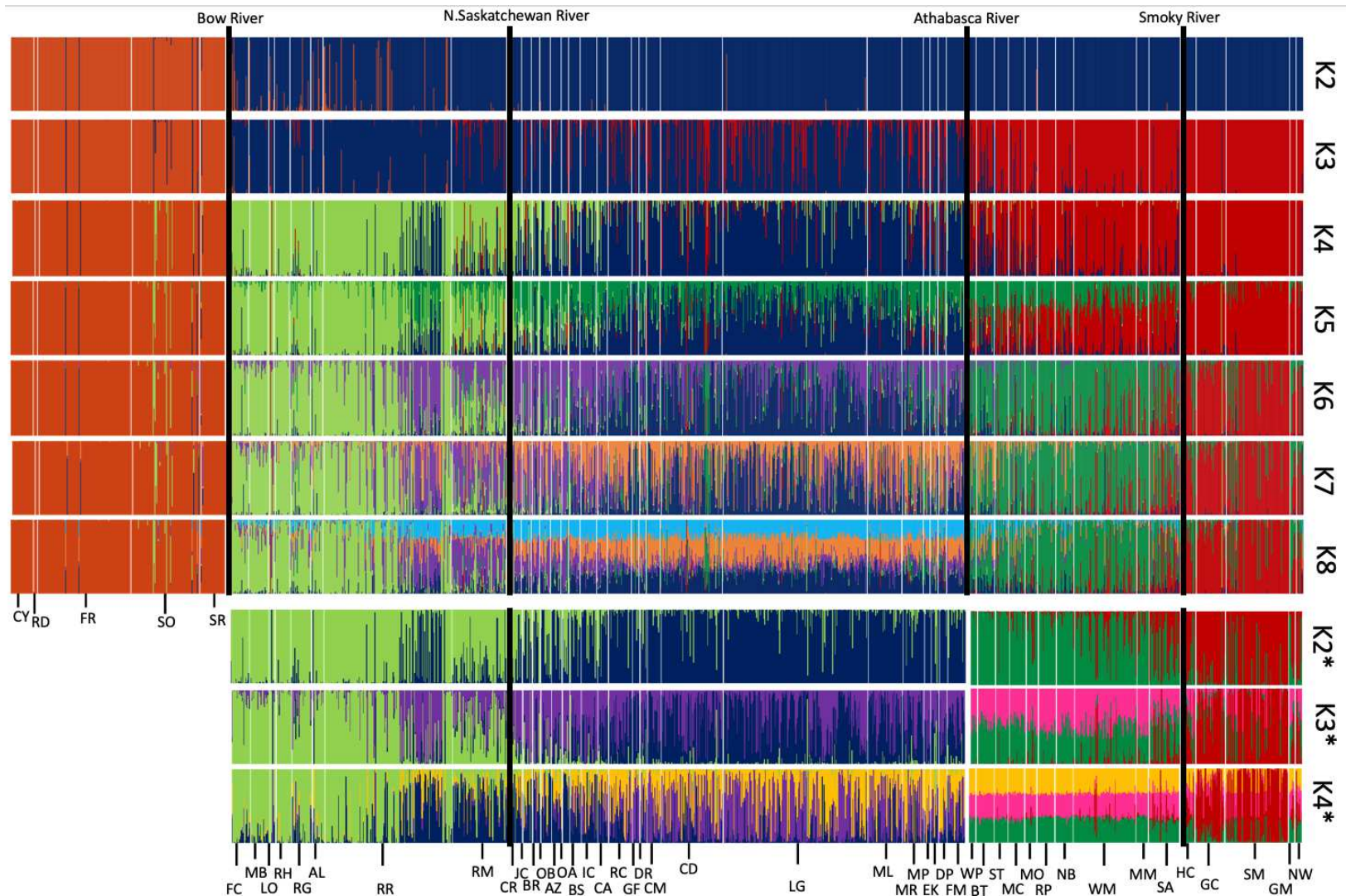
Figure 2-6 Admixture plots for K2-K8 for all individuals sampled (K2-K8), and K2-K4 for the region between the Bow and Athabasca river and the region north of the Athabasca river (K2*-K4*, each region analysed separately). The clusters represent south of the Bow River (dark orange), between the Bow and the North Saskatchewan rivers (light green), between the North Saskatchewan and the Athabasca rivers (a mix of dark blue and purple), between the Athabasca and the Smoky rivers (dark green), and north of the Smoky River (red). Black bars represent the major rivers.
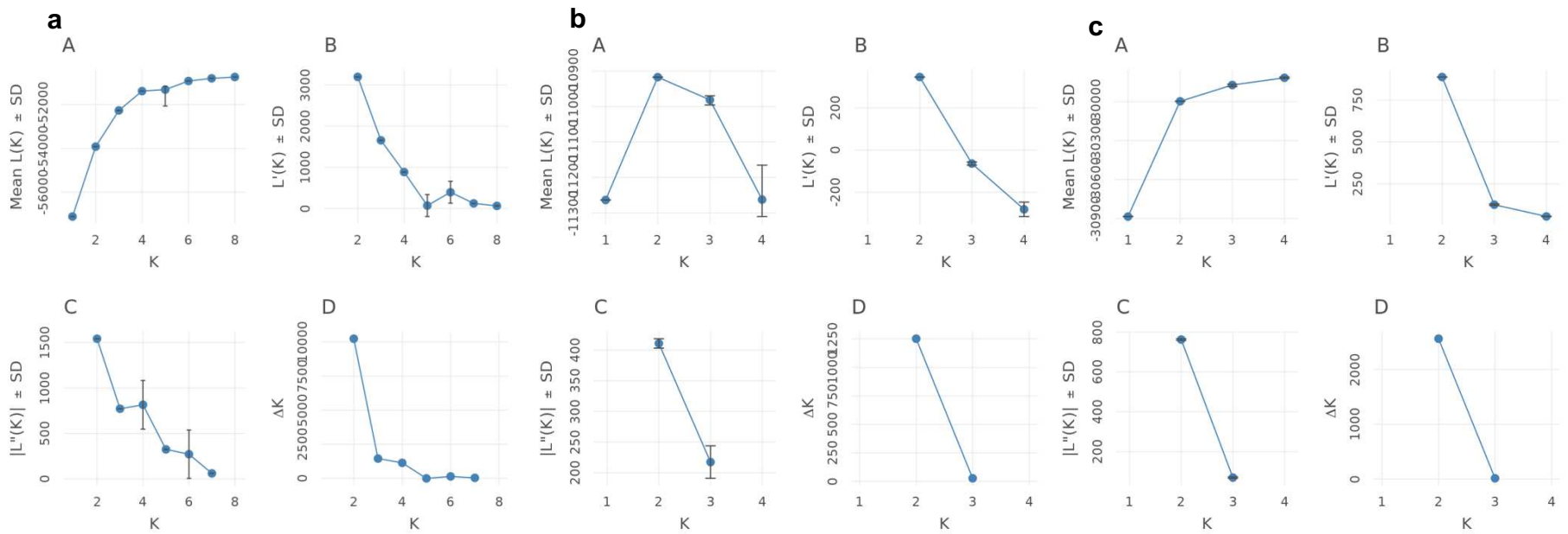
Figure 2-7 a. Likelihood plots for Structure runs of K1-K8 performed on all individuals sampled. **b.** Likelihood plots for admixtures of K1-K4 performed on individuals from sampling locations north of the Athabasca river. **c.** Likelihood plots for admixtures of K1-K4 performed on individuals from sampling locations between the Bow and Athabasca rivers.
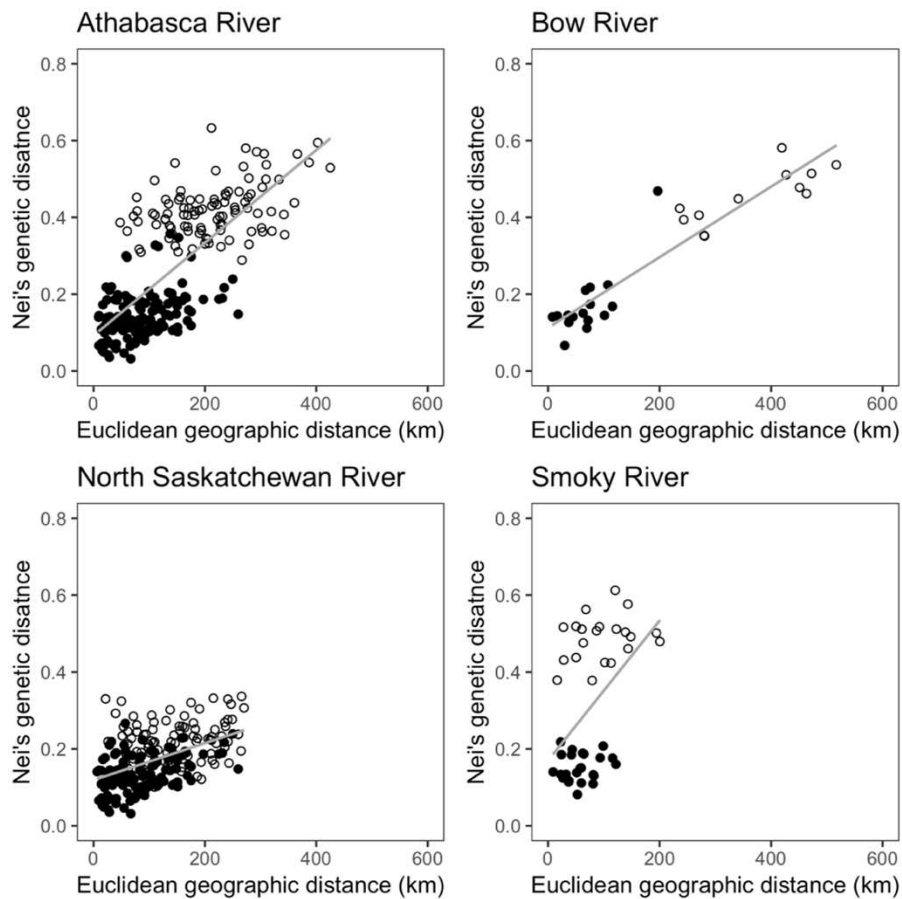
Figure 2-8 Regression of Nei's standard genetic distance (1972) on Euclidean geographic distance for sampling locations situated on the same and different sides of major rivers, represented by filled and unfilled circles respectively. The effect of distance on genetic distance in each region is as follows: Athabasca River, $r^2= 0.735$, p<0.001, slope y=0.0012, intercept =0.0923. Bow River, $r^2= 0.938$, p<0.001, slope y=0.0009, intercept =0.1130. North Saskatchewan River, $r^2= 0.487$, p<0.001, slope y=0.0005, intercept =0.1190. Smoky River, $r^2= 0.479$, p=0.007, slope y=0.0018, intercept =0.1700. The effect of each river on genetic distance when controlling for geographic distance is as follows: Athabasca River, $r^2= 0.811$, p<0.001. Bow River, $r^2=0.183$, p=0.234. North Saskatchewan River, $r^2= 0.551$, p =<0.001. Smoky River, $r^2= 0.953$, p<0.001.
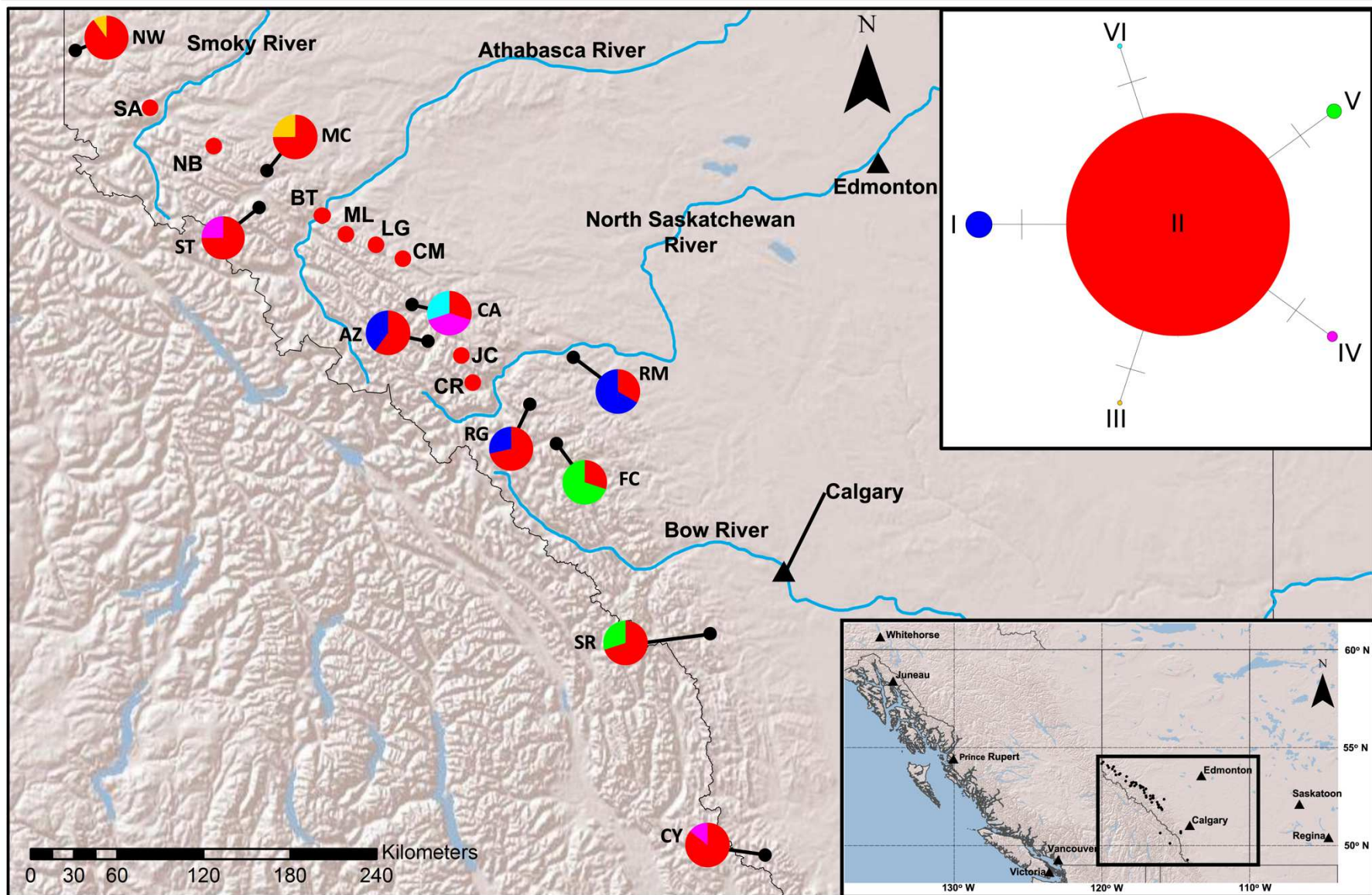
Figure 2-9 The haplotype network and the proportion of each haplotype at each sampling location. I, II, III, IV, V and VI represent haplotypes 131A (blue), M (red), 144G (yellow), 201C (pink), 186T (green), and 239C (cyan) respectively. Plain red circles indicate populations with no variation from the M haplotype. Major rivers highlighted in blue.

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Chapter 3 - Ewe are what ewe wear: bigger horns, better ewes and the potential consequence of trophy hunting on female fitness in bighorn sheep

3.1 Chapter summary

In polygynous species, secondary sexual traits such as weapons or elaborate ornaments have evolved through intrasexual competition for mates. In some species, these traits are present in both sexes but are underdeveloped in the sex facing lower intrasexual competition for mates. It is often assumed that these underdeveloped sexually selected traits are a vestige of strong sexual selection on the other sex. Here, we challenge this assumption and investigate whether the expression of secondary sexual traits is associated with fitness in female bighorn sheep. Analyses of 45 years of data revealed that female horn length at 2 years, while accounting for mass and environmental variables, is associated with younger age at primiparity, younger age of first offspring weaned, greater reproductive lifespan and higher lifetime reproductive success. There was no association between horn length and fecundity. These findings highlight a potential conservation issue. In this population, trophy hunting selects against males with fast-growing horns. Intersexual genetic correlations imply that intense selective hunting of large-horned males before they can reproduce can decrease female horn size. Therefore, intense trophy hunting of males based on horn size could reduce female reproductive performance through the associations identified here, and ultimately reduce population growth and viability.

3.2 Introduction

In polygynous species where males use secondary sexual traits to compete for mates, the evolution of those traits in females is usually attributed to one or more of the following mechanisms: shared genetic architecture (Tobias, Montgomerie and Lyon, 2012), intrasexual resource competition (Stankowich and Caro, 2009; Tobias, Montgomerie and Lyon, 2012), predator defence (Stankowich and Caro, 2009) or male-mate choice (Hare and Simmons, 2019).The shared genetic architecture hypothesis, first recognized and described by Darwin, (1872) as 'correlated inheritance', states that although directional selection only affects male traits, the underlying genetic architecture (the same genes affect the trait in both sexes) results in females exhibiting underdeveloped forms of the same traits, despite no obvious fitness benefit for females (Lande, 1980). Female traits maintained via shared genetic architecture, such as the long tail feathers of barn swallow (*Hirundo rustica*) (Cuervo, de Lope and Møiller, 1996), are thought to not affect female fitness. Alternatively, the intrasexual competition hypothesis holds that these traits may aid access to resources (Kraaijeveld, Kraaijeveld-Smit and Komdeur, 2007). For example, female dung beetles (*Onthophagus sagittarius*) use horns to compete for dung, where they raise their offspring (Watson and Simmons, 2010a, 2010b). Additionally, secondary sexual characteristics in females can be an anti-predation adaptation, such as the horns of many female bovids (Stankowich and Caro, 2009). Finally, female ornamentation may influence male-mate choice (Hare and Simmons, 2019).

A common, often extremely sexually dimorphic, secondary sexual trait is the cranial weaponry of many ungulates. The use of cranial weaponry in male–male competition is well documented (Coltman *et al.*, 2002; Emlen, 2008; Watson and Simmons, 2010b), but its function in females is debated. Stankowich and Caro (2009) theorize that the horns of female bovids are an anti-predator adaptation, with larger more conspicuous species more likely to exhibit headgear (Packer, 1983; Stankowich and Caro, 2009). Cranial weaponry may also function in female intrasexual competition; female reindeer (*Rangifer tarandus*) use antlers to compete for forage (Espmark, 1964), and female Soay sheep (*Ovis aries*) with more developed horns win more intrasexual interactions (Robinson and Kruuk, 2007). Alternatively, shared genetic architecture may explain female cranial weaponry as a vestige of strong sexual selection in males for that trait (Lande, 1980). Regardless of which mechanism is responsible for the development of horns in female ungulates,

individual variation in cranial weaponry size may either influence or indicate variation in female fitness. If cranial weaponry functions as an anti-predator trait, individuals with larger horns may be better able to defend themselves. If weaponry size simply reflects overall body condition or resource acquisition, it may be correlated with body mass and therefore with reproductive success. Alternatively, given the high cross-sex heritability of headgear length in some ungulates (Poissant *et al.*, 2012), females who inherit alleles for more exaggerated headgear may also inherit alleles for other traits associated with greater fitness potential. For example, heavier mass as well as disease and parasite resistance are all correlated with the expression of male secondary sexual traits in diverse species (Hamilton and Zuk, 1982; Coltman *et al.*, 2001, 2005). Here we investigate if the expression of cranial weaponry in females is associated with reproductive fitness in a wild ungulate population.

The functions of horns in female bighorn sheep (*Ovis canadensis*) and their association with reproductive fitness are largely unknown. Horn length is highly genetically correlated across the sexes (Poissant *et al.*, 2012), thus females with long horns are expected to be both progeny and parents of longer-horned, typically higher-fitness males (Coltman *et al.*, 2002). Jorgenson *et al.* (1993) found that female yearlings with larger horns were more likely to have offspring earlier in life, however, that study did not control for a possible effect of body mass, which is also associated with age at primiparity (Martin and Festa-Bianchet, 2012; Pigeon, Festa-Bianchet and Pelletier, 2017). Thus far, the relationships between horn size, body mass and age at primiparity have not been disentangled. Factors other than horn size known to be associated with reproductive traits include body mass (Festa-Bianchet, Gaillard and Jorgenson, 1998) and population density (Pigeon and Pelletier, 2018), with density during early life being particularly important for reproductive potential (Allendorf and Hard, 2009; Martin and Festa-Bianchet, 2012). Additionally, potential associations between female horns and reproductive fitness may lead to correlated, indirect effects on fitness via anthropogenic selective pressures on male horn length. Similar to other species, male bighorn sheep are subject to phenotype-based harvest (Carlson *et al.*, 2007; Allendorf and Hard, 2009; Campbell-Staton *et al.*, 2021). Intense selective harvest can result in an evolutionary response in horn size, leading to a reduction in breeding values for horn length in both sexes (Pigeon *et al.*, 2016). Hence, if an association between female horn length and reproductive fitness

exists in bighorn sheep, selective harvest of males may negatively affect population demography as seen in other species (Olsen *et al.*, 2004; Walsh *et al.*, 2006; Knell and Martínez-Ruiz, 2017).

Despite the reproductive benefits conferred by horns to male bighorn sheep (Coltman *et al.*, 2002) in the absence of selective harvest (Bonenfant *et al.*, 2009; Pigeon, Festa-Bianchet and Pelletier, 2017), and the functionality of female weapons in some ungulates (Espmark, 1964; Robinson and Kruuk, 2007), little is known about the relationship between horn size and fitness in most ungulate species. Given the fitness benefits potentially conferred by cross-sex shared genetic architecture (Coltman *et al.*, 2005; Poissant *et al.*, 2012), we hypothesize that females with longer horns may have higher reproductive fitness. We used approximately 45 years of longitudinal data to examine the effect of horn length, body mass, population density and other environmental variables on four components of reproductive fitness (age at primiparity, age at first offspring weaned, reproductive lifespan and fecundity) and lifetime reproductive success (LRS).

3.3 Methods

3.3.1 Study site/population

Ram Mountain, Alberta (52°80 N, 115°80 W, 1082–2173 meters above sea level) is a rocky outcrop encompassing approximately 38 km^2 of alpine and sub-alpine terrain, approximately 30 km east of the main Rocky Mountain range. The sheep population is geographically and genetically distant (Deakin *et al.*, 2020) from the main species range. Monitoring started in 1971 (Jorgenson *et al.*, 1993) and is ongoing. The Ram Mountain population was historically subjected to trophy hunting of males based on horn size (Pigeon *et al.*, 2016). In 1996, a more restrictive regulation was introduced, and a moratorium has stopped sport hunting since 2011.

3.3.2 Monitoring

Between late May and September, sheep were trapped in a corral baited with salt. At each capture, body mass was measured with a spring scale to the nearest 125g. Horn length and base circumference (cm) were measured using a flexible tape. At first capture, a tissue sample was taken for DNA analyses and pedigree construction. Reproductive status was determined by observing lactation at capture, or by mother–offspring interactions. Mother–offspring pairs were determined

by behavioural observations and confirmed by a genetic pedigree constructed using 26 microsatellite markers (Coltman *et al.*, 2002; Poirier *et al.*, 2019). For this study, we analysed data from 1973 to 2018 for females of the 1973–2016 cohorts.

3.3.3 Individual measurements

For females from the 1973 to 2016 cohorts, we recorded age at primiparity (years), age at first offspring weaned (years), yearly reproductive status (to examine reproductive lifespan), adjusted horn length (cm) and body mass (kg) to 15 September at two years to allow for comparison between individuals. Using repeated measurement of the same individual in the same season, horn length was adjusted using individual horn growth rates and mass was adjusted using mixed-effect models (Martin and Pelletier, 2011). Age at primiparity was defined as the first occasion in which a female lactated (assessed by inspection of the udder during captures). We defined weaning as rearing offspring to mid-September. Our measure of reproductive lifespan was the number of years between first and last reproductive activity, defined as the last occasion in which a female lactated. Fecundity and LRS were examined for females from the 1973 to 2012 cohorts for which entire reproductive histories were available. Fecundity was the proportion of reproductive years in which an offspring was weaned and LRS was the total number of offspring weaned by each female. To control for environmental variation, we recorded population density, winter temperature and winter precipitation for the first year of life for each female and for each year from age two to primiparity or first weaning of offspring. As in previous publications, population density was the number of adult females in June. Mean winter temperature (°C) and mean winter precipitation (cm) were the average of monthly temperatures and precipitation recorded at the Environment Canada meteorological station in Nordegg (52°300 N, 116°030 W, elevation: 1320 m, about 20 km west of Ram Mountain) from November to March (see also (Pigeon, Festa-Bianchet and Pelletier, 2017)). Prior to analysis all measurements were standardized by subtracting the mean and dividing by the standard deviation (s.d.), to compare the effect of variables with differing units of measurement.

3.3.4 Statistical analysis

To analyse factors associated with age at primiparity, age at first offspring weaned and reproductive lifespan, we used R v. 4.0.3 (R Core Team, 2013), packages survival v. 3.2.13

(Therneau, 2019) and coxme v. 2.2.16 (Therneau, 2015) to calculate Cox mixed-effect survival models. We considered 12 models, four for each of the three sets of observations, to examine how environmental effects, adjusted horn length at age two and adjusted mass at age two were associated with age at primiparity, age at first weaning success and reproductive lifespan (Tables 3-1 and 3-3). Female cohort, observation year and individual ID were included in all models as random effects. We obtained the hazard ratios for each fixed effect from coxme. We performed model selection on Cox mixed-effect survival models by ranking each set of four models by their Akaike information criterion corrected for small samples size (AICc) and selecting the model with the lowest AICc (Table 3-1). We tested the validity of the best fitting models by their relationship between the Schoenfeld residuals (Schoenfeld, 1982) and time using the survival package (Therneau, 2019). Schoenfeld residuals are the difference between observed and predicted values for any covariate at any given event time and should have a non-significant relationship with time in valid models.

To analyse factors associated with fecundity and LRS, we used R package *lme4* v. 1.1.27.1 (Bates *et al.*, 2014) to fit generalized linear mixed-effect models (GLMM). We considered eight models in total, four for each of the two sets of observations, to examine how environmental effects, adjusted horn length at age two and adjusted mass at age two were associated with fecundity and LRS (Tables 3-2 and 3-4). Female cohort, grouped into 4-year periods to avoid overfitting, was included in all models as the sole random effect. Gaussian and Poisson error families were used for the fecundity and LRS models, respectively. We used r2glmm v. 0.1.2 (Nakagawa and Schielzeth, 2013) to calculate r^2 values for each fixed effect in Gaussian model. We calculated pseudo r^2 values for fixed effects in Poisson models by subtracting the marginal r^2 value of the model with the target variable from the marginal r^2 value of the whole model. Marginal r^2 values were calculated using the R package *Mumin* v. 1.43.17 (Barton, 2009). We performed model selection on GLMMs by selecting the model with the lowest AICc (Tables 3-1 and 3-2). We tested the fit of residuals from the best fitting models to a normal distribution by the Shapiro–Wilk normality test (Shapiro and Wilk, 1965).

3.4 Results

Age at primiparity and age at first successful weaning datasets included 217 females, where horn length at 2 years ranged from 9.8 to 28.6 cm and body mass from 41.0 to 71.6 kg, with means of 19.0 cm (3.0 s.d.) and of 57.8 kg (5.7 s.d.), respectively. Age at primiparity ranged from 2 to 7 years, and age at first offspring weaned ranged from 2 to 9 years. The median age was 3 years for both variables (Appendix B Figure B1). The reproductive lifespan dataset included 160 females, whose average horn length and body mass at 2 years were 19.6 cm (3.0 s.d.) and 58.8 kg (4.9 s.d.), respectively. Reproductive lifespan ranged from 1 to 15 years, with a median of six (Appendix B Figure B1). LRS and fecundity were known for 152 females and ranged from 1 to 12 lambs weaned and 20% to 100% annual fecundity, with a median of four and mean of 74.6%, respectively (Appendix B Figure B1). The average horn length and body mass at 2 years in these datasets were 19.2 cm (3.1 s.d.) and 58.3 kg (5.5 s.d.), respectively. Temporal trends for horn length, fecundity, reproductive lifespan and LRS were characterized by a decline until the early to mid-1990s followed by an increase (Figure 3-1). Conversely, age at primiparity and at first offspring weaned increased until the 1990s then declined. Appendix B Figure B2 reports temporal trends in environmental variables.

For factors associated with age at primiparity and age at first offspring weaned, the best model contained adjusted horn length at age two, adjusted mass at age two and all environmental effects (Tables 3-1 and 3-3). In these models, adjusted horn length and adjusted mass at age two had hazard ratio of 1.34 and 1.40, respectively, for the likelihood of transitioning from a nonparous to a primiparous state at any age, and 1.57 and 1.51, respectively, for the likelihood of transitioning from a non-weaning to a weaning state at any age. Thus, for every additional 3.0 cm (1 s.d.) of horn length and 5.5 kg (1 s.d.) of mass, the likelihood of being primiparous increased by 39.8% and 34.0%, respectively, and for every additional 3.0 cm (1 s.d.) of horn length and 5.5 kg (1 s.d.) of mass, the likelihood of first weaning an offspring increased by 57.4% and 51.1%, respectively (Figure 3-2). These likelihoods increased exponentially with every year spent nonparous or non-weaning.

For factors associated with reproductive lifespan, the best model contained only environmental variables (Tables 3-1 and 3-3). This model, however, was only weakly supported over one

containing horn length at age two and environmental variables, with a delta AICc of 0.76. The Schoenfeld residuals of Cox survival models (Appendix B Table B1) revealed a significant relationship between birth density and age (time) in the primiparity model, as expected due to temporal trends in density. We subsequently repeated this model series twice, once with birth density removed and again with an interaction between birth density and age included (Appendix B Table B2). These models showed the same pattern as the original models when ranked by AICc; therefore, age at primiparity was associated with both horn length and mass at 2 years in addition to environmental effects.

For factors associated with fecundity, the best model contained only environmental effects (Tables 3-2 and 3-4). For LRS, the best model contained adjusted horn length at 2 years and all environmental effects (Tables 3-2 and 3-4; Figure 3-3). In this model, r^2 value for adjusted horn length was 0.050. The residuals of these models were normally distributed according to Shapiro–Wilk normality tests (Shapiro and Wilk, 1965).

3.5 Discussion

Variation in age at primiparity and age at first offspring weaned in female bighorn sheep was associated with both horn length and body mass when controlling for environmental factors. Additionally, we found an association between LRS and horn length when controlling for environmental factors. Both female mass and environmental factors were associated with age at primiparity and age at first offspring weaned (Jorgenson *et al.*, 1993; Martin and Festa-Bianchet, 2012; Pigeon, Festa-Bianchet and Pelletier, 2017). However, horn size appeared to explain more variation than body mass in age at first offspring weaned, and to be the most explanatory variable of variation in LRS. Thus, larger horns correlate with higher reproductive fitness in both male (Coltman *et al.*, 2002) and female bighorn sheep.

The relationship between horn length and female reproductive fitness may result from shared genetic architecture, if alleles for greater fitness are associated with alleles for larger headgear. Female horn length could indicate variation in condition or other aspects of individual reproductive potential through its association with other traits linked to the shared genetic architecture for horns (Coltman *et al.*, 2005). Therefore, due to the high degree of cross-sex heritability of horn length

(Poissant *et al.*, 2012), females who inherit alleles for larger horns are also likely to inherit alleles for greater fitness phenotypes, a positive relationship already observed in other fitness-related traits in this population (Coltman *et al.*, 2005), and similar to correlations observed in other species (Hamilton and Zuk, 1982; Coltman *et al.*, 2001). This hypothesis suggests that selective harvesting of males based on horn size could deplete alleles for traits associated with greater female reproductive fitness in bighorn sheep.

We observed temporal changes in reproductive traits that appeared to be associated with trophy hunting activity in this population. Female horn length at age 2 years decreased and age at primiparity and first weaning success increased until the near cessation of trophy hunting in 1996, after which horn length at age 2 years increased and age at primiparity and at first weaning success decreased. These patterns mirror the trends of expected breeding value for horn length in both sexes and male horn circumference observed by Pigeon et al. (Pigeon *et al.*, 2016) and further exemplify how reducing hunting pressure can mitigate human-mediated evolutionary changes (Carlson *et al.*, 2007). Additionally, reproductive lifespan decreased from a peak in about 1980 until the cessation of trophy hunting. Many populations of mountain sheep in Canada are subjected to similar trophy hunting regimes as that experienced by the Ram Mountain population prior to 1996. Across Alberta, declining male horn length has been observed over the past approximately 40 years (Festa-Bianchet *et al.*, 2014; Morrissey, Hubbs and Festa-Bianchet, 2021). Assuming other populations also exhibit cross-sex heritability of horn length (Poissant *et al.*, 2012), harvested populations may also experience delayed age at primiparity and the subsequent reduction in population growth as a cross-sex correlated response to harvest selection.

While plausible, we cannot conclusively determine that the correlation between horn length and reproductive fitness results from shared genetic architecture. Individual variation in nutritional intake, parasite load, non-additive genetic effects or other unmeasured environmental effects could also mediate the correlation between horn growth and reproductive fitness. However, if environmental variability was the main driver of the relationships we reported, we would have expected body mass to play a greater role than horn length, as body mass directly affects female reproductive potential (Festa-Bianchet, Gaillard and Jorgenson, 1998).

Our findings suggest that secondary sexual traits, particularly cranial weaponry, in females of sexually dimorphic species may be more than ornamental vestiges of intense selection in males, as they are associated with fitness variation in females. These traits might confer direct benefits in intrasexual competition or indirect benefits via shared genetic architecture. Female horn length could increase intrasexual competitiveness, given that the use of horns in intrasexual resource competition has been observed in numerous other species (Espmark, 1964; Robinson and Kruuk, 2007; Watson and Simmons, 2010a, 2010b). However, there is no evidence that horn size determines the outcome of dominance interactions in female bighorn sheep (Favre, Martin and Festa-Bianchet, 2008). Regardless of the underlying mechanism, the association of female horn length at 2 years with early reproductive success and LRS highlights a potential important conservation issue for trophy-hunted populations of bighorn sheep. Intense selective harvest has been linked to an evolutionary decline in horn length in both sexes (Pigeon *et al.*, 2016). Although female horn length was not a target of selective hunting, it declined because of cross-sex heritability (Poissant *et al.*, 2012). Here, we show that females with smaller horns have delayed primiparity and age at first successful weaning, leading to a reduction in LRS. Therefore, selective harvest may indirectly select for females with lower overall reproductive fitness, which may negatively affect population dynamics. Our study exemplifies how species subjected to selective harvesting regimes may exhibit decreases in overall population fitness (Carlson *et al.*, 2007; Campbell-Staton *et al.*, 2021) with demographic and evolutionary effects (Olsen *et al.*, 2004; Walsh *et al.*, 2006; Knell and Martínez-Ruiz, 2017) extending beyond changes in the trait targeted by trophy hunters (Allendorf and Hard, 2009).

3.6 Bibliograpy

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Table 3-1 Results of mixed effect models testing the association of female horn length and body mass at two years with age at primiparity, age at first offspring weaned, and reproductive lifespan for female bighorn sheep at Ram Mountain, Alberta, Canada 1973 to 2018. Each model included cohort, ID, and year as random effects. Best model for each trait is in bold.

Model	Age at primiparity			Age at first offspring weaned			Reproductive lifespan		
	df	AICc	ΔAICc	df	AICc	ΔAICc	df	AICc	ΔAICc
Environment + horn length + body mass	**82.06**	**1707.26**	**0**	**117.14**	**1752.14**	**0**	**137.76**	**1332.33**	**45.89**
Environment + body mass	83.58	1712.98	5.72	116.26	1754.47	2.33	141.28	1292.87	6.43
Environment + horn length	83.76	1719.83	12.57	119.96	1759.49	7.35	141.88	1287.20	0.76
Environment	85.99	1729.67	22.41	119.15	1767.65	15.51	141.63	1286.44	0

Table 3-2 Generalized mixed effect models testing the association of horn length and body mass at two years with fecundity and lifetime reproductive success (LRS) for female bighorn sheep at Ram Mountain, Alberta, Canada cohorts 1973 to 2012. Each model included cohort, ID, and year as random effects. Best model for each trait is in bold.

Model		Fecundity			LRS	
	df	AICc	ΔAICc	df	AICc	Δ AICc
Environment + horn length	**7**	**1343.38**	**1.00**	**7**	**701.23**	**0**
Environment + horn length + body mass	8	1345.49	3.11	8	701.80	0.57
Environment + body mass	7	1344.38	2.00	7	703.19	1.96
Environment	6	1342.38	0	6	706.30	5.07

Table 3-3 Coefficients, hazard ratios (HR), and *p* values from best Cox mixed effect survival models of associations of environmental variation, body mass and horn length on age at primiparity and of first offspring weaned for female bighorn sheep at Ram Mountain, Alberta, Canada between 1973 and 2018.

Variable	Age at primiparity			Age at first lamb weaned			Reproductive lifespan		
	Coefficient (se)	HR	*p*	Coefficient (se)	HR	*p*	Coefficient (se)	HR	*p*
Fixed effects									
Adjusted mass at two years	0.335 (0.133)	1.398	0.012	0.413 (0.166)	1.511	0.013			
Adjusted horn length at two years	0.293 (0.133)	1.340	0.028	0.454 (0.169)	1.574	0.007			
Density experienced as a lamb	-0.508 (0.425)	0.601	0.230	-0.943 (0.344)	0.389	0.006	-0.696 (0.389)	0.499	0.073
Winter precipitation experienced as a lamb	-0.024 (0.324)	0.976	0.940	-0.075 (0.284)	0.927	0.790	0.587 (0.409)	1.799	0.150
Winter temperature experienced as a lamb	0.386 (0.303)	1.471	0.200	0.070 (0.250)	1.073	0.780	0.779 (0.364)	2.180	0.032
Yearly density	-0.163 (0.470)	0.849	0.730	0.391 (0.337)	1.478	0.250	-0.680 (0.269)	0.507	0.011
Yearly winter precipitation	0.437 (0.256)	1.548	0.088	-0.021 (0.152)	0.979	0.890	-0.037 (0.169)	0.964	0.830

Yearly winter temperature	0.068 (0.275)	1.070	0.810	-0.203 (0.165)	0.812	0.220	0.079 (0.164)	1.082	0.630
	Variance			Variance			Variance		
Random effects									
Cohort	1.564			1.057			0.016		
ID	0.282			1.283			0.147		
Year	1.327			0.165			>0.001		

Table 3-4 Best fitting generalised mixed effect models examining associations of environmental variation and horn length with reproductive lifespan, environmental variation with fecundity, and horn length, body mass, and environment with lifetime reproductive success (LRS) for female bighorn sheep at Ram Mountain, Alberta, Canada, cohorts 1973 to 2012.

Variable	Fecundity			LRS		
	Coefficient (se)	p	r^2	Coefficient (se)	p	r^2
Fixed effects						
Adjusted horn length at two years				0.131 (0.049)	0.007	0.050
Density experienced as a lamb	-0.271 (0.127)	0.032	0.057	-0.027 (0.077)	0.721	0.006
Winter precipitation experienced as a lamb	-0.002 (0.317)	0.994	0	-0.014 (0.055)	0.797	0.007
Winter temperature experienced as a lamb	-1.422 (0.930)	0.126	0.020	-0.071 (0.055)	0.198	0.035
	Variance			Variance		
Random effects						
Grouped cohort	62.45			0.041		
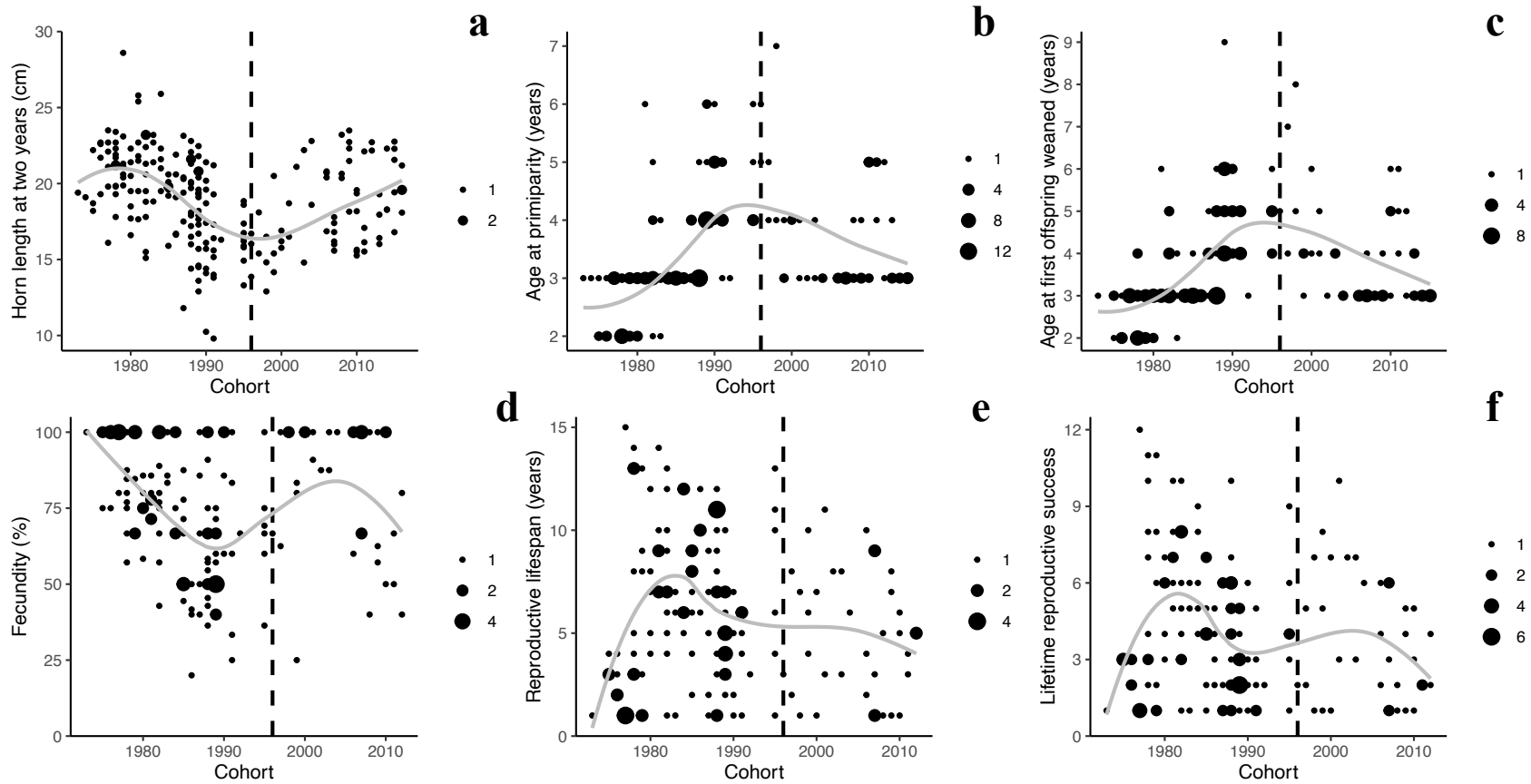
Figure 3-1 (a) Horn length at 2 years and (b) age at primiparity for female bighorn sheep from cohorts 1973 to 2015, and (c) age at first offspring weaned, (d) fecundity, (e) reproductive lifespan and (f) LRS for female bighorn sheep from cohorts 1973 to 2012 at Ram Mountain, Alberta, Canada. Dashed vertical line represents the near cessation of trophy hunting in 1996. Smooth line was fitted using loess. Point size represents overlapping data points.
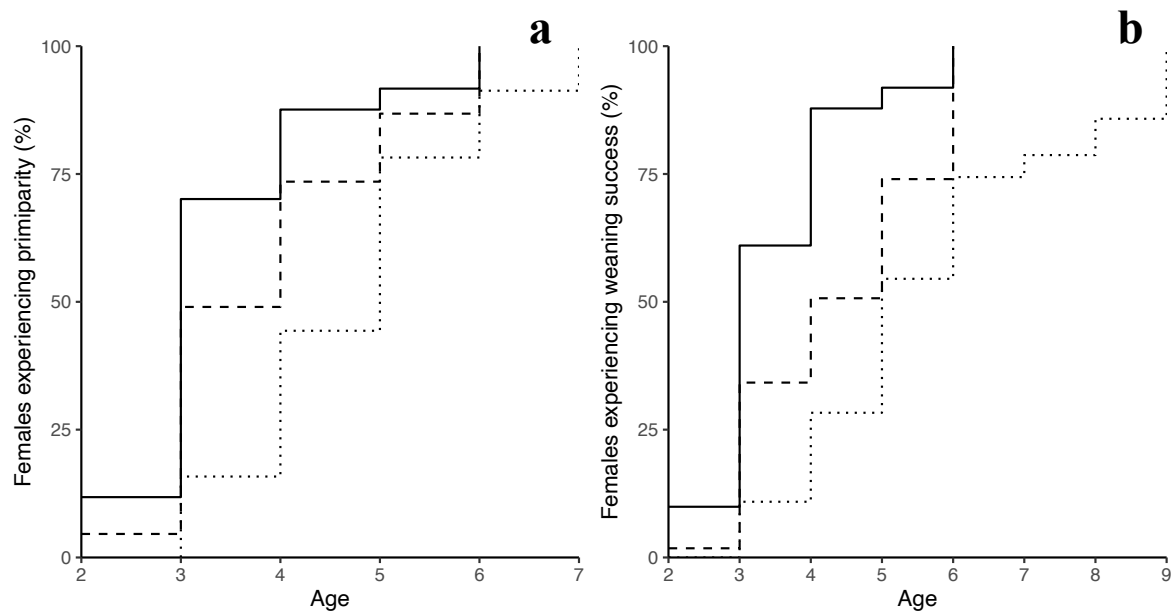
Figure 3-2 Associations of three different classes of female horn length at 2 years with (a) proportion of primiparous females and (b) proportion of females that weaned their first offspring across ages. Dotted line, dashed line and solid line represent short, medium and long horn classes, respectively. Females with standardized horn lengths less than−0.5 s.d, between−0.5 and 0.5 s.d. or greater than 0.5 s.d., respectively, were assigned to the short, medium and long horn length classes, respectively. Only females that experienced primiparity or weaned at least one offspring were included: (a)n= 189 and (b)n= 182
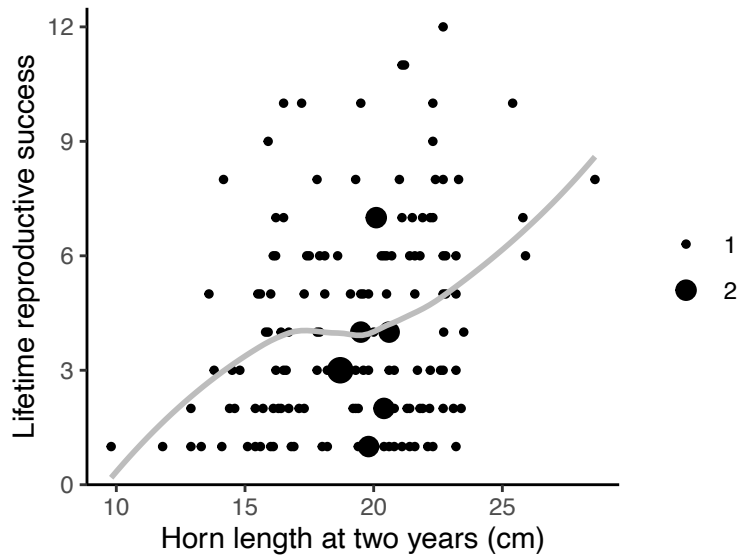
Figure 3-3 Associations of horn length at 2 years with LRS for female bighorn sheep at Ram Mountain, Alberta, Canada, cohorts 1973–2012. Grey lines fitted with loess, for ease of interpretation.

Chapter 4 - Development of a high-density species-specific targeted SNP assay for Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*)

4.1 Chapter summary

Due to their abundance and relative ease of genotyping, single nucleotide polymorphisms (SNPs) are a commonly used molecular marker for contemporary population genetic and genomic studies. A high-density and cost-effective way to type SNP loci is Allegro targeted genotyping (ATG), which is a form of targeted genotyping by sequencing developed and offered by Tecan genomics. One major drawback of this technology is the need for a reference genome and information on SNP loci when designing a SNP assay. However, for some non-model species genomic information from other closely related species can be used. Here we describe our process of developing an ATG assay to target 50,000 SNPs in Rocky Mountain bighorn sheep, using a reference genome from domestic sheep and SNP resources from prior bighorn sheep studies. We successfully developed a high accuracy, high-density, SNP assay for genotyping Rocky Mountain bighorn sheep that genotyped ~45,000 SNP loci. These loci were relatively evenly distributed throughout the genome. Furthermore, the assay produced genotypes at tens of thousands of SNP loci when tested with Desert bighorn sheep, Sierra Nevada bighorn sheep, Dall sheep, and Stone sheep.

4.2 Introduction

Molecular markers are the basis of many contemporary studies in the field of molecular ecology. Due to their abundance and relative ease of genotyping, single nucleotide polymorphisms (SNPs) are a common marker-type of choice for contemporary population genetic and genomic studies (Grover and Sharma, 2016). Despite typically being biallelic and therefore less informative than other markers such as microsatellites (Aitken *et al.*, 2004), their relative ease of discovery in both model and non-model organisms (Baird *et al.*, 2008; Peterson *et al.*, 2012; Narum *et al.*, 2013), abundance (Aitken *et al.*, 2004), and ability to be sequenced on extremely high throughput next-generation sequencing (NGS) platforms (Metzker, 2010) make SNPs an ideal high-density marker for genotyping large numbers of individuals.

When designing a SNP based genetic or genomic study there are multiple approaches that can be taken. In non-model organisms or species without a reference genome sequence, *de novo* discovery by genotyping by sequencing (GBS) (Narum *et al.*, 2013) approaches such as restriction-site associated DNA sequencing (RAD-seq) (Baird *et al.*, 2008) and double digest restriction-site associated DNA sequencing (ddRAD-seq) (Peterson *et al.*, 2012) can be used. However, the random nature in which these methods target sequencing sites allows for no control over the regions of the genome targeted to be sequenced. An alternative to RAD-seq and ddRAD-seq is targeted-GBS, which allows user-specified specific regions of the genome to be sequenced (Kozarewa *et al.*, 2015; Meek and Larson, 2019; Scaglione *et al.*, 2019). A recently developed, high-density, cost-effective form of targeted GBS is Allegro targeted genotyping (ATG). ATG is developed and offered by Tecan genomics (Redwood City, California, United States) and is a form of Single Primer Enrichment Technology (SPET) (Barchi *et al.*, 2019; Scaglione *et al.*, 2019). This form of SPET uses a simplified single primer design and functions by sequencing flanking regions around a probe to sequence target regions of interest (Barchi *et al.*, 2019). Despite being a relatively new technology, it has proven applications in humans (Scolnick *et al.*, 2015; Nairismägi *et al.*, 2016; Saber *et al.*, 2017), non-human mammals (Andrews *et al.*, 2021; Gavriliuc *et al.*, 2022), plants (Barchi *et al.*, 2019; Scaglione *et al.*, 2019; Gramazio *et al.*, 2020), arthropods (Chang *et al.*, 2020; Vu *et al.*, 2023), and bacteria (Benjamino *et al.*, 2021; Homeier-Bachmann *et al.*, 2022).

One major drawback of all targeted-GBS technologies is the genomic information required to design an assay. Unlike RAD based methods, targeted-GBS requires prior knowledge of both polymorphic loci or genomic regions of interest and a reference genome applicable to the target species, and therefore may not be suitable for all species (Kozarewa *et al.*, 2015). However, for some non-model species genomic information from other closely related species can be used. For example, genomic resources developed for the domestic cat and sheep have been applied to their wild counterparts (Li *et al.*, 2019; Sim and Coltman, 2019; Santos *et al.*, 2021). Thus, for wild species for which a genome of a closely related species and SNP or genomic regions of interest are known for the target species or closely related species, targeted-GBS is a viable high-density SNP genotyping technology.

The Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) may be a good candidate for the application of Allegro targeted-GBS for two reasons. Firstly, a reference genome exists for the closely related domestic sheep (*O. aries*) which diverged from bighorn sheep ~3 mya (Bunch *et al.*, 2006), and previous cross-species applications of the domestic sheep reference genome to bighorn sheep genomic studies have been successful. Secondly, multiple studies have previously identified abundant SNP loci in Rocky Mountain bighorn sheep (Miller, Kijas, *et al.*, 2012; Miller, Hogg and Coltman, 2013; Kardos *et al.*, 2015; Miller *et al.*, 2015, p. 201; Miller, Festa-Bianchet and Coltman, 2018). Thus, all the resources required to develop a targeted-GBS assay are available. Additionally, another characteristic of Tecan's ATG which make it well suited for Rocky Mountain bighorn sheep genomic research, is the low quantity of input genomic DNA required. Therefore, this allows for the use of alternative biological sample types such as faeces, (Albaugh *et al.*, 1992; Gavriliuc *et al.*, 2022), which can be easier to obtain but typically contain less DNA than invasively collected tissue samples.

Here, we describe our process of developing a high-density SNP assay to be used with the ATG technology for Rocky Mountain bighorn sheep. We aim to design an assay to target 50k SNP loci for studying high-resolution population genetic structure and quantitative genetics. Upon development of the assay, we test the efficiency and accuracy of the technology by sequencing a number of individuals on a trial and full version of our assay. We include input DNA from differing biological materials to test how DNA source affects the accuracy of the assay. Finally, we also

examine the assay performance on two other sub-species of bighorn sheep; Desert (*O. c. nelsoni*) and Sierra Nevada (*O. c. sierrae) bighorn* sheep, and two other sub-species of thinhorn sheep; Dall (*Ovis dalli dalli*) and Stone (*O. d. stonei*) sheep.

4.3 Methods

4.3.1 Assay development

Variant site data were sourced from five studies; an implementation of a commercially available SNP-chip (Miller, Kijas, *et al.*, 2012), RAD-seq performed on individuals from Ram Mountain, Alberta and the National Bison Range, Montana (Miller, Hogg and Coltman, 2013), whole genome sequencing performed on one individual from Ram Mountain, Alberta (Miller *et al.*, 2015), pooled whole genome sequencing of 58 individuals from Montana and Wyoming (Kardos *et al.*, 2015), and the application of another commercially available SNP-chip (Miller, Festa-Bianchet and Coltman, 2018). We took the variant call data files (VCFs) from four of these studies; Kardos e *et al.* (2015), Miller *et al.* (2012), Miller *et al.* (2015), and Miller, Festa-Bianchet and Coltman (2018), as these studies were already mapped to the *Ovis aries 3.1* (OAR3.1) reference genome (Jiang *et al.*, 2014). The Miller, Hogg and Coltman (2013) study was mapped to an older domestic sheep reference genome, therefore we aligned its raw reads to the OAR3.1 reference genome, and then called variants from this alignment to produce a variant call file in a format consistent with that of the other studies. We produced indexes from the OAR3.1 genome using the Bowtie2 v2.3.5.1 (Langmead and Salzberg, 2012) build function and the SAMtools v1.10 (Li *et al.*, 2009) command "faidx". Then both forward and reverse reads were aligned as pairs to the indexed reference genome using the "—sensitive" pre-set options in Bowtie2. The SAMtools command "view" was then run, with the reference index specified, to convert the paired read alignments from un-indexed .sam files to indexed .bam files. Finally, the SAMtools command "sort" was used to sort the .bam files.

We subsequently filtered the variant site data generated by aligning the Miller, Hogg and Coltman (2013) reads and variant data from the other four studies through a series of steps to reduce the number of variants and obtain optimal loci for our assay. To filter the variant site data we used VCFtools v0.1.15 (Danecek *et al.*, 2011) and BCFtools v1.10.2 (Li *et al.*, 2009). See details of our filtering process in Figure 4-1. Most of the filtering processes were the same among all variants

sourced from all five studies. However, due to the geographic separation between the Kardos *et al.* (2015) study area and our intended study area, we altered the filtering parameters for Kardos *et al.* (2015) at two steps in an attempt to retain common SNPs, which in theory were less likely to be alleles unique to the Kardos *et al.* (2015) study sites. Thus, we altered our parameters to filter out alleles with a minor allele frequency <0.3 and only retained loci polymorphic in each of the three Kardos *et al.* (2015) sampling locations.

Our target was to submit 100,000 candidate SNPs to Tecan for probe design, as the probe design process had ~50% success rate in designing paired probes for target loci. For this 100,000 we retained all the Miller *et al.* (2012), Miller, Hogg and Coltman (2013), and Miller, Festa-Bianchet and Coltman (2018) SNPs that passed filtering and subsampled SNPs from the Kardos *et al.* (2015) and Miller *et al.* (2015) SNPs which passed filtering. Subsampling of the Kardos *et al.* (2015) and Miller *et al.* (2015) SNPs was done by using the VCFtools function "—thin" at increasingly larger increments until we reduced the candidate SNP list to ~100,000 (Figure 4-1). We submitted the target SNP list to Tecan as a regions file (bed format), targeting a 5bp region around our target SNPs (2bp upstream, 2bp downstream), from which they designed a genotyping assay for 50,000 loci across the Rocky Mountain bighorn sheep genome based upon the OAR3.1 domestic sheep genome. To visualise the distribution of these SNPs, we created a rainfall plot using the R (R Core Team, 2013) package karyoploteR (Gel and Serra, 2017). Additionally, we created two further plots in ggplot2 v3.3.5 (Wickham, 2016): a bar plot comparing proportion of SNPs contained in each chromosome and proportion of the genome contained in each chromosome, and a density plot for inter-SNP distances between SNPs in our 50,000 SNP assay.

As part of the development of the 50,000 loci assay (referred to as the 50k assay from herein) a 10,000 loci trial assay (referred to as the 10k assay from herein) was performed, this 10k assay was performed to test the ATG technology prior to confirming our 50k assay design. For assessing the performance of both the 10k SNP and 50k SNP assays, an identical set of individuals were genotyped with both assays. We genotyped DNA samples from 40 Rocky Mountain bighorn sheep and 16 individuals (four each) from each of the four sub-species of mountain sheep. All Rocky Mountain bighorn sheep samples were typed in duplicate to test the accuracy of the assays, resulting in a total number of samples genotyped of 96 (Table 4-2). To test the performance of the

assay on differing biological sample types, Rocky Mountain bighorn sheep DNA was sourced from blood, faeces, and skin or muscle samples (together referred to as tissue samples from herein) (Table 4-3).

We extracted DNA from differing biological material using different protocols for each material type. Tissue samples were digested and extracted using the DNeasy Blood and Tissue kit (Qiagen N.V., Venlo, the Netherlands), following the manufacturer's recommended procedure. Blood was treated with ammonium–chloride–potassium (ACK) (Brown, Hu and Athanasiou, 2016) prior to undergoing the same procedure as tissue samples. For faecal samples, three pellets from each sample were soaked in 1X phosphate-buffered saline (PBS) for 20 min before being swabbed around the exterior with a cotton-tip applicator, which in turn was washed with Aquastool (MoBiTec GmbH, Goettingen, Germany); the material was digested and extracted following the Aquastool protocol. Each sample was quantified and normalized to a concentration of 8ng/uL, resulting in a total of 80ng of DNA processed per sample during library preparation. For the library preparation we used two Allegro Targeted Genotyping V1 kits from Tecan Genomics. The first kit targeted and genotyped 10,000 SNP loci, which was a subset of SNP loci from the full 50k assay, and the second kit targeted and genotyped 50,000 SNP loci. We followed the manufacturer's protocol provided by Tecan with a slightly increased fragmentation digest time of 22.5 minutes rather than 15 minutes. Following library preparation, we sequenced a total of 96 samples: 80 Rocky Mountain bighorn sheep (40 individuals in duplicate), 4 desert bighorn sheep, 4 Dall sheep, 4 Sierra Nevada bighorn sheep, and 4 Stone sheep using both kits. The 10k SNP library was sequenced on an Illumina (San Diego, United States) NextSeq using a NextSeq V2.5 mid-output 300 cycle kit which provided 120,000,000 two-directional 150bp reads. Thus, each sample had an average coverage of 125X (120,000,000 reads ÷ 10k loci ÷ 96 samples = 125X coverage). Our 96 samples processed using the 50k SNP loci ATG kit were sequenced as part of a 384-individual library on a NextSeq V2.5 high-output 300 cycle kit providing 400,000,000 two-directional 150bp reads. Thus, each sample had an average coverage of 20.83X (400,000,000 reads ÷ 50k loci ÷ 384 samples = 20.83X coverage).

The raw reads generated by the two sequencing runs were analysed using FastQC v0.11.9 (Andrews, 2010). Raw reads were trimmed using the software TrimGalore v0.6.5 (Martin, 2011)

with the commands "—paired" and "—phred33" used to instruct the program that paired reads and the phred33 quality scale were being used. Additionally, the command "—adapter2" followed by adaptor sequence removed adaptors from the R2 read. The commands "—three_prime_clip_R1/R2 5" and "—quality 30" were used to trim five bp from the three-prime end of both the R1 and R2 reads, and trim anything with a quality less than 30. See appendix C for further details of trimming.We divided our reads by sequencing run (for the 10k or 50k assay) and by species and subspecies before calling variant sites.

To align the reads to the OAR3.1 genome and call variant sites, we used Bowtie2, SAMtools, and BCFtools following the same process and parameters as previously described for realigning the Miller, Hogg and Coltman (2013) reads. Subsequently, we filtered the 10 VCF files individually. We removed indels using VCFtools. Then removed loci with a quality score lower than 30 and a depth of less than 6 using BCFtools. We excluded loci with more than 30% missing data and loci with a minor allele frequency of less than 0.05 in our Rocky Mountain bighorn sheep VCF and 0.15 in all other species VCFs using VCFtools. Samples with more than 25% missing data were removed, and our resulting dataset was re-filtered based on the earlier minor allele frequency requirements to ensure all retained SNPs still had suitable minor allele frequencies using VCFtools. Finally, we filtered to retain loci with no more than 10% missing data using VCFtools.

4.3.2 Assay validation

To evaluate the efficiency of the two assays, we examined reads from the 56 genotyped individuals, 96 samples in total including duplicates, sequenced on our 10k trial assay and our full 50k assay. First, we examined the overall efficiency and on-target efficiency of each assay. We defined the overall efficiency as the proportion of SNPs recovered compared to the total number of loci targeted, which, due to the nature of the SPET technology, could be higher than 100% as novel SNPs may be discovered in the flanking regions of the targeted loci. We defined on-target efficiency as the total number of targeted SNPs recovered compared to total number of loci targeted. This was evaluated by examining the total remaining SNPs after filtering to only retain SNPs located at our target loci (+-2bp to account for error) using VCFtools.

To evaluate the accuracy of our assay, we compared genotypes between duplicate samples within and between the two assays. We used PLINK (Purcell *et al.*, 2007) to calculate the identity by state (IBS) for all genotypes, calculated as a proportion of identical loci between two samples excluding missing data. Rocky Mountain bighorn sheep duplicate samples were compared within and between the two assays. Two other sub-species of bighorn sheep and two sub-species of thinhorn sheep were only compared across the two assays at loci common between the 10k and 50k assays. To assess the accuracy of the genotyping assays on DNA sourced from differing biological materials we split our Rocky Mountain bighorn sheep samples into three groups; blood, faecal, and tissue derived DNA. We then examined the accuracy of those groups of genotypes separately. Finally, to further assess the ability of our assays to identify matching identical genotypes we compared IBS values between duplicate samples, samples from the same sampling location, and between samples from differing sampling locations.

We used PLINK to calculate observed (H_{obs}) and expected heterozygosity (H_{exp}) within sampling locations of the Rocky Mountain bighorn sheep, as well as the total sample set, using only one of the duplicated samples. We then calculated Wright's inbreeding coefficient $f = (H_{exp} - H_{obs})/H_{exp}$ (Wright, 1922). Additionally, we calculated minor allele frequencies for all SNPs using PLINK. To examine patterns of genetic distance between Rocky Mountain bighorn sheep sampling locations, we used adegenet (Jombart, 2008; Jombart and Ahmed, 2011) to calculate Nei's standard genetic distance (Nei, 1972) between sampling locations. Subsequently, we used the R package *vegan* v2.5.7 (Oksanen *et al.*, 2013) to perform simple Mantel tests on the genetic distances and geographic distances between sampling locations. Finally, to further asses the ability of the SNP assay to identify population structure, we ran two principal component analyses (PCA) on Rocky Mountain bighorn sheep data from each assay using the R package *adegenet*.

4.4 Results

4.4.1 Assay development

From the five studies we sourced a total of ~39.7 million variant sites. After filtering, we retained 100,000 SNP loci and submitted them to Tecan for assay development. From our list of 100,000 SNPs Tecan designed an assay which could target 50,000 loci. For more information on the numbers of loci sourced from each study and included in the 50k assay see Table 4-1, for full

details of the assay designed by Tecan see electronic material 1. The 50,000 loci were relatively evenly distributed within and between chromosomes (Figures 4-2 and 4-3) and had an average inter-locus distance of 53,339 bp (Figure 4-4).

4.4.2 Post-sequencing filtering

After trimming and alignment, variant calling identified 163,176 and 769,835 variant sites from the 10k and 50k assays, respectively, in our Rocky Mountain bighorn sheep. After filtering we retained 10,215 and 45,367 SNPs in the 10k and 50k datasets, respectively, of which 8,082 and 38,499 SNPs were "on-target" in the 10k and 50k SNP datasets, respectively (Table 4-4). For further details of SNPs retained throughout filtering steps in the other mountain sheep species and subspecies, see table 4-4.

4.4.3 Assay validation

Our first validation step was to examine the efficiency of the assays. The 10k and 50k assays had overall efficiencies 102% and 91%, respectively, for Rocky Mountain bighorn sheep samples. On-target efficiency was lower at 81% and 77% for the 10k and 50k loci assays, respectively, based on Rocky Mountain bighorn sheep samples. For other mountain sheep species and subspecies, overall efficiency for the 10k and 50k SNP assays ranged from 32% to 43% and 17% to 42%, respectively, and on-target efficiency ranged from 14% to 29% and 12% to 31% for the 10k and 50k SNP assays, respectively.

IBS values between Rocky Mountain bighorn sheep duplicate samples ranged from 96% to 97% within and between the 10k and 50k SNP assays (Table 4-4). The values of IBS between assays for other species and subspecies of mountain sheep ranged from 93% to 96% (Table 4-5). The IBS values of genotypes from DNA sourced from blood and tissue exceeded those for DNA sourced from faeces (Table 4-6). The only sample type to lose genotypes during filtering were those sourced from faecal material, with the 10k and 50k assays losing 21% and 56 % of samples, respectively (Table 4-6). To further assess the ability of our assays to identify matches between identical genotypes, we examined the distribution of IBS values for different classes of Rocky Mountain bighorn sheep samples; between duplicate samples, between samples from the same sampling location, and between samples from differing sampling locations (Figure 4-5).

We found the Rocky Mountain bighorn sheep samples to have an observed heterozygosity of 28% and 29%, and an expected heterozygosity of 33% and 33% for the 10k and 50k SNP assays, respectively. Wright's inbreeding coefficient for each overall sample set was 0.133 and 0.135 for the 10k SNP and 50k SNP assays, respectively (Table 4.7). We identified a strong pattern of isolation-by-distance between Nei's standard genetic distance and geographic distance among five sampling locations of Rocky Mountain bighorn sheep in both SNP assays (Figure 4-6), with geographic distance accounting for 88% and 84% of genetic distance between populations (10k assay r^2=0.88, p=0.008; 50k assay r^2=0.84, p=0.017), respectively. This isolation-by-distance was also reflected in the distribution of samples in PCA plots (Figure 4-7). Notably, the two points positioned between Cadomin Mountain and Ram Mountain is a genotype from the same individual who was a sheep with Cadomin-admixed ancestry from Ram Mountain according to pedigree data (Coltman *et al.*, 2002; Poirier *et al.*, 2019).

4.5 Discussion

We set out to develop and test the efficiency and accuracy of a species-specific high-density SNP genotyping assay for Rocky Mountain bighorn sheep. The final assay targeted 50,000 loci, which were evenly selected from chromosomes relative to chromosome size, and were evenly distributed throughout the genome with an average inter-SNP distance of 53,339 bp. The 50k assay used with the ATG technology yielded overall and on-target efficiencies of 91% and 77%, respectively, with an average accuracy of 97%. As expected, we found that genotype accuracies increased with better quality biological material for DNA extraction. Therefore, genotypes sourced from blood and tissue samples had a higher accuracy than those generated from faecal samples. Additionally, of the three sample types, only faeces derived genotypes were missing sufficient data for samples to be excluded from our final genotype dataset. Overall, the assay and technology yielded ample SNP data with a high degree of accuracy, with varying levels of success from both tissue samples, but with lower genotyping success for faecal derived samples.

4.5.1 Faecal genotyping

Our assays performed worse with DNA extracted from faecal samples than DNA extracted from other sample types. The 10k and 50k assays only retained ~79% and ~43% of faecal samples,

respectively, and had accuracies of 93% and 92%, respectively. Both these values are low when compared to the overall genotyping success of 85% and accuracy of 99.9% observed by Gavriliuc *et al.*(2022) for ATG genotyping faecal samples from domestic horses (*Equus caballus*). However, we should note some differences between the studies. First, Gavriliuc *et al.* (2022) used swabs from fresh faecal samples, whereas our faecal samples were in the environment for an undetermined amount of time prior to collection. Secondly, Gavriliuc *et al.* (2022) only typed 48 samples at 279 SNPs using a sequencing kit which provided ~800,000 reads, and thus had a sequencing coverage almost three times greater than the 50k assay. With greater sequencing coverage we may have observed higher success and accuracy.

Unlike Gavriliuc *et al.* (2022), our samples were exposed to the environment for an undetermined amount of time. During this time samples would have been exposed to ultra violet light and, despite most of our samples being collected in the winter, potentially above optimal temperatures for DNA preservation. Thus, our samples may have experience more environmental degradation than those of Gavriliuc *et al.*, (2022). Notably, we observed a high rate of microsatellite genotyping failures using the same samples, necessitating typing each sample in triplicate to generate reliable results (Chapter 2). However, despite these issues, sampling fresher samples is not easily done, given that bighorn sheep inhabit difficult to access terrain in remote locations.

There are two options for improving genotyping success and accuracy. The first is to sequence samples with more coverage. Samples in the 10k and 50k assay were sequenced with 125X and 20.8X, respectively, and faecal samples in the 10k assay had a 1.8% increase in accuracy and a 26% increase in genotyping success over faecal samples in the 50k assay. This trend is also reflected in the accuracies and genotyping success for the two other sample types and by the accuracy obtained by Gavriliuc *et al.* (2022) when using greater coverage. This suggests increasing sequencing coverage also increases genotyping rate and accuracy in faecal samples.

A challenge may be the presence of polymerase chain reaction (PCR) inhibitors in DNA samples sourced from faecal samples. PCR inhibitors may not be removed in DNA purification (Morin *et al.*, 2001) and thus may inhibit efficient amplification. The ATG protocol used in this paper uses PCR, so it is possible PCR inhibitors have contributed to the poor performance of our assays on

faecal material. To rectify this different methodological approaches such as DNA extraction using magnetic beads (Flagstad *et al.*, 1999), or a post extraction clean-up such as ethanol precipitation (Green and Sambrook, 2016) could be used.

4.5.2 Forensic matching

One of the original purposes of our assay was to forensically identify samples taken from the same individual. This was to be done by identifying matching genotypes from samples collected at differing times and locations. However, our assays only had an accuracy of ~97% between biological replicates and not the ~100% accuracy typically desired for forensic purposes (Johnson, Wilson-Wilde and Linacre, 2014). For future studies we suggest sequencing with a greater read depth, as greater read depth appears to improve the accuracy of ATG (see section 4.5.1). Alternatively, filtering for a greater genotyping depth post-sequencing may increase accuracy and should not limit forensic analysis, which typically require fewer loci than quantitative or fine-scale population genetics (Ogden, 2011).

If these two approaches do not increase accuracy to a level where perfect identity is reached between biological replicates, an alternative "fuzzy matching" approach to identifying matching genotypes may be used. There are noticeable gaps in the distribution of IBS values for duplicates, individuals within sampling locations, and between locations (Figure 4.5). One could use these gaps to set thresholds for classifying pairs of samples into those from same or different individuals. For example, only biological replicates had IBS values greater than ~88% (Figure 4-5), thus we would assume any sample pairs typed with an IBS greater than 88% to be from the same individual, if typed under the same conditions as these assays.

4.5.3 Population structure

To test the utility of the assays for population genomic studies of Rocky Mountain bighorn sheep, we visualized the genetic similarity among individuals using Principal Component Analysis and by plotting isolation by distance. As expected, Rocky Mountain bighorn sheep samples grouped together by sampling location, and sampling locations separated relative to geographic separation. We also observed a strong pattern of isolation-by-distance among sampling locations using both assays, with over 80% of variance explained by geographic distance alone. This high degree of

isolation-by-distance was expected, as previous analyses of bighorn sheep in this region identified a strong pattern of isolation-by-distance (Chapter 2).

4.5.4 Application to other mountain sheep

Our assays had varying efficiencies with other mountain sheep species and subspecies. The variation in efficiencies appears to be explained by the phylogenetics of North American wild sheep. The efficiency of the assays decreases as time since divergence increases between the trialed species or sub-species and Rocky Mountain bighorn sheep. This was expected, Miller *et al.* (2013) found that cross species applications of SNP assays yield exponentially less polymorphic SNPs as time to divergence increases. Of the sub-species of bighorn sheep, desert bighorn had the highest efficiency, which is expected given desert and Rocky Mountain bighorn sheep diverged ~94kya, whereas Rocky Mountain and Sierra Nevada bighorn sheep diverged ~315kya (Buchalski *et al.*, 2016). We see a further reduction in efficiencies for Dall and Stone sheep, with efficiencies of ~33% and ~32% for the 10k SNP assay and ~21% and ~17% for the 50k SNP assay, respectively, which is expected given that bighorn and thinhorn sheep diverged ~1mya (Rezaei *et al.*, 2010). Despite the reductions in efficiency, we found genotypes for all of these sub-species and species to have high accuracies in the range of 93%-96%.

4.5.5 Conclusion

In conclusion, we successfully developed a high-density and high-accuracy SNP assay for consistently genotyping Rocky Mountain bighorn sheep at ~45,000 SNP loci evenly distributed throughout their genome. The assay performs well on DNA sourced from tissue, but less so when used on DNA sourced from faeces. Thus, this assay enables us to perform high-throughput genotyping on Rocky Mountain bighorn sheep at a higher density than offered by previously used technologies (Miller, Kijas, *et al.*, 2012; Miller, Festa-Bianchet and Coltman, 2018) and will serve as a genomic resource for future studies on the species. Furthermore, this assay can be used to analyse many SNP loci in other species and sub-species of mountain sheep. The assay's efficiency decreased as the divergence times of these species and subspecies from Rocky Mountain bighorn sheep increased. However, the number of loci in the assay still allows for tens of thousands of SNP loci to be analysed. Thus, potentially having applications in other North American species and sub-species of mountain sheep.

This assay is another successful cross-species application of a genome developed for the domestic sheep (Bunch *et al.*, 2006) to Rocky Mountain bighorn sheep, further exemplifying how studies may use genomic resources with non-target, closely related species (Li *et al.*, 2019; Sim and Coltman, 2019; Santos *et al.*, 2021). Additionally, our study contributes to the growing number of successful applications of ATG and SPET in non-human mammals (Andrews *et al.*, 2021; Gavriliuc *et al.*, 2022) and other species (Saber *et al.*, 2017; Scaglione *et al.*, 2019; Vu *et al.*, 2023), proving to be a viable option for low-cost, high-throughput, high-density SNP genotyping. Furthermore, our study highlights how the targeted interrogation of genomes with targeted-GBS technology (Kozarewa *et al.*, 2015; Meek and Larson, 2019; Scaglione *et al.*, 2019) allows researchers to target specific loci throughout a genome. Thus, enabling researchers to obtain relatively evenly distributed genome wide SNP genotypes, which is required for quantitative genetic and genomic studies

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Table 4-1. Total variant sites sourced from each of the five studies and number of SNPS from each study included in our final 50,000 SNP assay.

Study	Total variant sites	SNPs retained in 50k assay
Kardos *et al.*, (2015)	20,117,094	21,121
Miller *et al.*, (2012)	40,843	147
Miller *et al.*, (2013)	290,287	4244
Miller *et al.*, (2015)	19,153.582	23,829
Miller *et al.*, (2018)	3,777	659

Table 4-2 Sample information for 96 validation individuals sequenced in our validation run. Including sampling location, biological material sample type, number of individuals genotyped, number of times each individual was replicated, and overall number of samples sequenced.

Species/sub-species	Sample type	Individuals genotyped	Number of replicates	Number of samples genotyped
Rocky Mountain bighorn sheep	Blood, Faeces, Tissue	40	2	80
Sierra Nevada bighorn sheep	Tissue	4	1	4
Desert bighorn sheep	Tissue	4	1	4
Dall sheep	Tissue	4	1	4
Stone sheep	Tissue	4	1	4
Total		56		96

Table 4-3 Details of the 40 Rocky Mountain bighorn sheep samples included and replicated within our 96-sample validation run. Including sampling location, biological material sample type, number of individuals sequenced, number of times each individual was replicated, and sampling location coordinates.

Sampling location	Sample type	Individuals genotyped	Number of replicates	Latitude (˚N)	Longitude (˚W)
Cadomin Mountain	Faeces	8	2	52.97	117.20
Castle Yarrow	Blood	8	2	49.27	114.20
Narraway	Tissue	8	2	54.27	119.91
Ram Mountain	Tissue	8	2	52.36	115.79
Stornoway Mountain	Faeces	8	2	53.29	118.39

Table 4-4 Variant sites retained after each stage of filtering and efficiencies for the five species/subspecies of mountain sheep genotyped on the 10,000 and 50,000 SNP assays.

Species/sub-species	10,000 SNP assay					50,000 SNP assay				
	RM bighorn	Dall	Desert	SN bighorn	Stone	RM bighorn	Dall	Desert	SN bighorn	Stone
All sites	163,176	67,212	65,849	64,838	62,102	769,835	140,008	131293	126914	128393
Remove indels	89,879	12,699	14,503	13,677	12,999	449,563	82,379	81474	79508	85747
Quality >30	49,815	9,977	11,476	10,814	9,770	183,799	35,471	39925	38182	31876
Depth >6	49,059	9,778	11,230	10,560	9,528	183,476	27,495	31515	29495	22923
Loci missing data <30%	45,736	9,682	11,106	10,445	9,395	148,161	26,556	28889	27208	21915
Minor allele frequency >5%	11,502	3,431	4,440	3,769	3,338	48,791	11,793	20882	18295	10448
Individual missing data <25%	73	4	4	4	4	62	4	3	3	4
Minor allele frequency >5%	11,448	3,431	4,440	3,769	3,338	48,355	11,793	20,881	18295	10448
Loci missing data <10%	10,215	3,345	4,334	3,688	3,225	45,367	10,329	20,880	18295	8485
On-target	8,082	1,445	2,872	2,384	1,465	38,499	5,939	15,597	13560	5338
Overall efficiency	102.15	33.45	43.34	36.88	32.25	90.73	20.66	41.76	36.59	16.97
On-target efficiency	80.82	14.45	28.72	23.84	14.65	77.00	11.88	31.19	27.12	10.68

Table 4-5 Average identity by state (± standard deviation) between replicates within the 10,000, within the 50,000, and between the 10,000 and 50,000 SNP assays for all species/subspecies of mountain sheep genotyped.

Species/subspecies	Within 10,000 SNP assay	Within 50,000 SNP assay	Between 10,000 and 50,000 SNP assays				
	RM bighorn	RM bighorn	RM bighorn	Dall	Desert bighorn	SN bighorn	Stone
IBS	97.02 ± 0.031	95.99 ± 0.022	96.51 ± 0.035	94.84 ±0.012	95.50 ± 0.020	95.06 ± 0.007	92.69 ± 0.012

Table 4-6 Average identity by state (± standard deviation) within the 10,000, within the 50,000, and between the 10,000 and 50,000 SNP assays and their respective genotyping successes for differing sample types.

Sample type	Within 10,000 SNP assay		Within 50,000 SNP assay		Between 10,000 and 50,000 SNP assays	
	IBS (%)	Samples lost (%)	IBS (%)	Samples lost (%)	IBS (%)	Samples lost (%)
Blood	98.94 ± 0.005	0	96.89 ± 0.009	0	97.88 ± 0.007	0
Faeces	93.48 ± 0.033	21.19	91.68 ± 0.024	56.25	88.55 ± 0.190	56.25
Tissue	98.58 ± 0.005	0	96.62 ± 0.012	0	97.56 ± 0.008	0

Table 4-7 Observed heterozygosity, expected heterozygosity, and Wrights inbreeding coefficient (f) for each sampling location of Rocky Mountain bighorn sheep.

Sampling location	10,000 SNP assay				50,000 SNP assay			
	Number of genotypes	H.Obs	H.exp	f	Number of genotypes	H.Obs	H.exp	f
Cadomin Mountain	12	0.283	0.333	0.151	6	0.272	0.334	0.185
Castle Yarrow	16	0.301	0.332	0.094	16	0.292	0.333	0.124
Narraway	16	0.288	0.332	0.132	16	0.278	0.333	0.165
Ram Mountain	16	0.325	0.332	0.019	16	0.334	0.333	-0.004
Stornoway Mountain	13	0.240	0.333	0.279	8	0.228	0.334	0.317
Overall	73	0.290	0.332	0.128	62	0.289	0.333	0.132
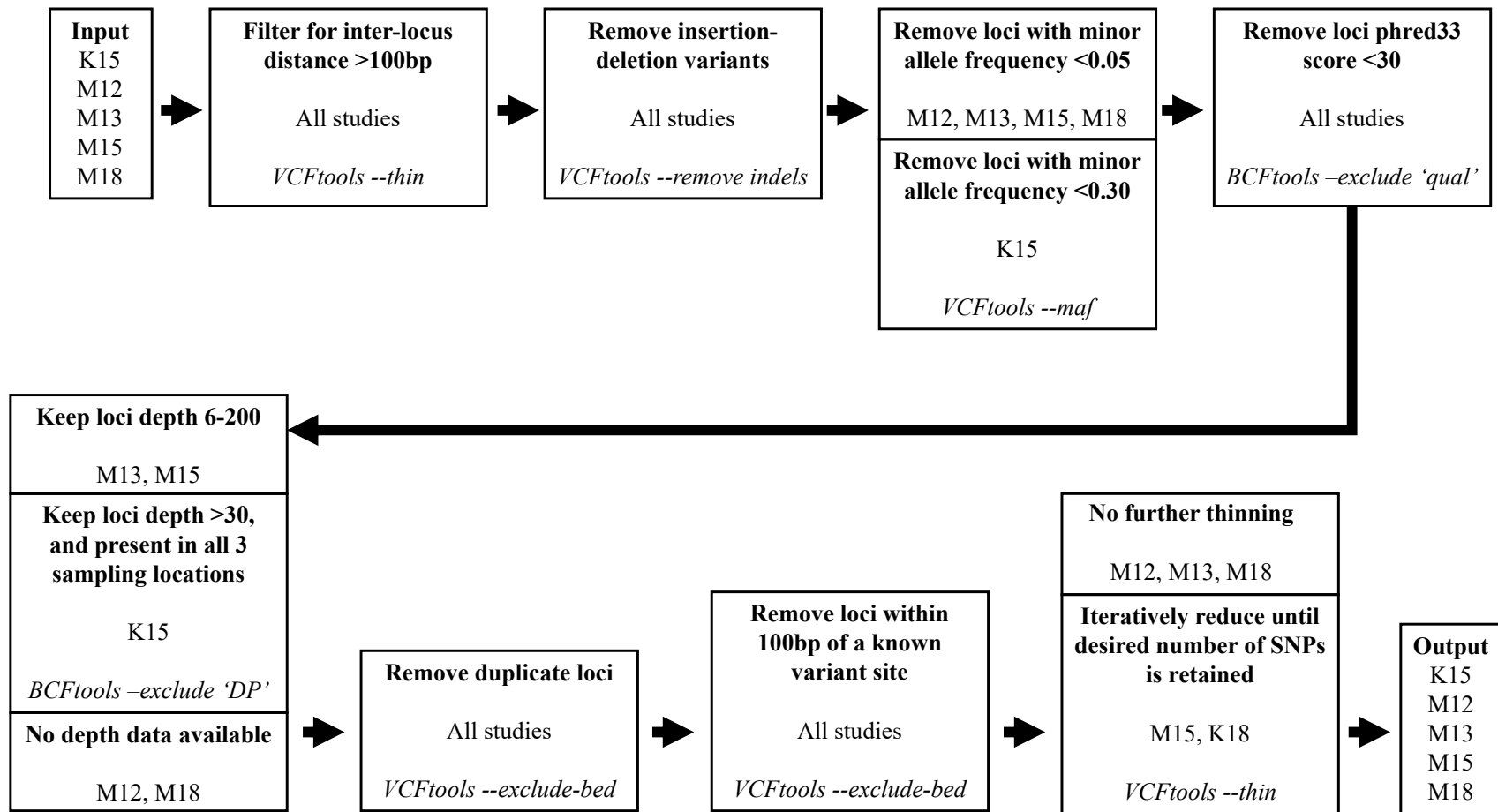
Figure 4-1. Filtering steps applied to variant data sourced from the five studies. The studies, Miller *et al*. (2012), Miller, Hogg and Coltman, (2013), Miller *et al*. (2015), Kardos et al. (2015), and Miller, Festa-Bianchet and Coltman (2018) are represented by M12, M13, M15, K15, and M18, respectively. Each step describes a process applied to data from the previous step, process is indicated in bold, source studies of variant data in plain text, and software and command used in italics.
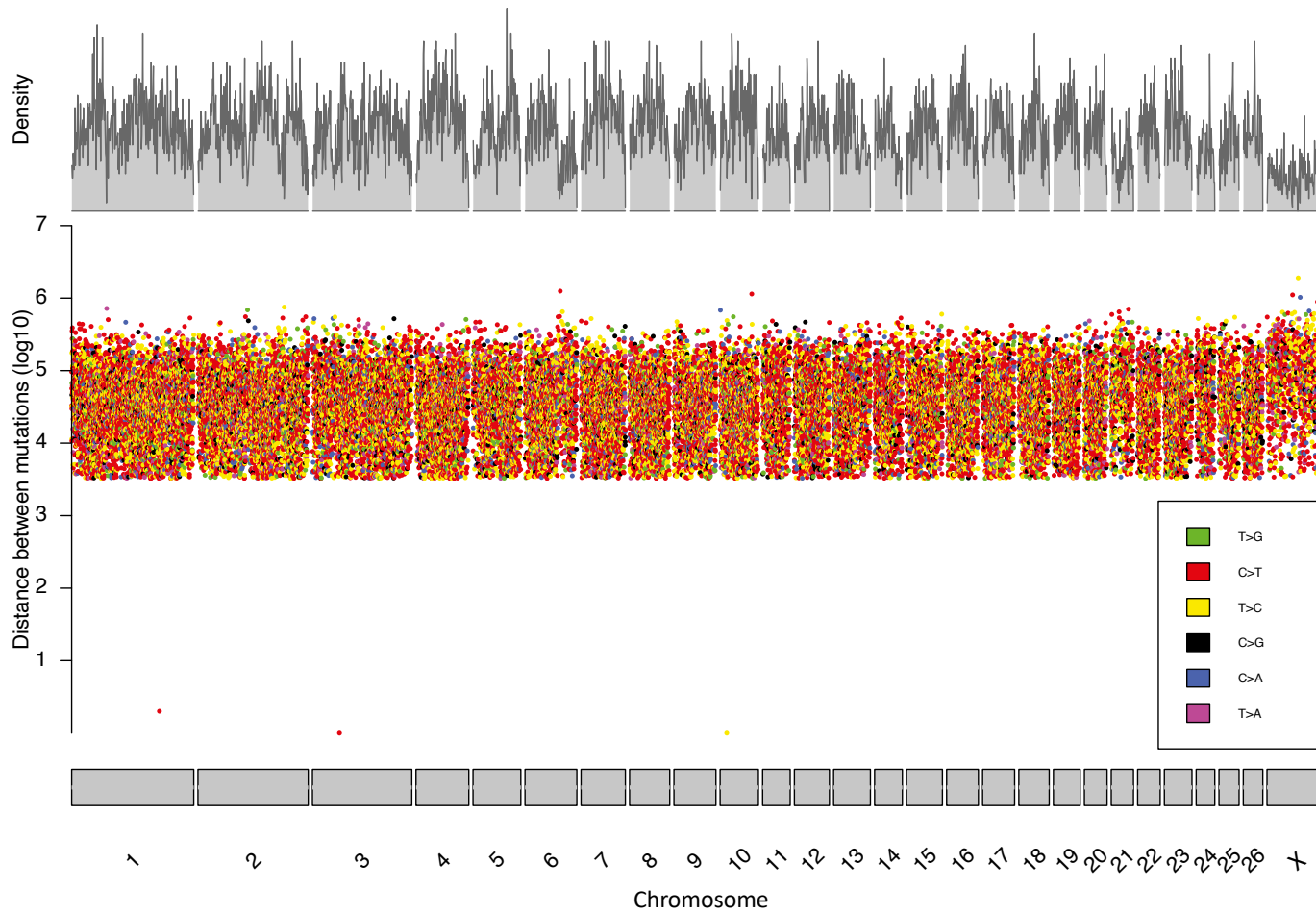
Figure 4-2 Rainfall plot characterising the position, distance between, and type of substitution for each of the 50,000 loci targeted by our assay. Density across chromosome regions shown in grey.
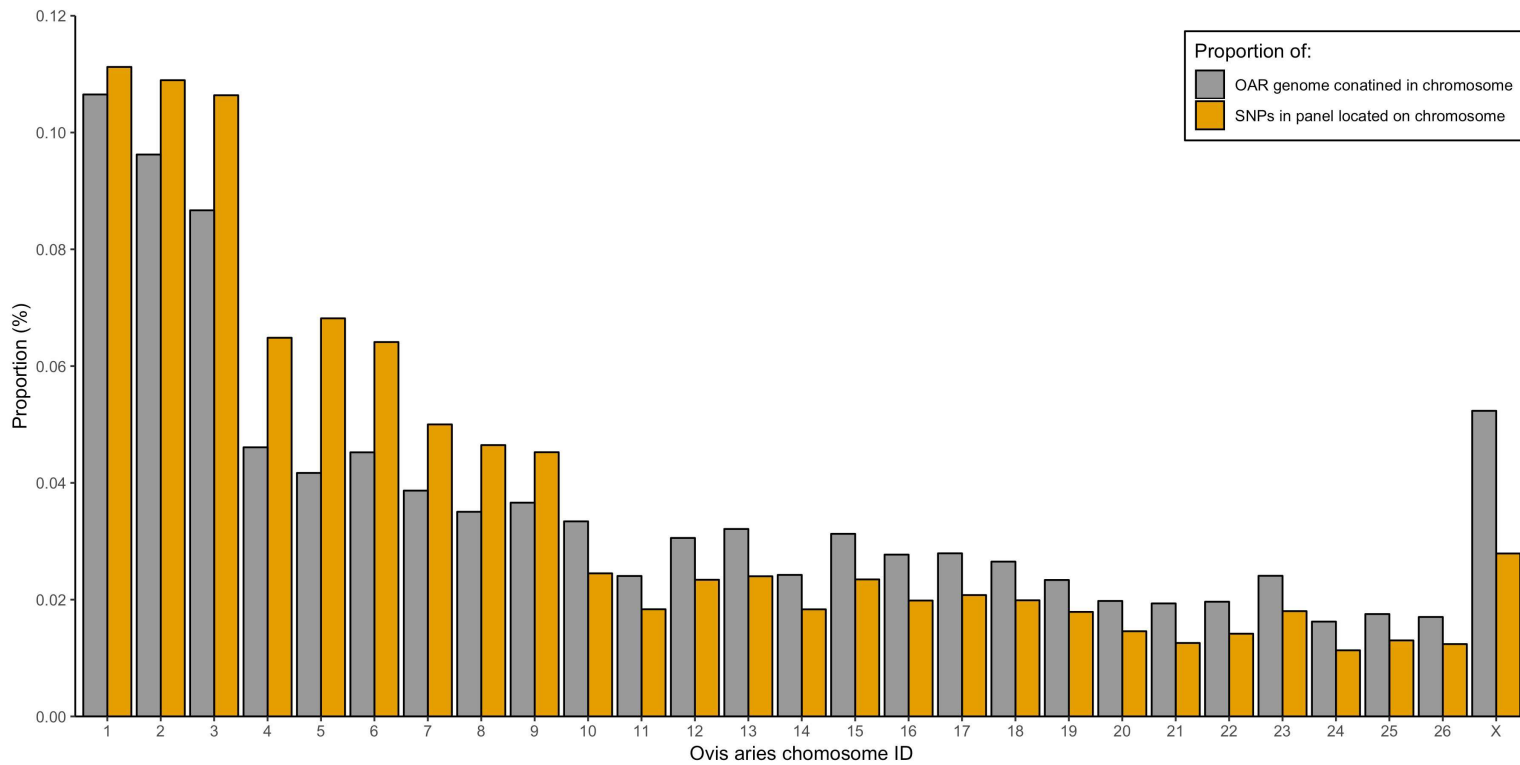
Figure 4-3 Proportion of the total *Ovis aries* 3.1 genome contained within each chromosome (grey) alongside the proportion of SNPs in the assay located on each chromosome (gold).
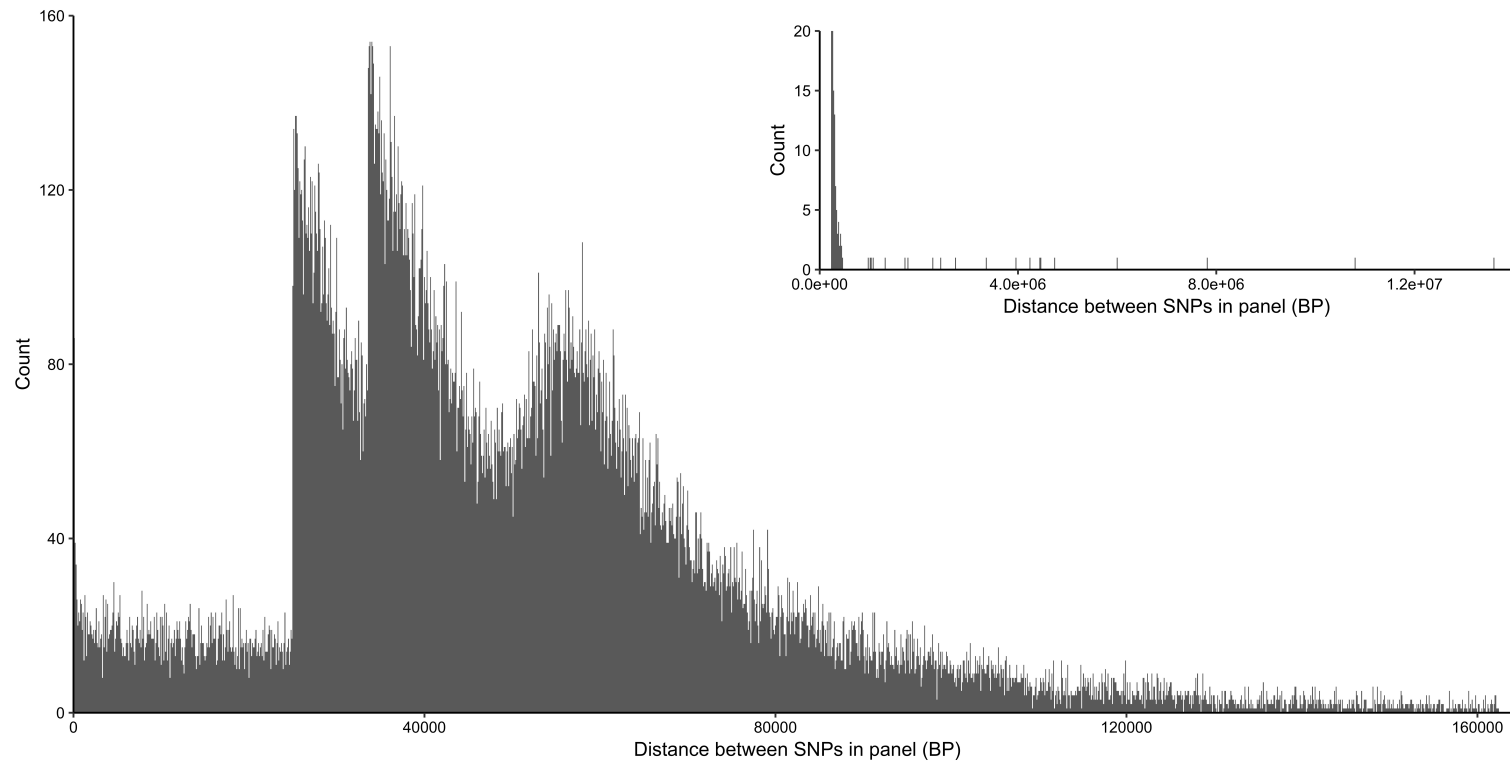
Figure 4-4 Frequencies of distances in base pairs between neighboring SNP loci in the 50,000 SNP assay designed for Rocky Mountain bighorn sheep. Main plot shows 99% of the data excluding the longest 1% of distances, insert (top right) shows all data.
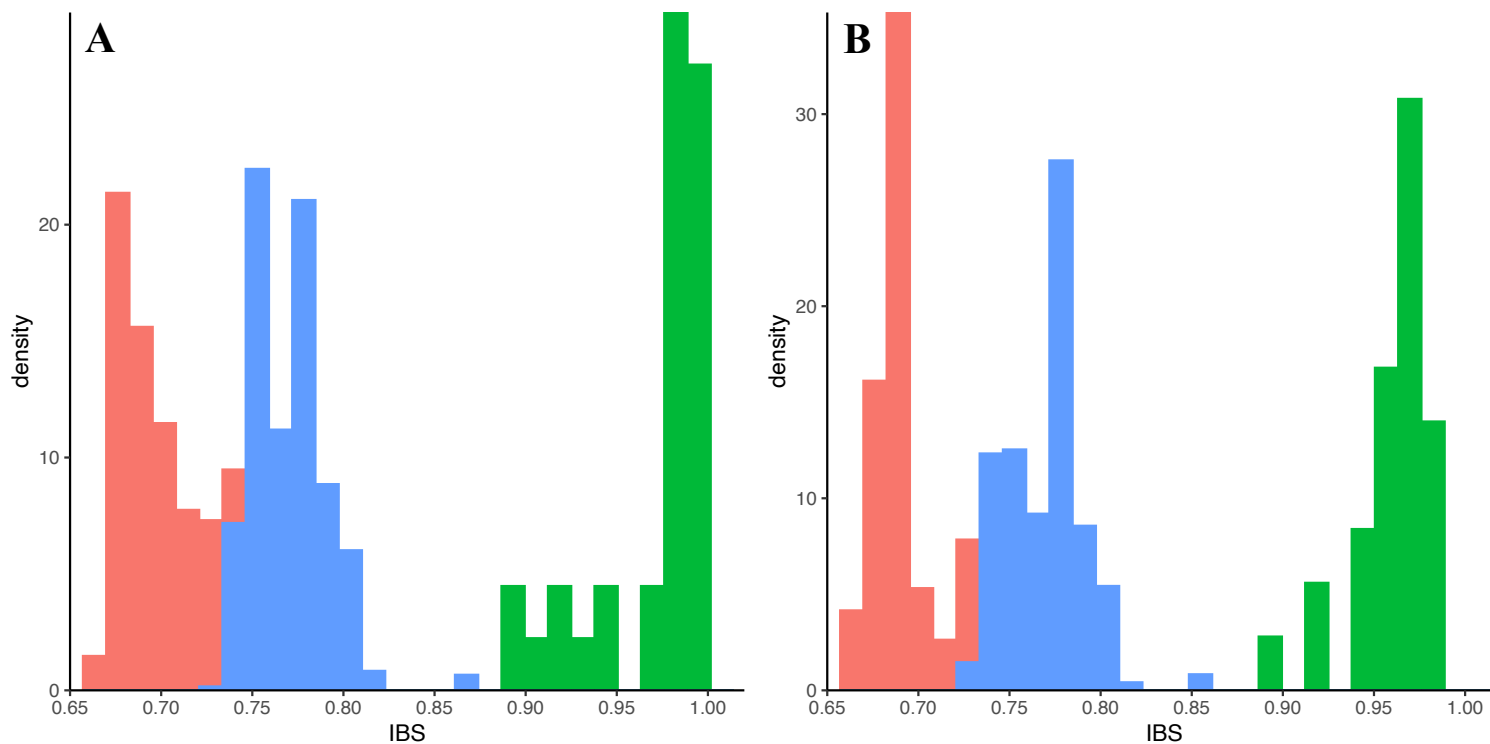
Figure 4-5 Distribution of values of identity by state (IBS) for our 10,000 SNP assay (assay A) and our 50,000 SNP assay (assay B) trialed on 40 Rocky Mountain bighorn sheep. Replicated sample IBS values shown in green, within sampling location IBS values shown in blue, and between sampling location IBS values shown in red.
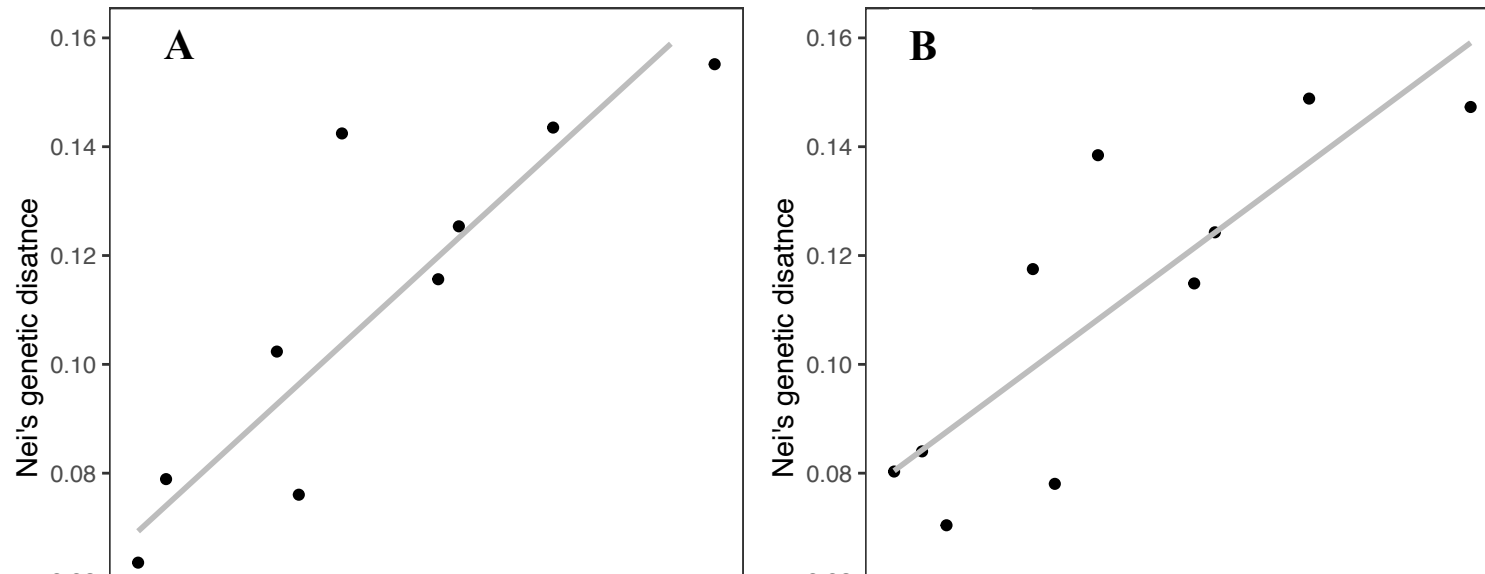
Figure 4-6 Patterns of isolation-by-distance for Nei's standard distance and geographic distance between the five Rocky Mountain bighorn sheep sampling locations genotyped by our 10,000 SNP assay (A) and 50,000 SNP assay (B).
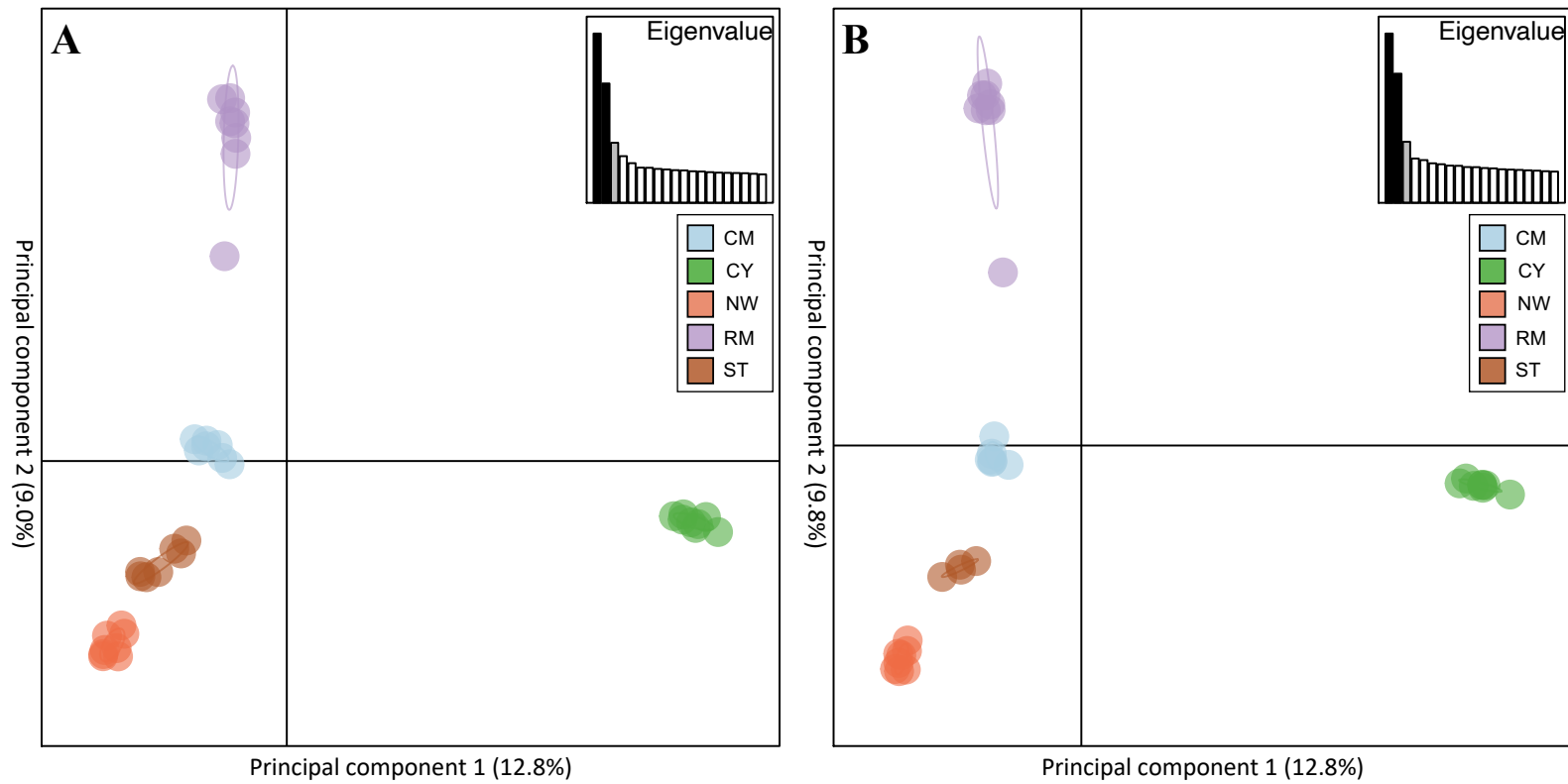
Figure 4-7 Principal component analysis of the five Rocky Mountain bighorn sheep sampling locations; Cadomin Mountain (CM), Castle Yarrow (CY), Narraway (NW), Ram Mountain (RM), Stornoway (ST), genotyped by our 10,000 loci SNP assay (A) and 50,000 loci SNP assay (B).

Chapter 5 - Quantitative trait loci and their response to anthropogenic selection in Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*)

5.1 Chapter summary

Genetic variance underpins variation in many traits. Thus, when selection acts upon a phenotype, an evolutionary response at loci associated with the trait may occur. In this study we set out to investigate the genetic basis of phenotype and examine how genomic regions we identified to be associated with phenotype responded to selective pressures. We performed genome-wide association studies (GWAS) using high density SNP genotypes for 305 Rocky Mountain bighorn sheep from the Ram Mountain, Alberta population to identify the genetic basis of six traits: male and female body mass, male and female horn length, female age at primiparity, female age at first weaning, female lifetime reproductive success, and female longevity. We identified 278 SNPs significantly associated with phenotype split relatively evenly between the eight traits. We further characterised eight SNP loci associated with male and female body mass, male and female horn length and female longevity. When examined over time, allele frequencies for five of these loci exhibited trend-shifts during differing temporal periods. The frequencies of an allele positively associated with male horn length at one of these loci declined from 1979 to 2001 under trophy hunting and then increased following the cessation of trophy hunting, suggesting this locus may have had an evolutionary response to selective harvest. This finding is consistent with the idea that intense trophy harvesting may cause evolutionary change in a population.

5.2 Introduction

Much phenotypic variation is underpinned by a genetic mechanism. Genotype-phenotype interactions can range from genotype being the sole determinant of phenotype, such as the case of melanistic colouration (Ritland, Newton and Marshall, 2001), or more complex interactions between genotype at single or multiple loci and environment influencing characteristics, such as breeding behaviour (Bourret and Garant, 2015). Understanding genotype-phenotype interactions is a central interest of conservation geneticists and genomicists (Stinchcombe and Hoekstra, 2008). From a conservation standpoint understanding the genotypes associated with certain phenotypes allows for a better understanding of how individuals, populations, and species are adapted to their environments at a genetic level, beyond the more apparent phenotypic level (Rudman *et al.*, 2018; Strickland *et al.*, 2022). Another area of conservation genetics that can be explored using genotype-phenotype association data is how selection influences allele frequencies at phenotype associated loci over time (Reid *et al.*, 2016; Campbell-Staton *et al.*, 2021; Salmón *et al.*, 2021).

The phenomena of phenotypic adaptation to different environments has been widely observed in many populations (Darwin, 1859; Williams and Moore, 1989; Campbell and Tishkoff, 2010). Typically, the rate of adaptive change in populations inhabiting stable environments is low. However, rapid shifts in phenotype have been observed in some wild populations as a result of strong selection caused by anthropogenic actions (Carlson *et al.*, 2007; Allendorf *et al.*, 2008; Allendorf and Hard, 2009; Chiyo, Obanda and Korir, 2015; Altermatt and Ebert, 2016) For example, during the industrial revolution, moth (*Leipdoptera*) populations in the UK experienced changes in the frequencies of their colour morphs; specifically, the darkening of moth habitats due to air pollution led to selection for melanism as an anti-predation adaptation (Kettlewell, 1961). Human induced selective forces that drive phenotypic changes can ultimately cause population level genetic changes at loci associated with traits under selection (Reid *et al.*, 2016; Campbell-Staton *et al.*, 2021; Salmón *et al.*, 2021).

One species in which these human induced evolutionary changes have been studied in is the Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*). In this species, in the absence of trophy hunting, both greater male horn length and greater body mass in both sexes are favoured by selection (Coltman *et al.*, 2002; Poissant *et al.*, 2008). Horn length is positively correlated with

male reproductive success (Coltman *et al.*, 2002). There is no correlation between horn length and female dominance (Favre, Martin and Festa-Bianchet, 2008), but females with longer horns do have greater reproductive fitness (Deakin *et al.*, 2022, Chapter 3). Body mass is correlated with reproductive success in males, as larger males have greater reproductive success (Coltman *et al.*, 2002), and mass is also positively correlated with female reproductive success (Poissant *et al.*, 2008; Deakin *et al.*, 2022)(Chapter 3). However, when subjected to trophy hunting under a harvest regime which imposes a minimum horn size for trophies, selective harvest selects against large horned males (Coltman *et al.*, 2003). This selection against large horned males has reduced male horn size and body mass over time (Coltman *et al.*, 2003; Pigeon *et al.*, 2016). Given the cross-sex heritability of these traits (Poissant *et al.*, 2012), hunting selection has indirectly reduced horn length in females (Pigeon *et al.*, 2016). These changes in female horns may have fitness consequences which extend beyond a reduction in horn size; as females with larger horns exhibit earlier age at primiparity and age at first weaning, and greater lifetime reproductive success (LRS)(Deakin *et al.*, 2022, Chapter 3), all of which are important traits for population growth.

Knowledge of the genetic architecture underlying traits in bighorn sheep is sparse and has so far only been examined for horn size and body mass (Poissant *et al.*, 2012; Kardos *et al.*, 2015; Miller, Festa-Bianchet and Coltman, 2018). Miller, Festa-Bianchet and Coltman (2018) identified a locus at OAR9_91647990 on the ninth chromosome which had a potential association with body mass. Poissant *et al.* (2012) located a quantitative trait locus on chromosome ten near the RXFP2 gene, which is known to affect horn morphology and size in feral domestic sheep *(Ovis aries)* populations(Johnston *et al.*, 2010, 2011). Kardos *et al.* (2015) identified a selective sweep at the RXFP2 gene in bighorn sheep, suggesting during the evolution of bighorn sheep sexual selection drove this region to fixation. In thinhorn sheep *(Ovis dalli),* two SNP loci, OAR2_43601714 and OAR3_134140997, located on the second and third chromosome, respectively, were found by Sim and Coltman (2019) to potentially be associated with horn length.

In this study we use our 50,000 single nucleotide polymorphism (SNP) loci assay designed in Chapter 4 to genotype 342 Rocky Mountain bighorn sheep from Ram Mountain, Alberta. Given overall efficiency of our genotyping panel at ~90% we expect to type ~45,000 SNPs for our 342 individuals selected for sequencing. In conjunction with these genotype data, we use 43 years of

longitudinal phenotypic data to investigate the genetic basis of both male and female body mass, male and female horn length, female age at primiparity, female age at first weaning, female LRS, and female longevity, as female longevity is associated with female LRS (Coltman *et al.*, 2005). Subsequently, we examine changes at loci of effect over time. The Ram Mountain bighorn sheep population was subject to trophy hunting until 1996. Thus, we expect to find the frequency of alleles associated with traits known to be selected against by trophy hunting; male body mass, male horn length and female horn length (Coltman *et al.*, 2003; Pigeon *et al.*, 2016), to decrease until ~1996. In the absence of trophy hunting after 1996, we expect these same alleles to increase in abundance, as the traits they are associated with are selected for under sexual selection. Additionally, due to the selection imposed on female horn length by trophy hunting (Pigeon *et al.*, 2016) and the association of female age at primiparity, age at weaning and LRS with female horn length (Chapter 3, Deakin *et al.* 2022), we expect to observe the same trend in frequencies for alleles associated with these female reproductive traits in the presence and absence of trophy harvest.

5.3 Methods

5.3.1 Study site/population

Ram Mountain, Alberta (52°8' N, 115°8' W, 1082-2173 meters above sea level), is a rocky outcrop encompassing ~38km^2 of alpine and sub-alpine terrain, ~30km east of the main Rocky Mountain range. The sheep population is geographically and genetically distant (Deakin *et al.*, 2020) from the main species range. Monitoring started in 1971 (Jorgenson *et al.*, 1993) and is ongoing. The Ram Mountain population was historically subjected to trophy hunting of males based on horn size (Pigeon *et al.*, 2016). In 1996, a more restrictive regulation was introduced, and a moratorium has stopped sport hunting since 2011. In an attempt to supplement and reduce inbreeding in the population a number of individuals from Cadomin, AB were transplanted into the Ram Mountain population during the mid to late 2000's (Poirier *et al.*, 2019).

5.3.2 Monitoring and trait data collection

Between late May and September, sheep were trapped in a corral baited with salt. At each capture, body mass was measured with a spring scale to the nearest 125g. Horn length and base circumference (cm) were measured using a flexible tape. At first capture, all individuals were

marked with ear tags and a tissue sample was taken for DNA analyses and pedigree construction. Reproductive status was determined by observing lactation at capture, or by observing mother-offspring interactions. Mother-offspring pairs were determined by behavioural observations and confirmed by a genetic pedigree constructed using 26 microsatellite markers (Coltman *et al.*, 2002; Poirier *et al.*, 2019). For this study we analyzed data from 1979-2022 for individuals from the 1979-2017 cohorts.

For all males and females, we calculated horn length (cm) and body mass (kg) adjusted to September 15th at two years. Horn length was adjusted to September 15th using growth rates calculated from repeated measures of the same individual in the same year. For individuals who were only captured once during their second year the average horn growth rate of their sex within the population was used to adjust their horn length. Body mass was adjusted using mixed-effect models (Martin and Pelletier, 2011). For the purpose of this chapter "adjusted body mass" and "adjusted horn length" refer to body mass and horn length adjusted to September 15th of year two. For females we recorded age at primiparity (years), age at first offspring weaned (years), LRS, and longevity (years). Age at primiparity was defined as the first occasion in which a female lactated (assessed by inspection of the udder during captures) and we defined weaning as rearing offspring to mid-September. Females were only given a trait value for either of these traits if they had experienced primiparity or weaning before the end of monitoring. LRS was defined as the number of lambs successfully weaned over the course of the female's lifetime. LRS was only examined for females who were dead at the end of monitoring and had complete reproductive histories. Female longevity was defined as the age at death and only females who were dead at the end of monitoring were included in our analysis. Additionally, using the constructed pedigree (Coltman *et al.*, 2002; Poirier *et al.*, 2019), we classified individuals based on two factors; the first being whether they were native to Ram Mountain or were Cadomin transplants, and the second being whether their genetic ancestry was pure Ram Mountain if they had full or partial Cadomin ancestry.

5.3.3 Sequencing and genotyping
We extracted DNA from tissue samples collected during captures using the DNeasy Blood and Tissue kit (Qiagen N.V., Venlo, the Netherlands), following the manufacturer's recommended procedure. Each sample was quantified and normalized to a concentration of 8ng/uL, resulting in

a total of 80ng of DNA processed per sample during library preparation. For the library preparation we used Allegro Targeted Genotyping kits from Tecan Genomics (Redwood City, United States). We followed the manufacturer's protocol provided by Tecan with a slightly increased fragmentation digest time of 22.5 minutes rather than 15 minutes. Following library preparation, we sequenced a total of 342 individuals from Ram Mountain in two libraries. We sequenced the first 304 of the 342 samples in a library containing 384 samples (the remaining 80 samples on this sequencing run were for a different study). This library was sequenced using an Illumina (San Diego, United States) NextSeq machine with a V2.5 high-output 300 cycle kit providing 400,000,000 two-directional 150bp reads. Thus, each sample had an average coverage of 20.83X (400,000,000 reads ÷ 50,000 loci ÷ 384 individuals = 20.83X coverage). The remaining 38 individuals and 58 repeats from the first sequencing run were sequenced in a library containing 288 samples. This second library was sequenced using an Illumina NextSeq machine and the same V2.5 high-output 300 cycle kit. Thus, each sample had an average coverage of 27.78X (400,000,000 reads ÷ 50,000 loci ÷ 288 individuals = 28X coverage).

The raw reads generated by these sequencing runs were analysed using FastQC v0.11.9 (Andrews, 2010). Raw reads were trimmed using the software TrimGalore v0.6.5 (Martin, 2011). The commands "—paired" and "—phred33" were used to instruct the program that paired reads and the phred33 quality scale were being used. The command "—adapter2" followed by adaptor sequence removed adaptors from the R2 read. The commands "—three_prime_clip_R1/R2 5" and "—quality 30" were used to trim five bp from the three-prime end of both the R1 and R2 reads, and trim anything with a quality less than 30. Subsequently, samples which were repeated were combined to maximize their number of reads and coverage; thus, reads were available for 342 individuals.

To expedite and reduce computational power required for alignment of these reads, prior to alignment we created a reduced version of the *Ovis aries* 3.1 genome (Jiang *et al.*, 2014).This reduced genome only included sequence data for a 400bp region, 200bp upstream, 200bp downstream of each of the 50,000 target loci. From this reduced genome we produced an index using the Bowtie2 v2.3.5.1 (Langmead and Salzberg, 2012) build function and the SAMtools v1.10 (Li *et al.*, 2009) command "faidx". Then both forward and reverse reads were aligned as pairs to

the index reference genome using the "—sensitive" pre-settings in Bowtie2. The SAMtools command "view" was then run with the reference index specified to convert the paired read alignments from un-indexed .sam files to indexed .bam files. Finally, the SAMtools command "sort" was used to sort the .bam files.

We subsequently filtered the variant site data generated by this variant calling. First, we removed insertion-deletion variants using the VCFtools v0.1.15 (Danecek *et al.*, 2011) command "—remove-indels". Then we removed loci with a Phred-33 quality score lower than 30 and a depth of less than 6 using BCFtools v1.10.2 (Li *et al.*, 2009). Subsequently loci with more than 30% missing data and loci with a minor allele frequency of less than 0.05 were removed using VCFtools. Individuals with more than 25% missing data were removed and our resulting dataset was re-filtered for minor allele frequency >0.05 using VCFtools to ensure all retained SNPs still had suitable minor allele frequencies. We then filtered to retain loci with no more than 10% missing data using VCFtools. Finally, we reduced this genotype dataset to only include individuals for which sufficient phenotypic data were available to perform analysis on at least one of our examined traits.

5.3.4 Statistical analysis

We used PLINK v1.90b6.9 (Purcell *et al.*, 2007) to calculate allele frequencies, observed heterozygosity, and expected heterozygosity for our filtered dataset. We then used the formula $f = (H_{exp} - H_{obs})/H_{exp}$ to calculate Wright's inbreeding coefficient (f) (Wright, 1922).To examine linkage disequilibrium, we used PLINK to calculate r^2 between syntenic markers on all chromosomes. From these values we calculate the linkage disequilibrium (LD) half-length (Reich *et al.*, 2001) for individuals in our dataset by taking the greatest inter loci distance for SNP pairs which had an r^2 of greater than half our maximum value of r^2.

To investigate the genetic basis of the eight traits we examined, we performed a series of genome-wide association surveys (GWAS) using the R v4.0.3 (R Core Team, 2013) package *statgenGWAS* v1.0.8 (van Rossum *et al.*, 2020).The GWAS were ran using an additive effect model, with homozygous reference allele, heterozygote, and homozygous alternative allele genotypes coded as 0,1, and 2, respectively. Prior to performing each of the eight GWAS, we reduced our genotype

datasets by only selecting individuals with values available for the focal trait and other required covariates for each GWAS. (See Table 5-1 for details of covariates in each analysis).

We generated kinship matrices for each of the eight genotype datasets using the "kinship" function in the R package *statgenGWAS* and the algorithm detailed in Astle and Balding (2009). Subsequently, to prepare our genotype datasets for GWAS analysis we removed loci or individuals missing more than 10% data, and then imputed random values for retained missing data using the "codeMarkers" function of *statgenGWAS*. Subsequently, we removed loci with a minor allele frequency of less than 0.05 after filtering an imputation using the "codeMarkers" function.

We performed single trait GWAS analyses on each trait using the *statgenGWAS* function "singletraitGWAS", while controlling for kinship and each series of covariates required for each of the traits we examined. Within the "singletraitGWAS" function we used Brzyski *et al.*'s (2017) false discovery rate algorithm to identify significant loci, which in short identifies SNPs representative of clusters of significant SNPs and assigns significance to them. R_{LR}^2 values, indicating phenotypic variance explained by each locus, was calculated according to Sun *et al.*, (2010)in the "singletraitGWAS" function. To display the output of each GWAS graphically QQ plots and Manhattan plots were generated using the plot function of the *statgenGWAS* package.

To examine the biological processes associated with genes situated within LD decay of SNPs significantly associated with a trait, we first identified genes with LD of our SNPs, we then examined which biological processes were enriched in this subset of genes when compared to genes from the rest of the *Ovis aries* 3.1 genome, and subsequently tested the significance of their enrichment. To do this we used the r package *GOfunR* v1.18.0 (Grote, 2018).

We selected focal SNP loci of large effect size and characterized their association with the phenotype. These SNPs were selected by examining Manhattan plots and the statistical output of the GWAS. Specifically, we first calculated the average phenotype for each genotype at the SNP and plotted this relationship using ggplot2 v3.3.5 (Wickham, 2016). Then, we performed a linear regression using the R function 'lm' to characterize the relationship between genotype and phenotype. In this linear regression we used an additive model with the value of the trait of interest

modeled as a function of the number of alleles positively associated with the trait value in each individual's genotype at that focal locus.

To examine allele frequency changes over time we calculated the allele frequencies of the focal loci in every cohort. For this we used all genotyped individuals rather than the subsets of individual genotypes used for examining each trait. We plotted the frequency of the allele which positively affected the associated trait over time using GGplot2. To visualise trends in these plots we fitted a smooth line using loess. To statistically test trends in allele frequencies at these loci over time, specifically if trends differed under different trophy hunting regimes, we fitted piecewise, or "broken stick", regressions. To do this we used the R package *segmented* v1.3.4 (Grote, 2018) and plotted the identified regressions using GGplot2. For models for which no break point (trend change) was identified, we fitted a linear regression using the R function 'lm'.

5.4 Results

5.4.1 Phenotypic data

Of our 342 sequenced individuals, 305 (132 males, 173 females) had phenotypic data to examine at least one of our traits of interest. Individual measurements ranged from 119 to 170 for each trait, for details and distributions of traits see Table 5-2 and Appendix D Table D1. Of our 305 individuals 14 were transplants and 46 had ancestry from the Cadomin population. We observed a significant association ($p <0.0001$) between longevity and LRS for 139 females, with longevity explaining 59.1% of variance in LRS (Table 5-3, Table D2).

5.4.2 Genotypic data

Of the 342 individuals sequenced, 338 yielded enough reads to proceed with trimming, alignment, and variant calling. Once trimmed and aligned, variant calling yielded a total of 1,365,560 variant sites for 338 individuals. When insertion/deletion variants were removed 1,018,039 SNPs remained. Removing loci with a quality score <30 reduced the dataset to 276,225 SNPs. Subsequently, filtering out loci with a depth <6 reduced the number of SNPs to 276,082. Removing loci with >30% missing data further reduced the dataset to 243,911 SNPs. Filtering out loci with a minor allele >0.05 reduced the dataset to 48,809 SNPs. Removing genotypes with >25% missing data reduced the number of individuals from 338 to 323. Re-filtering this 323-individual dataset

for loci with a minor allele frequency ≥0.05 reduced the number of SNPs to 48,762. Filtering out SNPs missing >10% of data retained 48,762 SNPs. Once we reduced the final genotype dataset to include only individuals for which we had sufficient phenotypic data to examine at least one trait, the dataset included genotypes for 305 individuals at 43,368 SNP loci. Thus, means the assay performed as expected, successfully interrogating genotypes at 43,368 SNP loci in 305 individuals, an efficiently of ~87% (comparable to the ~91% efficiency reported for the panel during its trials in Chapter 4).

We observed that loci had an average minor allele frequency of 25.6%, and the dataset had an observed heterozygosity of 34.9%, an expected heterozygosity of 34.7%, and Wright's inbreeding coefficient of -0.003. Analysis of LD generated 50,603,659 pairwise comparisons of r^2 values and from these values we found the Ram Mountain population to have a half-length, or LD decay, of 996,038 bp.

5.4.3 Genome wide association survey analysis

None of the eight traits we analysed had complete phenotypic and covariate datasets, thus we used a subset of the 305 individuals for analysis of each trait. All models were run on genotypes and phenotypes for 117-165 individuals and 42,428-43,090 SNP loci. For each model between 1.1% and 1.4% of genotypes were imputed across all individuals and loci. All models had genomic inflation factors of ~1 and identified between 26 and 46 SNPs associated with each trait with p-values ranging from 0.000015 to 0.0019. Heritability ranged from <1% to ~63%. From the Manhattan plots (Figures 5-1 to 5-8). we identified eight regions for five traits to further examine; regions associated with male adjusted body mass on chromosomes 1 and 20 (Figure 1), one region associated with female body mass on chromosome 2 (Figure 2), two regions associated with male adjusted horn length on chromosome 1 (Figure 3), one region associated with female horn length on chromosome 1 (Figure 4), and two regions associated with female longevity on chromosomes 7 and 17 (Figure 8). For further details of these GWAS and histograms of trait values see (Table 5-1, and Appendix D Figure D1).

5.4.4 Post-GWAS analysis

Each of our traits had between 8 and 971 known genes within LD of their respective significant SNPs. Between 7 and 232 biological processes were significantly enriched among each subset of genes for each trait. (Table 5-4). Six of the ~1200 biological processes significantly enriched around SNPs associated with our examined traits appeared to have functions which may be relevant to their associated trait; 4 biological processes were enriched around SNPs associated with male adjusted body mass, and male and female adjusted horn length each had a single enriched process around their associated SNPs. The 4 processes enriched around SNPs associated with male adjusted body mass were broadly involved in skeletal development or ossification. The process enriched around SNPs associated with male adjusted horn length was involved in ossification, as was the biological process enriched around SNPs for associated with female adjusted horn length (For further details of biological processes enriched for each trait see electronic material 2).

From the statistical output and visual examination of the Manhattan plots associated with each trait, we identified loci associated with phenotype to investigate further (See highlighted loci in Figures 5-9 to 5-13): two loci associated with adjusted male body mass, one locus associated with adjusted female body mass, two loci associated with adjusted male horn length, one locus associated with adjusted female horn length, and two loci associated with female longevity.

The two regions associated with male adjusted body mass were on chromosome one and twenty. There were five SNPs in the region between 49138872 and 69575994 bp on chromosome one associated with male adjusted body mass. For further analyses we focused on the SNP most centrally located in this region, OAR1_54952090 (Table 5-4 and Figure 5-9a). We observed a positive relationship between the number of A alleles at this locus and male adjusted body mass, which was significant when examined with linear regression ($r^2 = 0.11$, $p = 0.002$) (Figure 5-14a). There were three SNPs in the region between 25796186 and 26795935 bp on chromosome twenty associated with male adjusted body mass. For further analyses we focused on the SNP most significantly associated with male adjusted body mass in this region, OAR20_25796186 (Table 5-4, Figure 5-9b). We observed a positive relationship between the number of G alleles at this locus and male adjusted body mass, which was significant when examined with linear regression ($r^2 = 0.08$, $p = 0.004$) (Figure 5-14b).

The region associated with female adjusted body mass was on chromosomes two. There were four SNPs in the region between 105508317 and 162344074 bp associated with female adjusted horn body mass. The locus most significantly associated with female adjusted body mass in this region was OAR2_162344074 at 162344074 bp (Table 5-4 and Figure 5-10). We observed a positive relationship between the number of A alleles at this locus and female adjusted body mass, which was significant when examined with linear regression ($r^2 = 0.10$, $p < 0.001$) (Figure 5-14c).

There were two regions associated with male adjusted horn length on chromosome one. There were three SNPs in the region between 49138872 and 69575994 bp associated with male adjusted horn length. For further analyses we focused on the SNP most centrally located in this region, OAR1_58851106 (Table 5-5 and Figure 5-11a). We observed a positive relationship between the number of C alleles at this locus and male adjusted horn length, which was significant when examined with linear regression ($r^2 = 0.13$, $p < 0.001$) (Figure 5-14d). There were four SNPs in the region between 168744167 and 177484024 bp associated with male adjusted horn length. For further analyses we focused on the SNP most significantly associated with male adjusted horn length in this region, OAR1_171245188 (Table 5-5 and Figure 5-11b). We observed a positive relationship between the number of C alleles at this locus and male adjusted horn length, which was significant when examined with linear regression ($r^2 = 0.08$, $p = 0.002$) (Figure 5-14e).

The region associated with female adjusted horn length was on chromosomes one. There were seven SNPs in the region between 156561646 and 182631664 bp associated with female adjusted horn length. For further analyses we focused on the SNP most centrally located in this region, OAR1_167704342 at 167704342 bp associated with female horn length (Table 5-5 and Figure 5-12). We observed a positive relationship between the number of T alleles at this locus and female adjusted horn length, which was significant when examined with linear regression ($r^2 = 0.07$, $p = 0.008$) (Figure 5-14f).

There were two loci associated with female longevity. On chromosome seven there was one locus at 25168902 bp, OAR7_25168902, associated with female longevity (Table 5-6 and Figure 5-13a). We observed a positive relationship between the number of A alleles at this locus and female

longevity, which was significant when examined with linear regression ($r^2 = 0.10$, $p = 0.003$) (Figure 5-14g). On chromosome seventeen there were three SNPs in the region between 51486497 and 59277711 bp associated with female longevity. For further analyses we focused on the SNP most significantly associated with female longevity in this region, OAR17_55255290 (Table 5-6 and Figure 5-13b). We observed a positive relationship between the number of G alleles at this locus and female longevity, which was significant when examined with linear regression ($r^2 = 0.15$, $p < 0.001$) (Figure 5-14h).

We visualised temporal trends in allele frequency for the eight focal loci (Figures 5-15 and 5-16). From the piecewise regression we identified five loci within which allele frequencies exhibited differing linear trends over different time periods. Two loci, OAR1_167704342 and OAR7_25168902, exhibited a significant change in linear trend over time, these loci were associated with female adjusted horn length and female longevity, respectively (Table 5-7, Figures 15d and 15e). Three loci exhibited a near-significant change in linear trend over time; OAR1_54952090 and OAR20_25796186 which were associated with male adjusted body mass, and OAR1_58851106 which was associated with male adjusted horn length (Table 5-7, Figures 15a, 15b, and 15c). Piecewise regression could not identify a breakpoint in the temporal trends of allele frequencies at three of our eight loci. Therefore, we performed linear regression on these loci. None of these loci exhibited a significant linear trend (Appendix D Table D2 and Figure 5-16).

5.5 Discussion

We identified 278 SNPs of varying effect and significance that were associated with the eight traits: male and female adjusted body mass, male and female adjusted horn length, female age at primiparity, female age at first weaning, female LRS, and female longevity, using GWAS analyses on genotypes and phenotypes from over 300 bighorn sheep spanning 40 years at Ram Mountain, Alberta. These traits had a wide range of heritability values. Female longevity had the highest heritability at 63%, contrasting Coltman *et al.* (2005) who only found slight heritability for this trait which was not significantly different from zero. Male and female adjusted body mass had heritability values of 43% and 19% respectively, thus our estimate of male body mass is slightly higher and our estimate for female body mass is very similar to the most recent previous heritability

estimates for these traits (Poissant *et al.*, 2012). Male and female adjusted horn length had heritability values of 45% and 25% respectively, which align with the most recent heritability estimates for these traits (Pigeon *et al.*, 2016). Our heritability estimates of adjusted horn length and body mass indicate genetic variance influences phenotypic variation in these traits in males more than females, similar to the findings of Poissant *et al.* (2008, 2012) and Pigeon *et al.* (2016). Female age at primiparity, age at first weaning and LRS all had heritability values below 1%, thus aligning with the results of Coltman *et al.* (2005), and indicating these traits have little to no genetic basis. Twenty-two SNP loci were associated with more than one trait; eleven were associated with both male adjusted body mass and horn length, three were associated with both female adjusted body mass and horn length, and eight loci were associated with both female age at primiparity and female age at first weaning. These shared associations are likely due to the close relationship between these traits. None of our 278 significantly associated SNPs were found to be in regions previously known to affect any of the eight examined traits.

Of the eight loci we found significant genotype-phenotype interactions between: two loci and male adjusted body mass, one locus and female adjusted body mass, two loci and male adjusted horn length, one locus and female adjusted horn length, and two loci and female longevity. All eight of these loci exhibited patterns which suggested an additive model of effect. We observed changes in trend for allele frequencies over time at five of the focal eight loci. At two of these loci, one associated with male horn length and another associated with female longevity, alleles positively associated with each trait exhibited a decline under trophy hunting followed by an increasing trend shortly after the cessation of trophy hunting, similar to the patterns observed in breeding values for horn length by (Pigeon *et al.*, 2016)

5.5.1 Prior known associations

None of the SNPs significantly associated with adjusted body mass or horn were located in or within LD of loci or regions previously identified to be associated with similar traits in wild (Poissant *et al.*, 2012; Miller, Festa-Bianchet and Coltman, 2018; Sim and Coltman, 2019) or feral sheep (Johnston *et al.*, 2010, 2011). Miller, Festa-Bianchet and Coltman (2018) identified OAR9_91647990 to be potentially associated with body mass. Our closest genotyped SNP to this locus was 13,855 bp away and a further 41 SNPs were within LD of OAR9_91647990. However,

we found none of these 42 SNPs to be associated with adjusted body mass. Furthermore, there were no significantly associated SNPs on the entire of chromosome nine. Evidently, these results are non-corroborating, this could be due to differences between the two studies. Firstly, (Miller, Festa-Bianchet and Coltman, 2018) had a smaller sample size of 76 individuals and 3,777 SNPs rather than our 117 male and 154 female individuals and ~43,000 SNPs. Secondly, and perhaps more importantly, (Miller, Festa-Bianchet and Coltman, 2018) used a repeated measures method which factored in body mass at every age available for each individual included in their GWAS whereas we used body mass fixed at September 15th of year two. Therefore, suggesting the SNP loci identified by (Miller, Festa-Bianchet and Coltman, 2018) may be associated with mass gain in later life or over the course of the lifetime, whereas our GWAS only tested for association with early adult weight.

The RXFP2 gene and its surrounding region has been identified to be under selection (Kardos *et al.*, 2015) and associated with horn morphology in ovine species (Johnston *et al.*, 2010, 2011; Poissant *et al.*, 2012). None of our SNP loci were located directly within the RXFP2 gene. However, forty loci were within LD of this gene. None of the forty loci within LD of RXFP2 were significantly associated with adjusted horn length in either sex. A likely explanation for the lack of associations in this region comes from Kardos *et al.* (2015) who found the region around RXFP2 has been subject of a selective sweep likely as a result of sexual selection on male horn length. Our results are partially align with the findings of Kardos *et al.* (2015) as we found no SNPs explaining the variance horn length in either sex in this region. However, when we compare the expected heterozygosity of SNPs within LD of RXFP2 to SNPs from the entire of chromosome ten we find a 0.99% increase around RXFP2 suggesting an increase rather than decrease in genetic variance in this region. However, this is likely due to ascertainment bias when selecting SNPs for our assay, as we actively selected for variant loci (Chapter 4)

Finally, Sim and Coltman (2019) identified two SNPs; OAR2_43601714 and OAR3_134140997 to be potentially associated with horn length in thinhorn sheep. Our closest SNPs to OAR2_43601714 and OAR3_134140997 were 26,406 bp and 6,413 bp away, respectively, and there were 29 loci and 32 loci within LD of the two SNPs, respectively. None of these SNPs were

also associated with horn length in our population, likely as a result of species differences or population differences, or due to loci identified by prior analyses being false positives.

5.5.2 Gene ontology

Most biological processes enriched around SNPs significantly associated with our examined traits did not exhibit processes or mechanisms by which they obviously effected the examined trait. However, several enriched biological processes stood out as potentially biologically relevant. Three processes involved in skeletal development and one associated with ossification were enriched in genes within LD decay of the 35 SNPs associated with male adjusted body mass. Thus, suggesting these SNPs are associated with body mass due to their association with genes involved in bone growth and skeletal system development. A total of 13 genes responsible for these enriched biological processes were within LD decay of the 35 SNP loci associated with male adjusted body mass. TWSG1 is exclusively involved with ossification, HOXB1, HOXB2, HOXB3, HOXB5, HOXB6, HOXB7, HOXB8, HOXB9, HOXC6, and TEAD4 are exclusively involved with skeletal development, and COL1A1, SP3, and TWSG1 are involved with both ossification and skeletal development. One biological process involved in ossification was enriched in genes around the 33 SNPs associated with male adjusted horn length. Thus, suggesting these SNPs are associated with male horn length due to their association with genes involved in bone growth. One gene, RUNX2, was responsible for the ossifying biological process which was enriched within LD decay of the 33 SNP loci associated with male adjusted horn length. Another biological process involved in ossification was enriched in genes around the 37 SNPs associated with female adjusted horn length. Thus, suggesting these SNPs are associated with female horn length due to their association with genes involved in bone growth. Two genes, ADAMTS7 and ADAMTS12, were responsible for the ossifying biological process which were enriched within LD decay of the 37 SNP loci associated with female adjusted horn length.

5.5.3 Focal loci

Of the 278 SNP loci significantly associated with traits, we selected eight for further characterisation their genotype – phenotype interactions. Our GWAS analysis identified all these loci to be of additive genetic effect. Two of these loci, OAR1_54952090 an G/A transversion and OAR20_25796186 a C/G transition located on chromosome one and twenty, respectively, were

associated with male adjusted body mass. A male homozygous for the A allele at OAR1_54952090 is expected to be 3.3 kg heavier than a male homozygous for G, and a male homozygous for the G allele at OAR20_25796186 is expected to be 3.8 kg heavier than a male homozygous for C. We only characterised one locus associated with female adjusted body mass, OAR2_162344074 a A/C transversion located on chromosome two. A female homozygous for the A allele at this locus is expected to be 1.0 kg heavier than a female homozygous for C.

Three of our focal loci were associated with adjusted horn length. Two of these loci, OAR1_58851106 an T/C transversion and OAR1_171245188 another T/C transversion both located on chromosome one, were associated with male adjusted horn length. A male homozygous for the C allele at OAR1_58851106 is expected to have 3.1 cm longer horns than a male homozygous for T, and a male homozygous for the C allele at OAR1_171245188 is expected to have 2.8 cm longer horns than a male homozygous for T. We only characterised one locus associated with female adjusted horn length, OAR1_1627704342, a A/T transition located on chromosome one. At this locus a female homozygous for the A allele is expected to have 1.3 cm longer horns than a female homozygous for T.

Two of our focal loci were associated with female longevity, OAR7_25168902 an A/G transversion located on chromosome seven and OAR17_55255290 another A/G transversion located on chromosome 17. A female homozygous for the A allele at OAR7_25168902 is expected to live 1.3 years longer than a female homozygous for G. Whereas, the effect at OAR17_55255290 is much stronger, with a female homozygous for the G allele at this locus being expected to live 4.3 years longer than a female homozygous for A. Further significance is given to these association between genotype and longevity, given the association between longevity and LRS (Coltman *et al.*, 2005). Thus, the effect of alleles at these loci have influence which extend beyond individual longevity and effect female reproductive fitness, which consequently could influence overall population fitness and growth.

5.5.4 Changes at loci over time

As all phenotype has a genetic basis, we expect selection acting upon a particular trait to alter the underlying genetic basis of this trait (Allendorf *et al.*, 2008; Reid *et al.*, 2016; Salmón *et al.*, 2021).

In the Ram Mountain population of bighorn sheep trophy hunting selected for lower male body mass and horn size until the cessation of trophy hunting in 1996 (Coltman *et al.*, 2003; Pigeon *et al.*, 2016). Thus, we expect the frequencies of alleles positively associated with male adjusted body mass and horn size in both sexes to decrease up until 1996. After 1996 we expect the frequencies of these alleles to increase due to the fitness benefits conferred by them in intrasexual competition in the absence of selective harvest.

Alleles at the two loci, OAR1_54952090 and OAR20_25796186, associated with male body mass showed differing patterns both of which were near-significant with ($p = 0.09$ and $p = 0.10$, respectively). Alleles positively associated with body mass at OAR1_54952090 sharply declined in frequency from 1979 to 1984 and then gradually increased until 2017. Whereas alleles positively associated with body mass at OAR20_25796186 increased until 2006 and then declined until 2017. Neither of these patterns support our hypothesis that under trophy hunting alleles positively associated with body mass would decline and then increase with the cessation of selective harvest. Although male body mass and horn length have a genetic correlation (Poissant *et al.*, 2012), body mass is not a directly targeted trait of trophy hunting, thus selection on this trait and these loci may not be strong enough be detected in allele frequency changes over time.

Alleles positively associated with male adjusted horn length at OAR1_58851106 declined from 1979 to 2001 and then increased until 2017, this trend was near-significant ($p = 0.06$). This pattern is what we expected to see under the two different selective pressures in this population and is similar to those observed for the breeding values of horn length identified by Pigeon *et al.* (2016). This indicates that under trophy hunting, selection against large horned males depleted the alleles associated with horn length at this locus and then with the cessation of trophy hunting sexual selection selected for male horn length, therefore increasing the frequency of the alleles at this locus. Thus, suggesting the selection imposed by trophy hunting was sufficiently intense to potentially induce rapid evolutionary change in horn length in this population. Thus, further highlighting how selective harvest can cause evolutionary change in the desirable trait (Allendorf and Hard, 2009; Campbell-Staton *et al.*, 2021).

We observed the frequency of alleles positively associated with female body mass at the locus OAR1_167704342, to decline from 1979 to1984 and then increase until 2017 (*p* = 0.04). The mechanism behind this trend is unknown but indicates that the population may have greater capacity to produce heavier females towards the end of monitoring than it did in 1984. Additionally, alleles positively associated with female longevity at OAR7_25168902 declined from 1979 to 2005 and then increased until 2017, this trend was significant (*p* = 0.04). Again, the mechanism behind this trend is unknown but indicates that the population may have greater capacity to produce longer lived females towards the end of monitoring than it did in 2005. Finally, other loci which we examined were OAR2_162344074, OAR1_171245188, and OAR17_55255290 associated with female adjusted body mass, male adjusted horn length, and female longevity, however these loci showed no significant trend over time.

5.5.5 Conclusion

In conclusion high density genotyping of individuals from cohorts spanning ~40 years from the Ram Mountain, AB population identified 278 SNPs associated with phenotype in our studied traits. From examining the genes and their associated biological processes enriched within LD decay of these 278 SNPs we identified 16 genes responsible for six biological processes which potentially effect male body mass and male and female horn length. Of these 278 loci we selected a subset to further examine based visual examinations of Manhattan plots and statistical significance, eight of which were found to be significant when further examining genotype phenotype interactions. We identified changes in allele frequencies at one locus of effect which suggested selective harvest was having an evolutionary effect on male horn length at this locus. We did not observe changes in allele frequencies over time which suggested trophy harvesting was having an evolutionary effect on male body mass or female horn length, likely due to human mediated selection being less intense on these traits. This, study adds to the sparse literature available on GWAS studies in wild populations and has added significant data on quantitative trait loci for its focal species which open up further areas of study. We were fortunate enough to have DNA samples from individuals spanning ~40 years which enabled us to investigate adaptation at the molecular level over time, in a relatively consistent environment, and while under stark differences in selective pressure.

5.6 Bibliography

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Table 5-1 Characteristics and results of GWAS analysis performed on each trait. Including: covariates included in analysis; number of Rocky Mountain bighorn sheep from Ram Mountains, Alberta, Canada for which genotype, focal trait data, and covariate data were available; individuals retained as they had less than 10% missing data; SNPs retained as they had less than 10% missing data and minor allele frequency greater than 5%; percentage of imputed alleles across all individuals and loci; genomic control inflation-factor (GCIF); number of SNPs identified as being significantly associated with phenotype using Bryski *et al.*'s (2017) false discovery rate algorithm; and the heritability of the trait (%).

Trait examined in GWAS	Covariates	Individuals available	Individuals retained	SNPs retained	Imputed alleles (%)	GCIF	Significant SNPs	Heritability (%)
Male adjusted body mass	Cohort, source population, genetic ancestry	119	117	42,428	1.4	1.003	35	43.1
Female adjusted body mass	Cohort, source population, genetic ancestry	170	165	43,090	1.2	0.976	46	18.6
Male adjusted horn length	Cohort, source population, genetic ancestry, adjusted body mass	119	117	42,428	1.4	1.017	33	45.0
Female adjusted horn length	Cohort, source population, genetic ancestry, adjusted body mass	159	154	43,057	1.2	1.009	37	25.4

Female age at primiparity	Cohort, source population, genetic ancestry, adjusted body mass	142	139	42,934	1.1	0.944	32	<1
Female age at first weaning	Cohort, source population, genetic ancestry, adjusted body mass	135	132	42,960	1.2	0.946	31	<1
Female LRS	Cohort, source population, genetic ancestry, adjusted body mass	136	133	42,983	1.2	0.888	26	<1
Female longevity	Cohort, source population, genetic ancestry, adjusted body mass	137	134	42,986	1.2	1.012	38	62.8

Table 5-2 Phenotypic data for our 305 Rocky Mountain bighorn sheep (132 males, 173 females) from Ram Mountain, Alberta, Canada. Information included is trait, number of individual measurements, range of measurements, and average measurement with standard deviation where applicable (* indicates median value was calculated rather than mean).

Trait	Number of individuals	Range	Average
Male adjusted body mass	119	44.8 – 84.5 kg	67.2 ± 7.5 kg
Female adjusted body mass	170	41.0 – 70.0 kg	57.0 ± 5.6 kg
Male adjusted horn length	132	18.7 – 49.0 cm	36.8 ± 5.4 cm
Female adjusted horn length	162	11.8 – 28.6 cm	18.3 ± 2.8 cm
Female age at primiparity	145	2 – 7 years	3 years*
Female age at first weaning	138	2 – 8 years	4 years*
Female LRS	139	0 – 10 lambs	3 lambs*
Female longevity	134	2 – 27 years	7.8 ± 3.7 years

Table 5-3 Number of genes located and processes enriched within LD decay of each of the focal SNPs identified for traits studied in Rocky Mountain bighorn sheep, at Ram Mountain, Alberta, Canada.

Trait	Number of genes in LD decay of significantly associated SNPs	Number of enriched biological processes
Male body mass	536	147
Female body mass	14	224
Male horn length	971	24
Female horn length	842	213
Female age at primiparity	792	171
Female age at first weaning success	629	232
Female LRS	556	174
Female longevity	8	7

Table 5-4 Information on further characterised loci of association with male and female adjusted body mass for Rocky Mountain bighorn sheep, at Ram Mountain, Alberta, Canada. Including: trait, loci name, chromosome (Chr), position on chromosome (bp), the effect the SNP has on phenotype (values given as increase in body mass (kg) expected for an individual homozygous for the positively associated allele), with *p*-value, genotypes present, number of genotypes, and adjusted body mass with standard deviation (kg).

Trait	Locus	Chr	Position (bp)	effect	*p*	Genotype	Number of genotypes	Average adjusted body mass (kg)
Male adjusted body mass	OAR1_54952090	1	54952090	3.3	0.0004	G-G	26	63.1 ± 8.9
						G-A	50	68.2 ± 6.5
						A-A	39	69.2 ± 6.2
Male adjusted body mass	OAR20_ 25796186	20	25796186	3.8	0.0006	C-C	57	65.3 ± 7.2
						C-G	43	70.2 ± 6.7
						G-G	6	68.5 ± 5.9
Female adjusted body mass	OAR2_162344074	2	162344074	1.0	<0.0001	C-C	1	52.4
						A-C	36	53.9 ± 5.4
						A-A	128	58.0 ± 5.2

Table 5-5 Information on further characterised loci of association with male and female adjusted horn length for Rocky Mountain bighorn sheep, at Ram Mountain, Alberta, Canada. Including: trait, loci name, chromosome (Chr), position on chromosome (bp), the effect the SNP has on phenotype (values given as increase in horn length (cm) expected for an individual homozygous for the positively associated allele), with *p*-value, genotypes present, number of genotypes, and adjusted horn length with standard deviation (cm).

Trait	Locus	Chr	Position (bp)	effect	*p*	Genotype	Number of genotypes	Average adjusted horn length (cm)
Male adjusted horn length	OAR1_58851106	1	58851106	3.1	0.0004	T-T	82	35.1 ± 5.0
						T-C	27	40.0 ± 4.8
						C-C	5	40.0 ± 5.6
Male adjusted horn length	OAR1_171245188	1	171245188	2.8	0.0001	T-T	10	32.3 ± 5.9
						T-C	51	36.0 ± 4.7
						C-C	53	37.8 ± 5.5
Female adjusted horn length	OAR1_167704342	1	167704342	1.3	0.0010	A-A	9	17.6 ± 3.5
						A-T	56	18.5 ± 2.6
						T-T	87	19.2 ± 2.7

Table 5-6 Information on further characterised loci of association with female longevity for Rocky Mountain bighorn sheep, at Ram Mountain, Alberta, Canada. Including: trait, loci name, chromosome (Chr), position on chromosome (bp), the effect the SNP has on phenotype (values given as increase in longevity (years) expected for an individual homozygous for the positively associated allele), *p*-value, genotypes present, number of genotypes, and female longevity with standard deviation (years).

Trait	Locus	Chr	Position (bp)	effect	*p*	Genotype	Number of genotypes	Average longevity (years)
Female longevity	OAR7_25168902	7	25168902	1.3	<0.0001	G-G	26	5.8 ± 2.9
						A-G	100	7.9 ± 3.7
						A-A	5	12.0 ± 3.5
Female longevity	OAR17_55255290	17	55255290	4.3	<0.0001	A-A	22	5.7 ± 3.3
						A-G	79	7.4 ± 3.6
						G-G	33	10.1 ± 3.5

Table 5-7 Details of piecewise regressions examining the frequencies of alleles positively associated with traits in cohorts of Rocky Mountain bighorn sheep from 1979 – 2017 from the Ram Mountain, Alberta population. Data including trait, loci name, the breakpoint (year) identified by the model, intercept, coefficient and its standard error prior to the breakpoint, coefficient and its standard error after the breakpoint, and *p*-value.

Trait	Locus	Breakpoint	Intercept	Coefficient (se) prior to break point	Coefficient (se) after break point	*p*	r^2
Male adjusted body mass	OAR1_54952090	1984	195.5	-0.0982 (0.057)	0.0971 (0.057)	0.092	0.218
Male adjusted body mass	OAR20_25796186	2006	-12.3	0.0063 (0.004)	-0.0154 (0.013)	0.100	0.104
Male adjusted horn length	OAR1_58851106	2001	18.9	-0.0094 (0.005)	0.0226 (0.009)	0.060	0.165
Female adjusted horn length	OAR1_167704342	1984	138.4	-0.0694 (0.032)	0.0743 (0.032)	0.035	0.252
Female longevity	OAR7_25168902	2005	13.5	-0.0066 (0.003)	0.0235 (0.009)	0.037	0.213
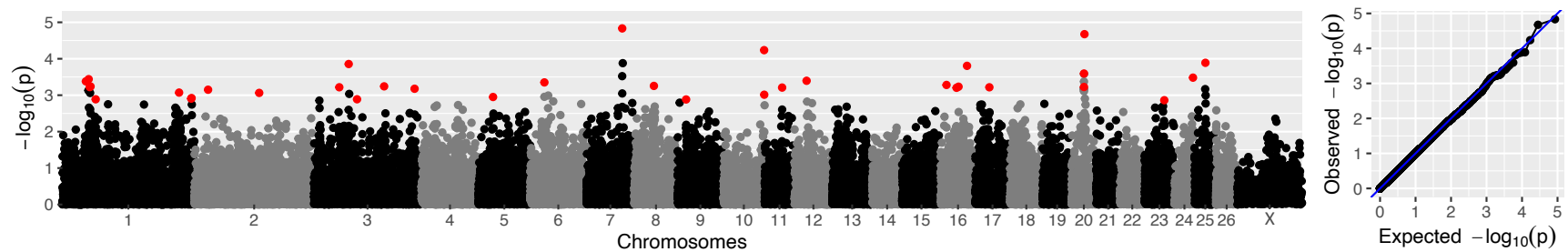
Figure 5-1 Significance of associations between SNP loci and male body mass adjusted to September 15th at age two. Manhattan plot (left) and corresponding Q-Q plot (right) of 42,428 SNP loci and the significance of their association with adjusted body mass at age two for males (n = 117) from the 1979 – 2017 cohorts in the Ram Mountain, Alberta population. Loci significantly associated with adjusted body mass at age two (n = 35) are shown in red. Blue line on Q-Q plot indicates a 1:1 correspondence.
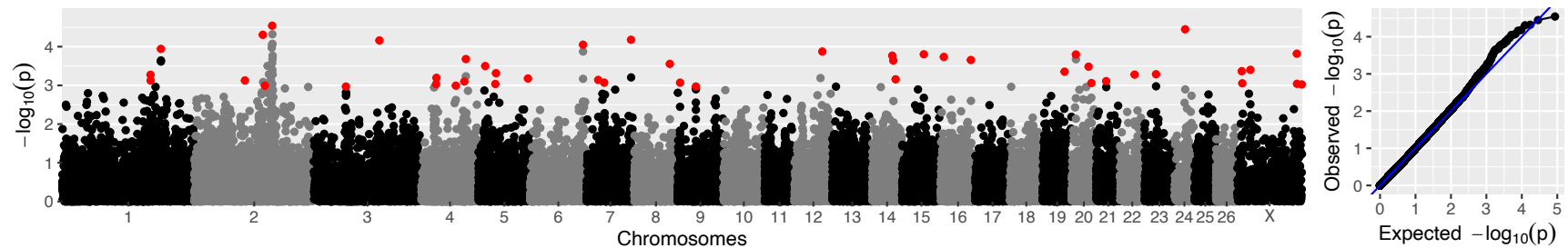
Figure 5-2 Significance of associations between SNP loci and female body mass adjusted to September 15th at age two. Manhattan plot (left) and corresponding Q-Q plot (right) of 43,090 SNP loci and the significance of their association with adjusted body mass at age two for females (n = 165) from the 1979 – 2017 cohorts in the Ram Mountain, Alberta population. Loci significantly associated with adjusted body mass at age two (n = 46) are shown in red. Blue line on Q-Q plot indicates a 1:1 correspondence.
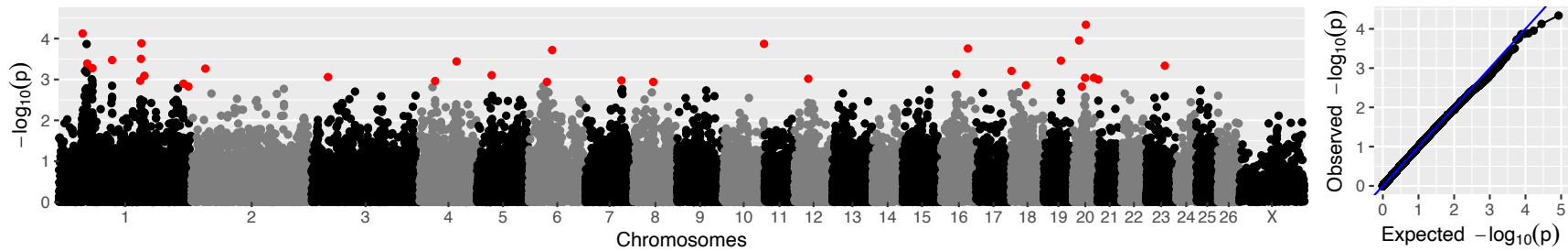
Figure 5-3 Significance of associations between SNP loci and male horn length adjusted to September 15th at age two. Manhattan plot (left) and corresponding Q-Q plot (right) of 42,428 SNP loci and the significance of their association with adjusted horn length at age two for males (n = 117) from the 1979 – 2017 cohorts in the Ram Mountain, Alberta population. Loci significantly associated with adjusted horn length at age two (n = 33) are shown in red. Blue line on Q-Q plot indicates a 1:1 correspondence.
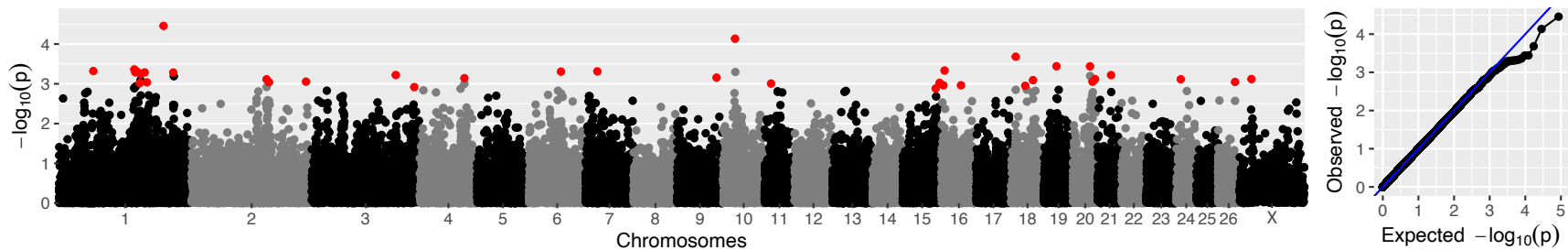
Figure 5-4 Significance of associations between SNP loci and female horn length adjusted to September 15th at age two. Manhattan plot (left) and corresponding Q-Q plot (right) of 43,057 SNP loci and the significance of their association with adjusted horn length at age two for females (n = 154) from the 1979 – 2017 cohorts in the Ram Mountain, Alberta population. Loci significantly associated with adjusted horn length at age two (n = 37) are shown in red. Blue line on Q-Q plot indicates a 1:1 correspondence.
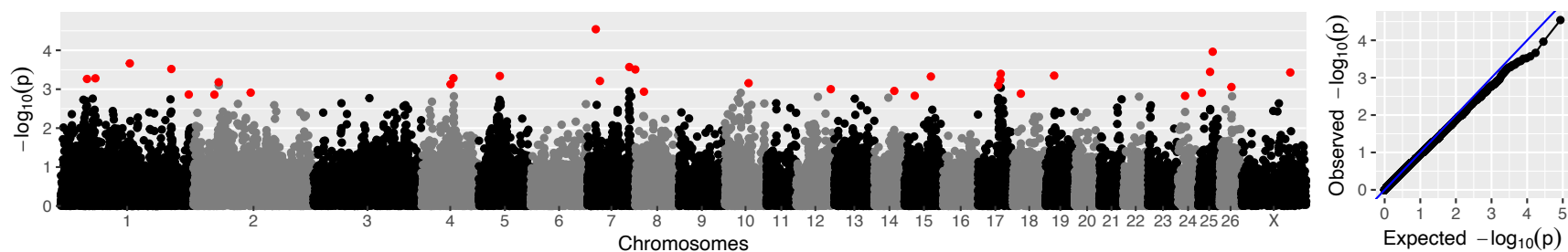
Figure 5-5 Significance of associations between SNP loci and female age at primiparity. Manhattan plot (left) and corresponding Q-Q plot (right) of 42,934 SNP loci and the significance of their association with female age at primiparity for females (n = 139) from the 1979 – 2017 cohorts in the Ram Mountain, Alberta population. Loci significantly associated with female age at primiparity (n = 32) are shown in red. Blue line on Q-Q plot indicates a 1:1 correspondence.
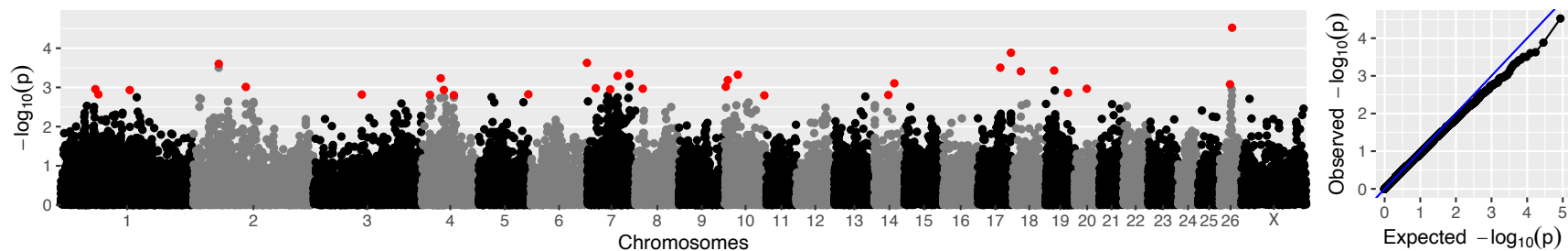
Figure 5-6 Significance of associations between SNP loci and female age at first weaning success. Manhattan plot (left) and corresponding Q-Q plot (right) of 42,960 SNP loci and the significance of their association with female age at first weaning success for females (n = 132) from the 1979 – 2017 cohorts in the Ram Mountain, Alberta population. Loci significantly associated with female age at first weaning success (n = 31) are shown in red. Blue line on Q-Q plot indicates a 1:1 correspondence.
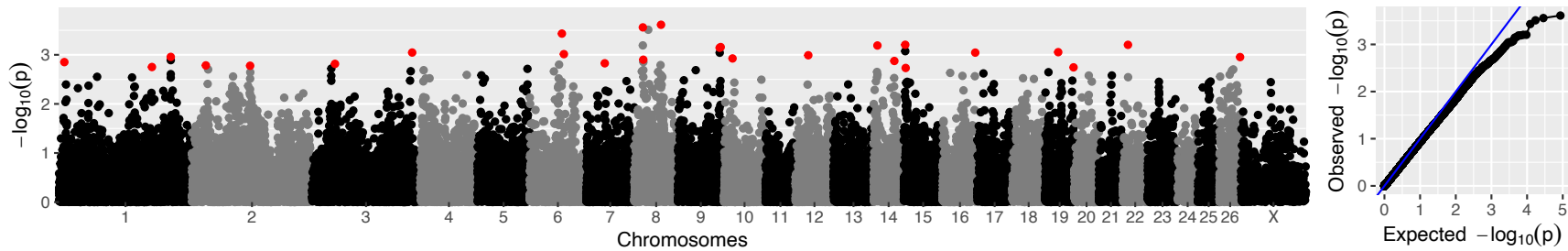
Figure 5-7 Significance of associations between SNP loci and female lifetime reproductive success. Manhattan plot (left) and corresponding Q-Q plot (right) of 42,983 SNP loci and the significance of their association with female lifetime reproductive success for females (n = 132) from the 1979 – 2017 cohorts in the Ram Mountain, Alberta population. Loci significantly associated with female lifetime reproductive success (n = 26) are shown in red. Blue line on Q-Q plot indicates a 1:1 correspondence.
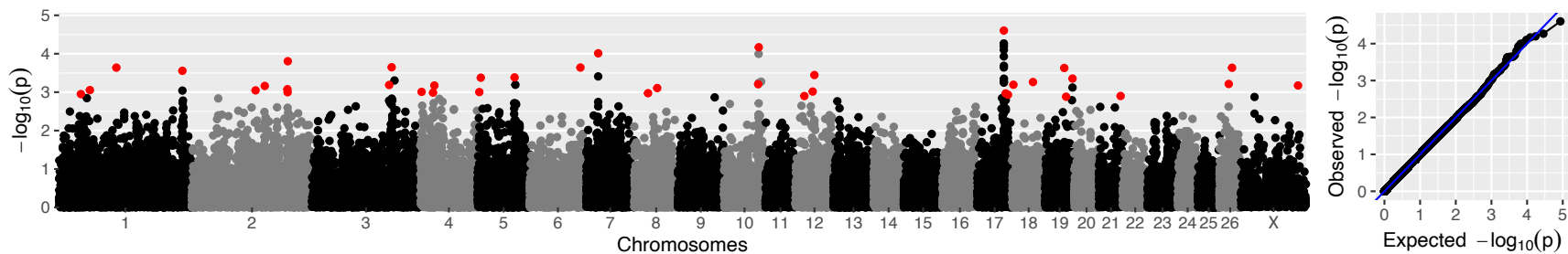
Figure 5-8 Significance of associations between SNP loci and female longevity. Manhattan plot (left) and corresponding Q-Q plot (right) of 42,986 SNP loci and the significance of their association with female longevity for females (n = 134) from the 1979 – 2017 cohorts in the Ram Mountain, Alberta population. Loci significantly associated with female longevity (n = 38) are shown in red. Blue line on Q-Q plot indicates a 1:1 correspondence.
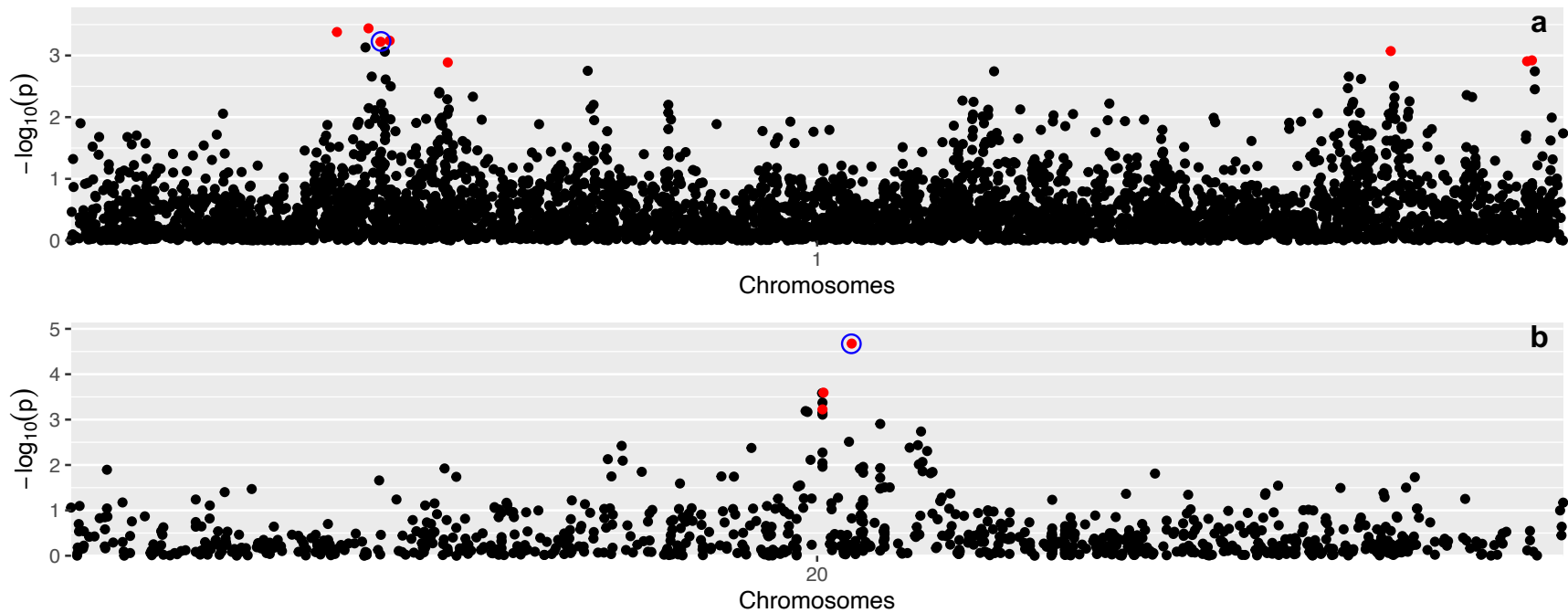
Figure 5-9 Significance of associations between SNP loci located on chromosome 1 (a) and 20 (b), and adjusted male body mass for males (n = 117) from the 1979 – 2017 cohorts in the Ram Mountain, Alberta population. Loci significantly associated with adjusted horn length are shown in red. Focal loci OAR1_54952090 and OAR20_25796186 circled in blue.
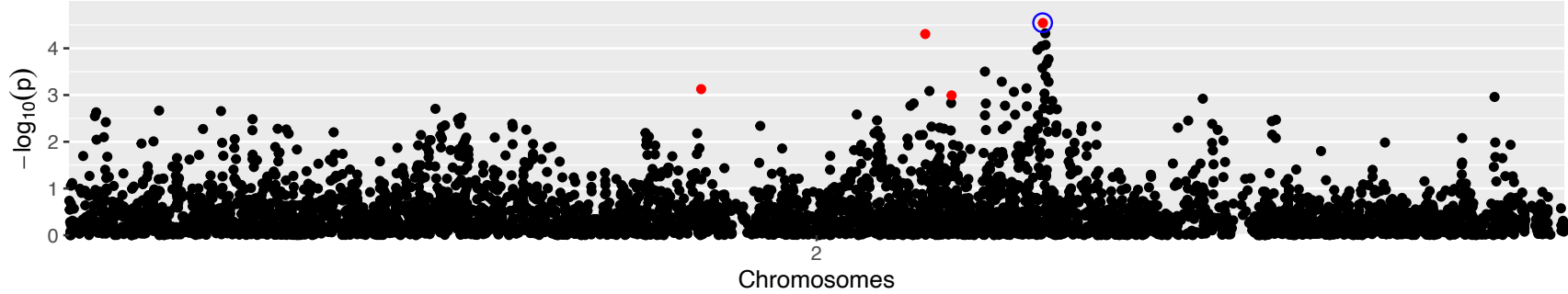
Figure 5-10 Significance of associations between SNP loci located on chromosome 2 and adjusted female body mass for females (n = 165) from the 1979 – 2017 cohorts in the Ram Mountain, Alberta population. Loci significantly associated with adjusted horn length are shown in red. Focal locus OAR2_162344074 circled in blue.
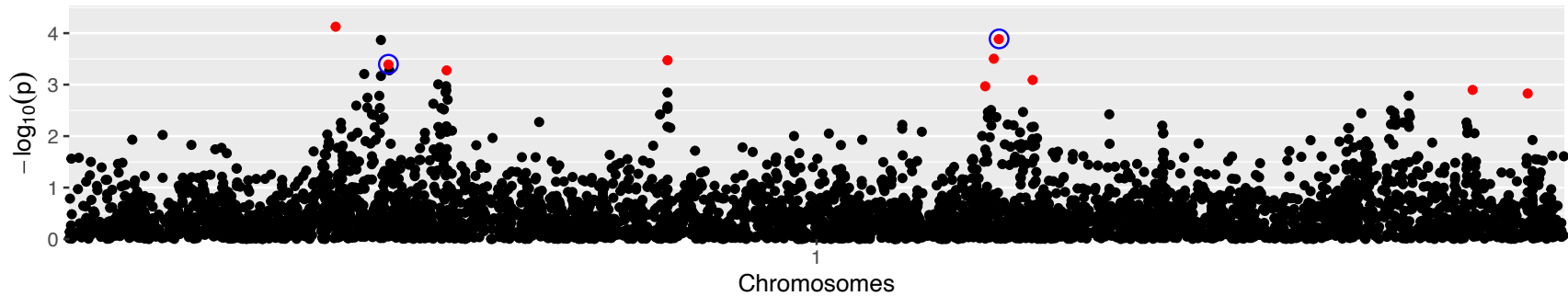
Figure 5-11 Significance of associations between SNP loci located on chromosome 1 and male adjusted horn length for males (n = 117) from the 1979 – 2017 cohorts in the Ram Mountain, Alberta population. Loci significantly associated with adjusted horn length are shown in red. Focal loci OAR1_58851106 and OAR1_171245188 circled in blue.
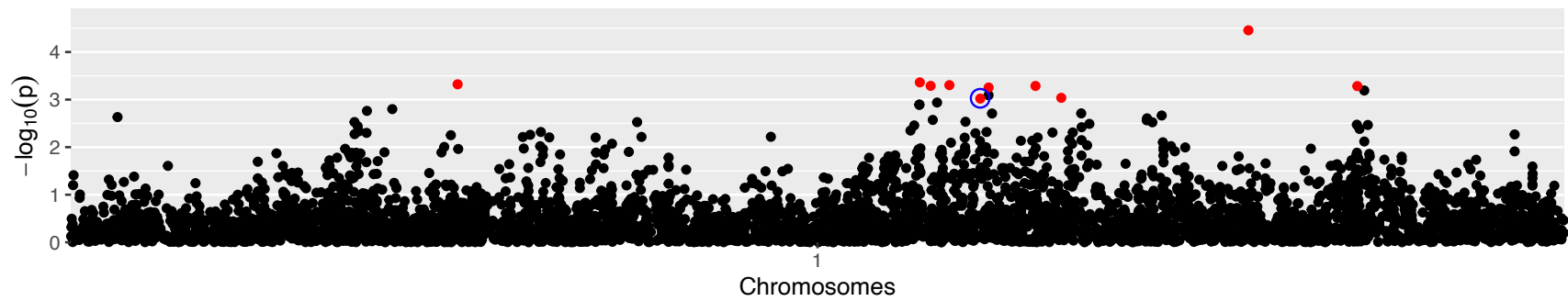
Figure 5-12 Significance of associations between SNP loci located on chromosome 1 and female adjusted horn length for females (n = 154) from the 1979 – 2017 cohorts in the Ram Mountain, Alberta population. Loci significantly associated with adjusted horn length are shown in red. Focal locus OAR1_167704342 circled in blue.
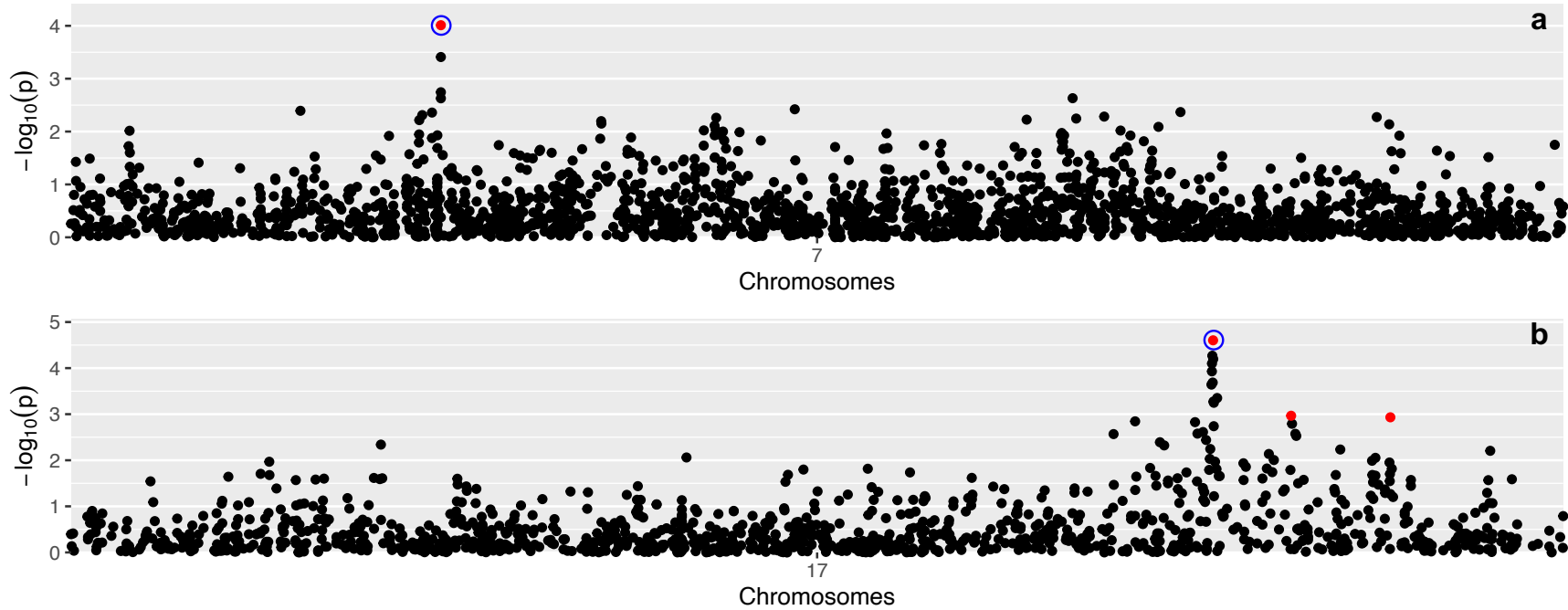
Figure 5-13 Significance of associations between SNP loci located on chromosome 7 (a) and 17 (b) and female longevity for females (n = 134) from the 1979 – 2017 cohorts in the Ram Mountain, Alberta population. Loci significantly associated with adjusted horn length are shown in red. Focal loci OAR7_25168902 and OAR17_55255290 circled in blue.
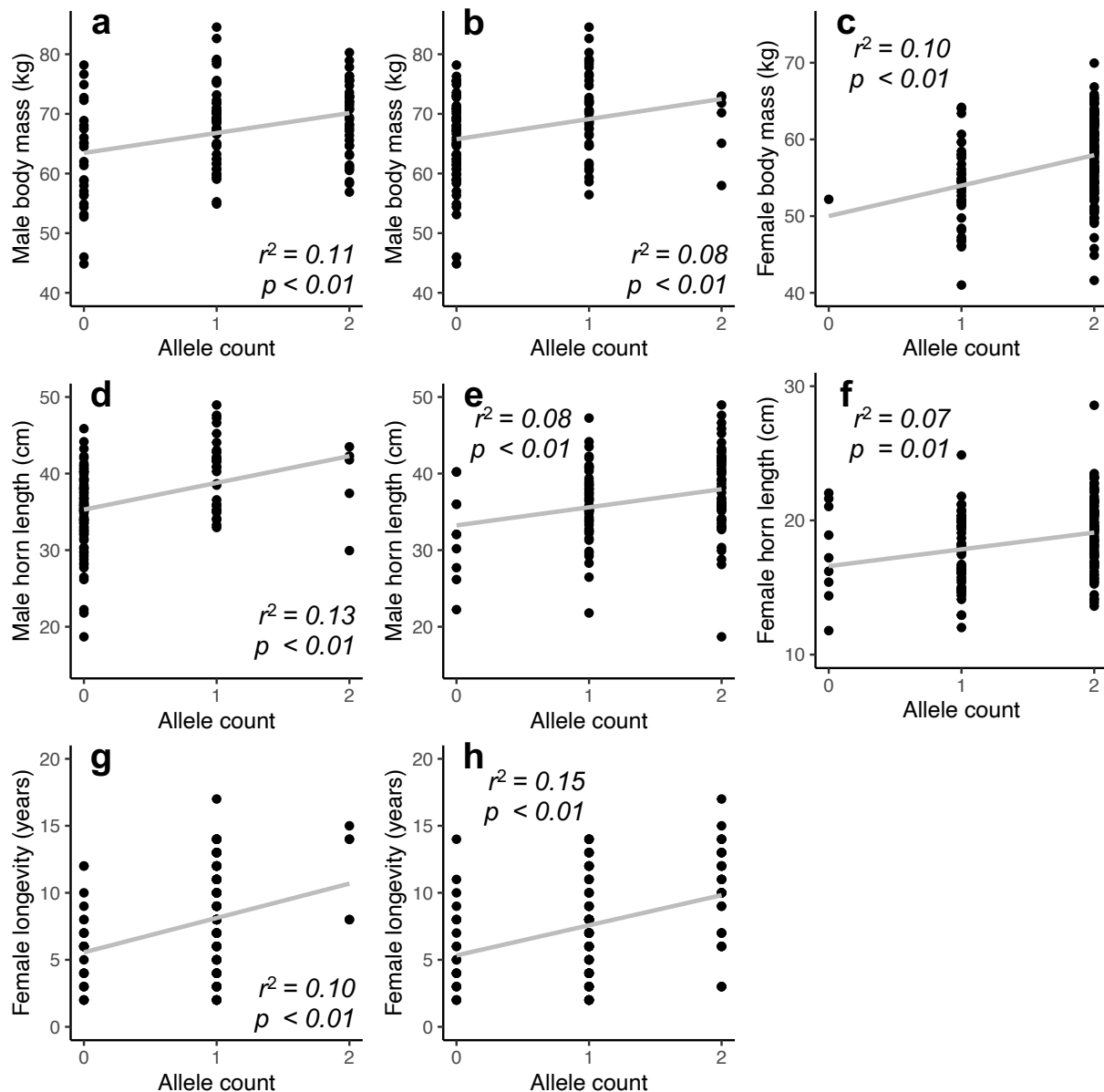
Figure 5-14 Association of phenotype and genotype at characterised loci. The loci-trait interactions shown are as follows; OAR1_54952090 and OAR20_ 25796186 with male adjusted body mass (n = 117) (a and b, respectively), OAR2_162344074 with female adjusted body mass (n = 165) (c), OAR1_58851106 and OAR1_171245188 with male adjusted horn length (n = 117) (d and e, respectively), OAR1_167704342 with female adjusted horn length (n = 135) (f), and OAR7_25168902 and OAR17_55255290 with female longevity (n = 134) (g and h, respectively).Plots show phenotype for individuals with 0, 1, or 2 copies of the allele found to be positively associated with the focal trait.
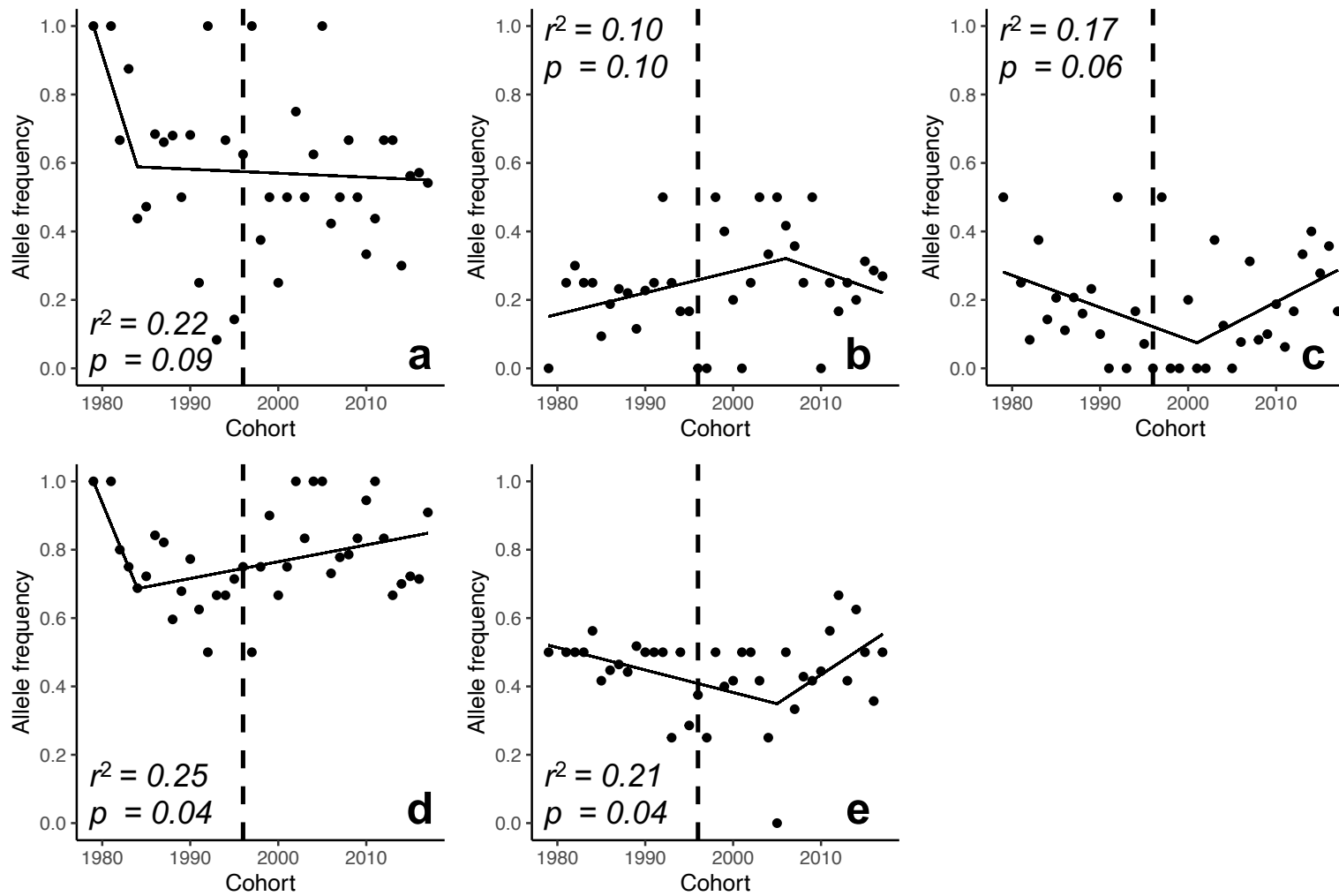
Figure 5-15 The frequencies of alleles identified to be positively associated with traits in cohorts from 1979 – 2017 from the Ram Mountain, Alberta population. Trendlines identified with piecewise regression. Dashed line represents cessation of trophy hunting in 1996. Loci include: OAR1_54952090 and OAR20_25796186 associated with male adjusted body mass (a and b), respectfully, OAR1_58851106 associated with male adjusted horn length (c), OAR1_167704342 associated with female adjusted horn length (d), and OAR7_25168902 associated with female longevity (e).
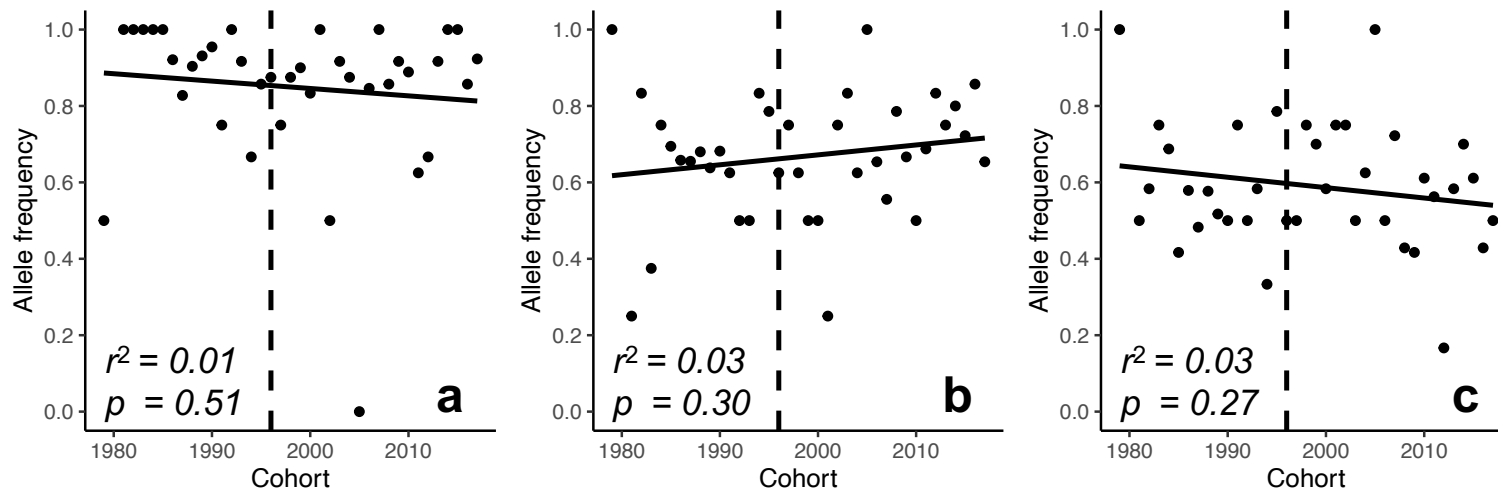
Figure 5-16 The frequencies of alleles identified to be positively associated with traits in cohorts from 1979 – 2017 from the Ram Mountain, Alberta population. Line fitted using linear model. Dashed line represents cessation of trophy hunting in 1996. Loci include: OAR2_162344074 associated with female adjusted body mass (a), OAR1_171245188 associated with male adjusted horn length (b), and OAR7_25168902 associated with female longevity (c).

Chapter 6 - Conclusion

6.1 Conclusion

During the course of my doctoral research, I characterised patterns of genetic and phenotypic variation in Alberta's Rocky Mountain bighorn sheep. First I characterised the spatial genetic structure of Alberta's Rocky Mountain bighorn sheep (*Ovis canadensis canadensis*) (Deakin *et al.*, 2020, Chapter 2), examined the relationship between different female traits and their associations with fitness (Deakin *et al.*, 2022, Chapter 3), and developed a high-density SNP assay (Chapter 4) which I used to examine the genetic basis of traits in Rocky Mountain bighorn sheep (Chapter 5).

In **Chapter 2** I examined the spatial genetic structure of bighorn sheep in the northern portion of their range in Alberta. I characterised the spatial genetic structure of bighorn sheep inhabiting a highly heterogenous vast landscape which is relatively unimpacted by anthropogenic factors and provided insights into the natural history of populations in this range. To characterise the spatial genetic structure of Alberta's bighorn sheep I examined 1,495 microsatellite genotypes and 188 mitochondrial haplotypes. I identified population genetic structure correlated with inter-river regions, and a strong pattern of isolation-by-distance within and between inter-river regions. Additionally, I identified patterns of spatial genetic structure which indicated gene flow occurs equally between the sexes up to 100km. Finally, I identified a reduction in genetic diversity moving northwards which indicates the post-glacial recolonization of the northern Rocky Mountains by bighorn sheep was sourced from the Southern refugium in a stepping-stone fashion. This chapter confirmed the Ram Mountain, Alberta population was a random mating population genetically distant from all other studied populations. Thus, examining selection and evolution within this population in Chapters 3 and 5 is appropriate as there is minimal phenotypic or genetic variance introduced into the population from elsewhere.

In **Chapter 3** I investigated the association between female secondary sexual traits and female reproductive fitness. To examine these associations, I examined 45 years of phenotypic data for 217 females Rocky Mountain bighorn sheep from the Ram Mountain, Alberta population. Using survival and generalised linear mixed effect models I identified female horn length to be associated with female reproductive fitness; with longer horned females reproducing and successfully raising offspring earlier in life, and therefore producing more lambs over their lifetimes. This highlighted

how these presumed vestiges of strong sexual selection on males can indicate individual fitness in females, similar to their male counterparts (Coltman *et al.*, 2002). The study also highlighted a potential conservation issue in this species as Rocky Mountain bighorn sheep are subject to selective harvest on horn length. It is understood intense trophy hunting has reduced male horn size in this population of bighorn sheep (Coltman *et al.*, 2003; Pigeon *et al.*, 2016), which in turn, due to the cross sex heritability of the trait (Poissant *et al.*, 2012), has also reduced female horn length (Pigeon *et al.*, 2016). In Chapter 3, I identified an association between female horn length and female reproductive fitness, thus selective harvesting males may ultimately be affecting female reproductive capabilities, and therefore potentially affecting population growth and viability. This potential conservation issue may be occurring across Alberta, or other bighorn sheep populations subject to a similar harvesting regime, given that other bighorn sheep populations in Alberta have shown declines in male horn length (Festa-Bianchet *et al.*, 2014).

In **Chapter 4** I developed and validated a high-density, species-specific SNP assay for Rocky Mountain bighorn sheep for high resolution analysis of population genetic structure and investigating the genetic variance associated with phenotypic traits identified in Chapter 3 (Deakin *et al.*, 2022) and other studies to be associated with individual fitness (Coltman *et al.*, 2002; Coltman *et al.*, 2005; Poissant *et al.*, 2008). To develop the assay I utilized six genomic resources; a genome for the domestic sheep (*Ovis aries*) (Jiang *et al.*, 2014) and variant loci data for Rocky Mountain bighorn sheep (Miller *et al.*, 2012, 2015; Miller, Hogg and Coltman, 2013; Kardos *et al.*, 2015; Miller, Festa-Bianchet and Coltman, 2018). From these five studies I sourced ~40 million variant sites which I iteratively reduced through a number of filtering steps to retain 50,000 SNPs, sourced from all of the five studies. Overall, my trials found the assay was highly efficient and accurate, however the assay performed less well on DNA samples sourced from faecal DNA. To my knowledge and at the time of writing, this panel is the largest, high-throughput, SNP assay specifically designed for Rocky Mountain bighorn sheep, which given its coverage of the genome and its performance on tissue and blood samples will be a valuable resource for future genomic studies on this species.

In **Chapter 5** I identified the genetic basis of several traits in Rocky Mountain bighorn sheep. To do this I performed genome wide association studies (GWAS) between high-density genotypes

(from the assay developed in Chapter 4) and phenotypes for 305 individuals from the Ram Mountain population. The GWAS identified 278 SNPs associated with eight traits: male and female body mass, male and female horn length, female age at primiparity and age at first weaning, female lifetime reproductive success (LRS), and female longevity. For these eight traits heritability values ranged from <1% to 63%, with female reproductive traits being the least and female longevity being the most heritable. Notably male body mass and horn length were around twice as heritable as the same traits in their female conspecifics; with heritability values for male body mass and horn length being 43% and 45%, respectively, whereas female body mass and horn length had heritability values of 19% and 24% respectively. I also found the effect sizes of SNPs I further characterised to show a similar pattern between males and females. The SNPs characterised for male body mass had effect sizes of 3.3 and 3.8, whereas the effect size of the locus characterised for female body mass only had an effect size of 1.0. The SNPs characterised for male horn length had effect sizes of 3.1 and 2.8, whereas the effect size of the locus characterised for female horn length only had an effect size of 1.3. However, the greatest effect size, relative to phenotypic variance, was found for female longevity with one locus having an effect size of 4.3 and another having an effect size of 1.3. I examined my eight focal SNPs to examine if any loci showed trends associated with trophy hunting. Of these eight, loci I identified significant or near significant trends over time for five loci. Of these five, I identified two loci which showed trends potentially associated with the presence and absence of trophy hunting. One locus was associated with male horn length and another associated with female longevity, these loci showed declines in their allele positively associate with phenotype under trophy hunting, followed by an increase when trophy hunting was ceased.

For my thesis, I had intended to produce a fifth data chapter investigating if male bighorn sheep were leaving protected areas during the rut and subsequently being harvested. To do this I was going to examine high-density genotypes sourced from faecal samples collected within protected areas during the summer and horn cores collected from harvested rams and look for matching genotypes between the two sample types. Thus, examining the proportion of rams from protected populations being harvested outside of protected populations in Alberta. Unfortunately, due to a machine malfunction and poor-quality DNA from faecal samples, I did not have the required genotypes to complete the study. However, some information can be gleaned from this failure with

regards to poor quality faecal samples. This was the first of my studies to do a post-extraction clean-up on my faecal DNA, based on the recommendations made in of Chapter 4. However, in this study the faecal samples performed even worse than in Chapter 4. I hypothesise this is due to my faecal sample collection taking place in the summer months. I believe two mechanisms affected my faecal samples, each acting upon samples collected at different levels of freshness. Firstly, old samples would have been subject to much greater temperatures and UV radiation than the faecal samples used in Chapter 2, thus degrading much of the host DNA contained within mucus membrane around the sample. The second factor affected very fresh samples, many of my samples were collected within minutes of being deposited by the host, and thus when frozen many bags contained a moisture build up, I believe this moisture condensed within the bags, disrupted, and effectively diluted the mucus layer around the faecal pellet which contained most of the host DNA (Albaugh *et al.*, 1992). Thus, I believe future studies using this kind of faecal sampling method should carefully examine if summer sampling is required, as winter faecal sampling may avoid some of the poor genotyping performance I encountered.

My research presented here in this thesis contributes to both species-specific knowledge and more broadly to the fields of conservation genetics and genomics. Chapter two was the first to examine the spatial genetic structure of Canada's Rocky Mountain bighorn sheep at a resolution which enabled inferences about population structure and historical recolonization. This also provides insights into the population structure of other alpine species and patterns of post glacial recolonization in North America. Chapter three was the first study to examine the association between female horn length and female reproductive fitness in bighorn sheep, while controlling for the effect of female body mass. In the study, I identified how secondary sexual traits, often presumed to be vestiges of intense sexual selection on males, can be indicative of overall female fitness in bighorn sheep, which may be the case for other species in which possess sexually dimorphic secondary sexual traits (Cuervo, de Lope and Møiller, 1996). In Chapter 4 I develop what is, to my knowledge and at the time of writing, the largest, high-throughput, SNP assay specifically designed for Rocky Mountain bighorn sheep, which given its coverage of the genome and its performance on tissue and blood samples will be a valuable resource for future genomic studies on Rocky Mountain bighorn sheep. This further exemplifies how genomic resources can be applied between closely related species, if genomic resources are sparse in the target species

(Li *et al.*, 2019; Sim and Coltman, 2019; Santos *et al.*, 2021). Finally, in Chapter 5, the study I conducted identifies 278 loci in the Rocky Mountain bighorn sheep genome associated with eight traits. This study is the first to identify this volume of SNPs of effect in Rocky Mountain bighorn sheep. These loci provide a valuable resource for further studies into the mechanisms, evolutionary histories, and local adaptation of these traits. Furthermore, the study provides insights into how intense selective harvest can cause evolutionary changes at loci associated with phenotypes, similar to other species (Allendorf and Hard, 2009; Campbell-Staton *et al.*, 2021).

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Appendix A

Information A1. Rocky Mountain bighorn sheep fecal genotyping.

Multiplex Primer L1 = Loci: *MAF64*, *OarCP26*, *BM4505*, *BM1225*

Multiplex Primer L2 = Loci: *MAF65*, *MAF209*, *MAF36*, *BMC1222*

Multiplex Primer L3 = Loci: *FCB266*, *OarAE16*, *TGLA387*

Solo loci are *Rt9* (co-load with L1), *TGLA122* (co-load with L3), *Sheep AML* (co-load with L3)

Multiplex primers L1, L2, and L3 need to run in Qiagen Type-it master mix for fecal DNA. Can use house mix if blood or skin extract. Single loci (*Rt9* and *TGLA122*) amplify best in house mix regardless of DNA extract material.

RECIPE 1. For blood samples run in house buffer regardless of loci.

House mix	1× (10 µL)	115× (full plate)
10× Buffer (blue)	1	115
2 mmol/L dNTP (green)	1	115
25 mmol/L MgCl$_2$ (red)	0.7	80.5
10 µmol/L forward and reverse primers	1	115
MilliQ H$_2$O	3.2	368
House *Taq* (Tp)	0.1	11.5
Template DNA	3	—

RECIPE 2. House mix PCR recipe for Rt9 and TGLA122 using fecal DNA.

House mix	1× (10 µL)	115× (full plate)
10× Buffer (blue)	1	115
2 mmol/L dNTP (green)	1	115
25 mmol/L MgCl$_2$ (red)	0.8	92
10 µmol/L forward and reverse primers	1	115
MilliQ H$_2$O	5.1	587
House *Taq* (Tp)	0.1	11.5
Template DNA	1	—

RECIPE 3. Qiagen Type-it master mix recipe for B1, B2, and B3 when using fecal DNA.

House mix	1× (10 µL)	115× (full plate)
2× Master Mix	5	575
2 µmol/L Multiplex Primer	1	115
RNA-free H$_2$O	3	345
Template DNA	1	—

Thermocycler Profiles

RECIPE 1

Primers L1 and L3 run at "Multiplex 60", B2 runs at "Multiplex 58"

Rt9 runs on "Standard Micro54", *TGLA122* runs on "Standard Micro60".

RECIPE 2

Rt9 runs at "Fecal Micro54" (45 cycles)

TGLA122 runs at "Fecal Micro60" (45 cycles)

Sheep AML runs at "Fecal Micro69" (45 cycles)

RECIPE 3

Multiplex primers L1, L2, and L3 runs on "Fecal Type-it56"

CLEAN UP of SOLOs

Sheep AML should have an annealing T_m of 69 °C when run solo in house mix. Add 30 µL water to PCR product, transfer 2 µL, top up to 30 µL, transfer 2 µL to final plate.

Post-PCR dilutions for fragment analysis

RECIPE 2 and 3 (FECAL DNA)

1. Add 10 µL MilliQ water to *TGLA122* PCR product.

2. Transfer 2 µL to "Dilution Plate 3".

3. Transfer 2 µL of B3 PCR product to "Dilution Plate 3" to co-load.

4. Transfer 2 µL of co-load to new plate labeled "B3" for 3730.

5. Transfer 2 µL of *Rt9* to "Dilution Plate 1".

6. Transfer 2 µL of B1 PCR product to "Dilution Plate 1" to co-load.

7. Transfer 2 µL of co-load to new plate labeled "B1" for 3730.

8. Add 2 µL of MilliQ water to empty "Dilution Plate 2".

9. Transfer 2 µL of B2 PCR product to "Dilution Plate 2" to dilute.

10. Transfer 2 µL of dilution to new plate labeled "B2" for 3730.

11. Mix 20 µL of TAMRA500 with 885 µL HiDi (one tube for each plate).

12. Add 8.5 µL of HiDi/TAMRA500 mix to new plates.

13. Place septa on new plates and quick spin at 900 r/min.

14. Heat at 95 °C for 2 min and snap cool on ice.

RECIPE 1 (BLOOD DNA)

1. Add 10 µL MilliQ water to all PCR products.

2. Transfer 2 µL of each to co-load B1 and *Rt9*, B3 and *TGLA122*, and B2 by itself.

3. Top up to 20 µL total volume with MilliQ water.

4. Transfer 2 µL to final plate.

5. Add 885 µL HiDi and 20 µL TAMRA500.

THERMOCYCLE PROFILES

Fecal Micro54	Fecal Micro60	Fecal Micro69	Standard Micro54
94 °C 2 min	94 °C 2 min	94 °C 2 min	94 °C 2 min
94 °C 30 s	94 °C 30 s	94 °C 30 s	94 °C 30 s
54 °C 20 s × 3 times	60 °C 20 s × 3 times	69 °C 20 s × 3 times	54 °C 20 s × 3 times
72 °C 5 s	72 °C 5 s	72 °C 5 s	72 °C 5 s

94 °C 15 s 54 °C 20 s × 42 times 72 °C 1 s 72 °C 10 min	94 °C 15 s 60 °C 20 s × 42 times 72 °C 1 s 72 °C 10 min	94 °C 15 s 69 °C 20 s × 42 times 72 °C 1 s 72 °C 10 min	94 °C 15 s 54 °C 20 s × 30 times 72 °C 1 s 72 °C 10 min
Fecal Type-it56 95 °C 5 min 95 °C 30 s 56 °C 90 s × 34 times 72 °C 30 s 60 °C 30 min	Type-it56 95 °C 5 min 95 °C 30 s 56 °C 90 s × 30 times 72 °C 30 s 60 °C 30 min	Multiplex58 95 °C 2 min 94 °C 30 s 58 °C 90 s × 33 times 72 °C 60 s 72 °C 10 min	Standard Micro60 94 °C 2 min 94 °C 30 s 60 °C 20 s × 3 times 72 °C 5 s 94 °C 15 s 60 °C 20 s × 30 times 72 °C 1 s 72 °C 10 min

Information A2. Bighorn mitochondrial DNA (mtDNA) D-loop sequencing.

1. Perform a PCR to amplify the complete or partial mtDNA D-Loop region, depending on the source of your DNA.

For Tissue: For Fecal:

Nuclease-free H$_2$O	5.65 μL	14.125 μL
10× Buffer	1.00 μL	2.500 μL
2 mmol/L dNTPs	1.00 μL	2.500 μL
25 mmol/L MgCl$_2$	0.40 μL	1.000 μL
10 μmol/L Fecal1F/Fecal4F	0.30 μL	0.750 μL
10 μmol/L Fecal4RC	0.30 μL	0.750 μL
BSA (20 mg/mL)	0.25 μL	0.625 μL
InHouse *Taq*	0.10 μL	0.250 μL
DNA Template	<u>1.00 μL </u>(undiluted)	<u>2.500 μL</u> (dilute by 1/10)
TOTAL	10.00 μL	25.000 μL

Run on thermocycler program: 1 cycle of 2 min at 94 °C; 9 cycles of 15 s at 94 °C, 30 s at 45 °C to 49 °C (temperature increase +0.5 °C per cycle), 45 s at 72 °C; 20 cycles of 15 s at 94 °C, 30 s at 50 °C, 45 s at 72 °C (time increase +5 s per cycle); and 7 min at 72 °C.

2. After checking the PCR Product on a 1% agarose gel (using 5 μL), clean up using ExoSAP. The 1000 bp band of the 1 kb ladder is 60 ng and the bands under 1000 bp are 25 ng.

NEB rSAP (1 U/μL)	1.00 μL
1/10 Diluted NEB Exonuclease I (2 U/μL)	1.00 μL
PCR Product	8.00 μL (Fecal) or 2.00 μL (Tissue)
Nuclease-free H$_2$0	<u>0.00 μL</u> (Fecal) or 6.00 μL (Tissue)
TOTAL	10.00 μL

Incubate at 37°C for 45 min followed by 80 °C for 20 min in a thermocycler.

3. Set up two sequencing reactions per sample using the forward or reverse primer.

Big Dye Terminator version 3.1	0.25 μL
5× Sequencing Buffer	1.50 μL
Big Dye Enhancing Buffer	0.75 μL
10 μmol/L Fecal1F/Fecal4F/Fecal4RC	0.25 μL
Nuclease-free H$_2$O	to total volume of 10 μL
ExoSAP PCR Product (~50–200 ng)	<u>up to 7.25 μL</u>
TOTAL	10.0 μL

Run on a thermocycler following the program: 1 cycle of 3 min at 96 °C; 35 cycles of 10 s at 96 °C, 10 s at 50 °C and 5 s at 60 °C; and 1 cycle of 2 min at 60 °C.

Table S1. Microsatellite genetic distance matrix. Pairwise F_{ST} distances (upper diagonal) and Nei's standard genetic distances (lower diagonal) between the 49 sampling locations calculated from the 13 microsatellite loci. **, $p < 0.01$; *, $p < 0.05$; ns, $p > 0.05$.

	CY	RD	FR	SO	SR	FC	MB	LO	RH	RG	AL	RR	RM	CR	JC
CY	0	0.18**	0.06**	0.09**	0.17**	0.17**	0.16**	0.13**	0.15**	0.14**	0.16**	0.13**	0.18**	0.16**	0.17**
RD	0.39	0	0.06**	0.09**	0.19**	0.20**	0.22**	0.15**	0.20**	0.17**	0.22**	0.16**	0.22**	0.19**	0.21**
FR	0.16	0.16	0	0.02**	0.10**	0.11**	0.12**	0.08**	0.11**	0.09**	0.11**	0.09**	0.11**	0.11**	0.11**
SO	0.21	0.21	0.05	0	0.10**	0.08**	0.10**	0.06**	0.09**	0.07**	0.09**	0.07**	0.10**	0.09**	0.09**
SR	0.45	0.43	0.28	0.28	0	0.16**	0.18**	0.13**	0.18**	0.15**	0.18**	0.14**	0.18**	0.18**	0.18**
FC	0.41	0.42	0.29	0.2	0.46	0	0.04**	0.01*	0.03**	0.03**	0.06**	0.03**	0.07**	0.06**	0.06**
MB	0.38	0.49	0.33	0.26	0.52	0.08	0	0.01*	0.00ns	0.03**	0.04**	0.01**	0.04**	0.03**	0.05**
LO	0.35	0.36	0.26	0.18	0.38	0.02	0	0	0.03**	0.03**	0.03**	0.01ns	0.04**	0.03**	0.02**
RH	0.35	0.43	0.28	0.22	0.51	0.08	0	0.05	0	0.04**	0.03**	0.02**	0.07**	0.05**	0.07**
RG	0.34	0.38	0.24	0.18	0.45	0.07	0.06	0.07	0.08	0	0.06**	0.02**	0.06**	0.05**	0.07**
AL	0.38	0.48	0.29	0.23	0.53	0.14	0.08	0.05	0.06	0.13	0	0.03**	0.06**	0.06**	0.08**
RR	0.35	0.42	0.25	0.19	0.46	0.07	0.03	0.03	0.06	0.05	0.08	0	0.03**	0.01*	0.03**
RM	0.43	0.49	0.28	0.25	0.52	0.16	0.08	0.07	0.15	0.12	0.13	0.07	0	0.04**	0.08**
CR	0.41	0.44	0.32	0.25	0.59	0.13	0.07	0.07	0.11	0.13	0.12	0.03	0.08	0	0.03**
JC	0.45	0.49	0.34	0.27	0.53	0.14	0.09	0.05	0.15	0.17	0.2	0.08	0.2	0.06	0
BR	0.42	0.39	0.27	0.23	0.49	0.17	0.09	0.07	0.13	0.09	0.18	0.04	0.07	0.02	0.12
OB	0.36	0.51	0.3	0.24	0.58	0.15	0.11	0.07	0.13	0.12	0.14	0.07	0.18	0.08	0.08
AZ	0.44	0.46	0.34	0.29	0.58	0.19	0.11	0.06	0.17	0.13	0.2	0.1	0.14	0.06	0.09
OA	0.43	0.58	0.36	0.32	0.49	0.15	0.12	0.09	0.15	0.16	0.14	0.11	0.15	0.15	0.2
BS	0.5	0.52	0.4	0.38	0.57	0.26	0.18	0.19	0.23	0.19	0.27	0.13	0.16	0.09	0.13
IC	0.42	0.47	0.3	0.23	0.52	0.15	0.09	0.09	0.13	0.15	0.11	0.08	0.12	0.04	0.08
CA	0.36	0.46	0.28	0.22	0.49	0.15	0.05	0.06	0.08	0.08	0.09	0.02	0.05	0.06	0.07
RC	0.43	0.49	0.31	0.27	0.52	0.24	0.13	0.09	0.16	0.17	0.17	0.1	0.1	0.1	0.09
GF	0.41	0.43	0.29	0.24	0.54	0.26	0.17	0.14	0.18	0.15	0.18	0.11	0.14	0.14	0.15
DR	0.36	0.39	0.24	0.18	0.46	0.18	0.13	0.12	0.11	0.15	0.13	0.07	0.12	0.08	0.07
CM	0.45	0.43	0.31	0.25	0.57	0.24	0.18	0.13	0.19	0.21	0.15	0.1	0.14	0.07	0.1
CD	0.39	0.37	0.27	0.23	0.49	0.23	0.15	0.11	0.16	0.15	0.19	0.09	0.13	0.08	0.1
LG	0.42	0.36	0.28	0.24	0.44	0.25	0.18	0.14	0.18	0.18	0.2	0.1	0.13	0.11	0.11
ML	0.43	0.39	0.28	0.24	0.53	0.24	0.15	0.11	0.17	0.17	0.17	0.09	0.1	0.1	0.12
MR	0.42	0.37	0.29	0.25	0.54	0.23	0.15	0.12	0.18	0.16	0.18	0.1	0.13	0.12	0.1
MP	0.53	0.49	0.38	0.32	0.63	0.28	0.14	0.2	0.22	0.22	0.19	0.13	0.15	0.06	0.07
EK	0.45	0.58	0.39	0.36	0.6	0.28	0.19	0.21	0.28	0.22	0.26	0.17	0.21	0.13	0.17
DP	0.44	0.43	0.32	0.28	0.53	0.27	0.14	0.2	0.19	0.16	0.22	0.13	0.15	0.08	0.11
FM	0.44	0.41	0.31	0.23	0.51	0.2	0.1	0.09	0.15	0.11	0.2	0.06	0.1	0.1	0.09
WP	0.43	0.32	0.26	0.27	0.6	0.32	0.19	0.12	0.25	0.19	0.26	0.19	0.2	0.18	0.19
BT	0.43	0.38	0.27	0.24	0.6	0.25	0.19	0.16	0.2	0.19	0.19	0.15	0.17	0.17	0.15
ST	0.54	0.44	0.37	0.35	0.68	0.33	0.27	0.27	0.32	0.26	0.3	0.23	0.18	0.2	0.26
MC	0.5	0.42	0.32	0.3	0.58	0.31	0.3	0.22	0.3	0.25	0.28	0.25	0.22	0.25	0.27
MO	0.49	0.42	0.3	0.27	0.51	0.26	0.28	0.22	0.29	0.22	0.25	0.24	0.22	0.22	0.2
RP	0.49	0.41	0.32	0.32	0.53	0.32	0.26	0.18	0.27	0.23	0.32	0.24	0.21	0.24	0.26
NB	0.49	0.45	0.32	0.28	0.58	0.23	0.24	0.18	0.25	0.19	0.24	0.2	0.17	0.21	0.25
WM	0.46	0.39	0.29	0.28	0.56	0.3	0.24	0.18	0.24	0.22	0.25	0.19	0.17	0.2	0.19
MM	0.44	0.4	0.29	0.29	0.52	0.31	0.25	0.17	0.23	0.21	0.26	0.2	0.19	0.28	0.22
SA	0.52	0.39	0.33	0.3	0.54	0.3	0.21	0.16	0.26	0.21	0.32	0.2	0.17	0.2	0.17
HC	0.51	0.42	0.38	0.35	0.63	0.34	0.27	0.16	0.32	0.3	0.33	0.25	0.28	0.24	0.24
GC	0.56	0.43	0.37	0.35	0.56	0.37	0.34	0.23	0.38	0.33	0.39	0.3	0.28	0.25	0.26
SM	0.55	0.39	0.36	0.35	0.56	0.37	0.33	0.22	0.36	0.31	0.4	0.29	0.27	0.25	0.25
GM	0.57	0.46	0.42	0.43	0.64	0.46	0.33	0.25	0.37	0.37	0.37	0.31	0.23	0.32	0.28
NW	0.57	0.48	0.43	0.4	0.64	0.38	0.29	0.2	0.34	0.31	0.38	0.27	0.24	0.27	0.22

	BR	OB	AZ	OA	BS	IC	CA	RC	GF	DR	CM	CD	LG	ML	MR	MP	EK
CY	0.17**	0.15**	0.17**	0.19**	0.20**	0.17**	0.15**	0.18**	0.17**	0.15**	0.19**	0.16**	0.17**	0.17**	0.17**	0.22**	0.18**
RD	0.19**	0.23**	0.20**	0.27**	0.24**	0.21**	0.21**	0.23**	0.21**	0.20**	0.22**	0.16**	0.16**	0.18**	0.17**	0.25**	0.27**
FR	0.10**	0.11**	0.12**	0.13**	0.15**	0.11**	0.10**	0.12**	0.11**	0.09**	0.12**	0.10**	0.10**	0.11**	0.11**	0.14**	0.13**
SO	0.09**	0.09**	0.11**	0.12**	0.15**	0.09**	0.08**	0.11**	0.09**	0.07**	0.10**	0.09**	0.09**	0.09**	0.09**	0.13**	0.12**
SR	0.16**	0.20**	0.19**	0.16**	0.20**	0.18**	0.16**	0.17**	0.18**	0.16**	0.20**	0.16**	0.14**	0.17**	0.18**	0.22**	0.20**
FC	0.07**	0.07**	0.08**	0.07**	0.12**	0.07**	0.06**	0.11**	0.11**	0.08**	0.11**	0.10**	0.11**	0.11**	0.10**	0.12**	0.11**
MB	0.05**	0.05**	0.05**	0.07**	0.09**	0.04**	0.03**	0.06**	0.08**	0.06**	0.09**	0.07**	0.08**	0.07**	0.07**	0.07**	0.08**
LO	0.03**	0.03**	0.03**	0.04*	0.09**	0.04**	0.02**	0.05**	0.05**	0.05**	0.07**	0.05**	0.06**	0.05**	0.05**	0.09**	0.07**
RH	0.06**	0.06**	0.08**	0.08**	0.11**	0.06**	0.04**	0.08**	0.08**	0.05**	0.09**	0.07**	0.08**	0.08**	0.08**	0.10**	0.11**
RG	0.04**	0.05**	0.06**	0.07**	0.08**	0.06**	0.03**	0.08**	0.07**	0.07**	0.10**	0.06**	0.08**	0.07**	0.07**	0.10**	0.08**
AL	0.08**	0.06**	0.09**	0.07**	0.12**	0.05**	0.04**	0.08**	0.09**	0.06**	0.07**	0.08**	0.09**	0.08**	0.08**	0.09**	0.11**
RR	0.01*	0.03**	0.03**	0.04**	0.05**	0.03**	0.01*	0.04**	0.04**	0.02**	0.04**	0.04**	0.04**	0.04**	0.04**	0.05**	0.05**
RM	0.03**	0.09**	0.07**	0.07**	0.08**	0.06**	0.02**	0.05**	0.07**	0.06**	0.07**	0.06**	0.06**	0.05**	0.06**	0.08**	0.09**
CR	0.01*	0.03**	0.02**	0.07**	0.04**	0.02**	0.02**	0.05**	0.06**	0.04**	0.04**	0.04**	0.05**	0.05**	0.05**	0.04**	0.04**
JC	0.05**	0.04**	0.04**	0.09**	0.06**	0.04**	0.03**	0.05**	0.07**	0.03**	0.05**	0.04**	0.05**	0.05**	0.04**	0.04**	0.06**
BR	0	0.04**	0.03**	0.06**	0.04**	0.04**	0.02*	0.04**	0.04**	0.05**	0.04**	0.03**	0.04**	0.04**	0.03**	0.05**	0.04**
OB	0.08	0	0.04**	0.04**	0.06**	0.03**	0.02**	0.04**	0.01**	0.04**	0.02**	0.03**	0.05**	0.04**	0.04**	0.05**	0.08**
AZ	0.06	0.08	0	0.05**	0.04**	0.03**	0.01*	0.03**	0.06**	0.05**	0.05**	0.03**	0.05**	0.04**	0.04**	0.04**	0.05**
OA	0.12	0.09	0.1	0	0.13**	0.03**	0.04**	0.05**	0.06**	0.06**	0.07**	0.06**	0.06**	0.05**	0.06**	0.10**	0.10**
BS	0.08	0.14	0.09	0.27	0	0.08**	0.04**	0.06**	0.06**	0.08**	0.06**	0.05**	0.05**	0.05**	0.07**	0.07**	0.07**
IC	0.09	0.05	0.06	0.07	0.17	0	0.00ns	0.03**	0.03**	0.02**	0.04**	0.02**	0.04**	0.03**	0.03**	0.03**	0.06**
CA	0.04	0.05	0.03	0.08	0.08	0.01	0	0.00ns	0.03**	0.01ns	0.03**	0.01*	0.02**	0.00ns	0.01*	0.04**	0.05**
RC	0.07	0.07	0.06	0.1	0.12	0.07	0	0	0.04**	0.01ns	0.02**	0.02**	0.03**	0.01*	0.02**	0.05**	0.05**
GF	0.08	0.02	0.14	0.13	0.14	0.07	0.06	0.06	0	0.02*	0.00ns	0.00ns	0.01*	0.00ns	0.01*	0.04**	0.07**
DR	0.1	0.09	0.11	0.13	0.18	0.05	0.01	0.01	0.03	0	0.01*	0.00ns	0.01ns	0.01ns	0.01ns	0.05**	0.03**
CM	0.07	0.04	0.11	0.12	0.13	0.07	0.05	0.04	-0.01	0.02	0	0.01*	0.01**	0.01*	0.01*	0.03**	0.08**
CD	0.06	0.07	0.08	0.12	0.1	0.05	0.02	0.04	0	0.01	0.02	0	0.00**	0.00*	0.01*	0.03**	0.05**
LG	0.08	0.1	0.1	0.13	0.1	0.08	0.04	0.06	0.03	0.02	0.02	0.01	0	0.01**	0.02**	0.04**	0.07**
ML	0.09	0.08	0.09	0.11	0.11	0.06	0.01	0.02	0.01	0.01	0.02	0.01	0.02	0	0.01*	0.03**	0.06**
MR	0.07	0.09	0.09	0.13	0.17	0.07	0.02	0.05	0.03	0.02	0.02	0.02	0.04	0.02	0	0.02**	0.07**
MP	0.09	0.09	0.07	0.18	0.13	0.05	0.08	0.08	0.08	0.08	0.04	0.06	0.07	0.06	0.05	0	0.06**
EK	0.14	0.21	0.13	0.25	0.19	0.18	0.15	0.12	0.19	0.09	0.19	0.15	0.2	0.18	0.19	0.15	0
DP	0.07	0.09	0.03	0.2	0.1	0.06	0.02	0.04	0.06	0.06	0.09	0.04	0.08	0.06	0.02	0	0.15
FM	0.06	0.08	0.09	0.13	0.14	0.06	0.01	0.04	0.04	0.03	0.05	0.02	0.04	0.03	0.02	0.04	0.19
WP	0.18	0.17	0.1	0.2	0.2	0.1	0.14	0.09	0.07	0.12	0.18	0.09	0.12	0.05	0.1	0.12	0.19
BT	0.17	0.08	0.1	0.17	0.24	0.09	0.09	0.08	0.06	0.04	0.09	0.08	0.1	0.06	0.06	0.11	0.19
ST	0.21	0.2	0.21	0.26	0.22	0.14	0.17	0.17	0.12	0.14	0.19	0.14	0.17	0.1	0.13	0.12	0.22
MC	0.25	0.19	0.22	0.18	0.29	0.18	0.22	0.17	0.12	0.17	0.2	0.17	0.2	0.13	0.17	0.21	0.25
MO	0.25	0.17	0.19	0.21	0.29	0.11	0.19	0.15	0.12	0.09	0.16	0.13	0.16	0.13	0.13	0.13	0.19
RP	0.19	0.17	0.15	0.15	0.23	0.16	0.16	0.13	0.13	0.19	0.19	0.12	0.14	0.11	0.12	0.16	0.3
NB	0.19	0.15	0.17	0.16	0.23	0.15	0.15	0.16	0.1	0.13	0.15	0.12	0.15	0.11	0.1	0.19	0.21
WM	0.17	0.14	0.16	0.13	0.22	0.11	0.14	0.1	0.06	0.09	0.12	0.08	0.1	0.07	0.08	0.11	0.23
MM	0.2	0.15	0.2	0.13	0.21	0.17	0.14	0.14	0.06	0.14	0.15	0.1	0.11	0.08	0.1	0.21	0.36
SA	0.14	0.17	0.13	0.18	0.2	0.16	0.15	0.11	0.11	0.14	0.16	0.1	0.12	0.1	0.1	0.11	0.25
HC	0.2	0.19	0.22	0.19	0.28	0.19	0.25	0.18	0.12	0.2	0.17	0.14	0.17	0.14	0.13	0.18	0.31
GC	0.21	0.23	0.23	0.22	0.28	0.2	0.27	0.19	0.14	0.21	0.19	0.15	0.17	0.17	0.17	0.19	0.28
SM	0.21	0.24	0.24	0.22	0.27	0.21	0.28	0.21	0.14	0.21	0.2	0.15	0.17	0.17	0.17	0.19	0.32
GM	0.25	0.28	0.28	0.22	0.28	0.2	0.25	0.16	0.11	0.19	0.2	0.14	0.16	0.14	0.14	0.21	0.34
NW	0.23	0.21	0.21	0.19	0.24	0.18	0.19	0.13	0.1	0.19	0.17	0.12	0.14	0.1	0.11	0.19	0.32

	DP	FM	WP	BT	ST	MC	MO	RP	NB	WM	MM	SA	HC	GC	SM	GM	NW
CY	0.18**	0.19**	0.18**	0.19**	0.25**	0.21**	0.21**	0.22**	0.21**	0.21**	0.20**	0.23**	0.23**	0.27**	0.28**	0.26**	0.25**
RD	0.21**	0.20**	0.17**	0.20**	0.26**	0.22**	0.24**	0.22**	0.22**	0.20**	0.22**	0.21**	0.25**	0.27**	0.25**	0.28**	0.28**
FR	0.12**	0.12**	0.10**	0.11**	0.16**	0.13**	0.12**	0.13**	0.13**	0.13**	0.12**	0.14**	0.16**	0.17**	0.18**	0.18**	0.17**
SO	0.11**	0.10**	0.11**	0.11**	0.16**	0.12**	0.12**	0.14**	0.12**	0.12**	0.13**	0.15**	0.17**	0.18**	0.19**	0.17**
SR	0.17**	0.18**	0.22**	0.22**	0.27**	0.22**	0.19**	0.21**	0.21**	0.21**	0.19**	0.21**	0.25**	0.24**	0.26**	0.24**	0.24**
FC	0.12**	0.09**	0.14**	0.12**	0.17**	0.14**	0.13**	0.16**	0.11**	0.15**	0.15**	0.15**	0.17**	0.21**	0.21**	0.23**	0.19**
MB	0.08**	0.06**	0.10**	0.10**	0.15**	0.14**	0.14**	0.13**	0.12**	0.12**	0.13**	0.11**	0.15**	0.20**	0.20**	0.19**	0.16**
LO	0.08**	0.05**	0.06**	0.09**	0.15**	0.10**	0.13**	0.10**	0.09**	0.10**	0.10**	0.09**	0.10**	0.16**	0.16**	0.14**	0.13**
RH	0.09**	0.07**	0.12**	0.10**	0.17**	0.14**	0.14**	0.14**	0.12**	0.12**	0.12**	0.13**	0.17**	0.21**	0.21**	0.20**	0.18**
RG	0.07**	0.05**	0.08**	0.09**	0.14**	0.11**	0.11**	0.12**	0.09**	0.11**	0.10**	0.11**	0.15**	0.18**	0.19**	0.19**	0.16**
AL	0.10**	0.10**	0.12**	0.10**	0.16**	0.13**	0.13**	0.16**	0.11**	0.13**	0.13**	0.15**	0.17**	0.22**	0.23**	0.20**	0.19**
RR	0.05**	0.03**	0.08**	0.07**	0.11**	0.10**	0.10**	0.10**	0.08**	0.09**	0.09**	0.09**	0.11**	0.14**	0.15**	0.14**	0.12**
RM	0.07**	0.05**	0.10**	0.09**	0.10**	0.10**	0.11**	0.11**	0.08**	0.09**	0.09**	0.09**	0.15**	0.17**	0.17**	0.11**	0.12**
CR	0.04**	0.05**	0.09**	0.09**	0.12**	0.11**	0.11**	0.12**	0.10**	0.10**	0.13**	0.10**	0.13**	0.15**	0.16**	0.17**	0.14**
JC	0.05**	0.05**	0.09**	0.08**	0.14**	0.12**	0.10**	0.13**	0.11**	0.10**	0.11**	0.09**	0.13**	0.16**	0.16**	0.15**	0.12**
BR	0.03**	0.03**	0.08**	0.09**	0.12**	0.12**	0.13**	0.10**	0.09**	0.09**	0.11**	0.08**	0.11**	0.14**	0.14**	0.15**	0.13**
OB	0.04**	0.04**	0.08**	0.05**	0.12**	0.09**	0.09**	0.09**	0.07**	0.08**	0.08**	0.09**	0.11**	0.14**	0.16**	0.15**	0.12**
AZ	0.02*	0.04**	0.05**	0.05**	0.12**	0.10**	0.10**	0.08**	0.08**	0.09**	0.11**	0.07**	0.12**	0.14**	0.16**	0.15**	0.12**
OA	0.09**	0.07**	0.10**	0.09**	0.16**	0.10**	0.11**	0.09**	0.08**	0.07**	0.07**	0.10**	0.12**	0.16**	0.17**	0.13**	0.12**
BS	0.05**	0.07**	0.10**	0.12**	0.13**	0.14**	0.15**	0.12**	0.11**	0.12**	0.11**	0.11**	0.15**	0.17**	0.17**	0.17**	0.14**
IC	0.03**	0.03**	0.05**	0.05**	0.09**	0.09**	0.06**	0.08**	0.07**	0.06**	0.09**	0.08**	0.10**	0.13**	0.14**	0.12**	0.10**
CA	0.01*	0.01ns	0.07**	0.05**	0.10**	0.10**	0.10**	0.09**	0.07**	0.08**	0.08**	0.08**	0.13**	0.16**	0.18**	0.14**	0.11**
RC	0.03**	0.02**	0.06**	0.04**	0.10**	0.09**	0.08**	0.07**	0.08**	0.05**	0.08**	0.06**	0.10**	0.12**	0.14**	0.10**	0.08**
GF	0.03**	0.02*	0.04**	0.03**	0.08**	0.06**	0.07**	0.08**	0.05**	0.04**	0.04**	0.06**	0.08**	0.10**	0.11**	0.08**	0.07**
DR	0.03**	0.02*	0.08**	0.03**	0.09**	0.09**	0.06**	0.10**	0.07**	0.05**	0.08**	0.08**	0.12**	0.14**	0.15**	0.12**	0.12**
CM	0.05**	0.03**	0.10**	0.05**	0.11**	0.10**	0.09**	0.10**	0.08**	0.07**	0.08**	0.09**	0.10**	0.12**	0.14**	0.12**	0.10**
CD	0.02*	0.01**	0.04**	0.04**	0.08**	0.08**	0.06**	0.06**	0.06**	0.04**	0.05**	0.05**	0.07**	0.09**	0.10**	0.08**	0.06**
LG	0.04**	0.02**	0.06**	0.05**	0.09**	0.09**	0.08**	0.07**	0.07**	0.05**	0.05**	0.06**	0.09**	0.10**	0.10**	0.09**	0.07**
ML	0.03**	0.01**	0.03**	0.03**	0.06**	0.06**	0.07**	0.06**	0.06**	0.04**	0.04**	0.06**	0.08**	0.10**	0.11**	0.08**	0.06**
MR	0.01ns	0.01**	0.05**	0.03**	0.08**	0.08**	0.07**	0.07**	0.05**	0.04**	0.05**	0.05**	0.07**	0.10**	0.12**	0.08**	0.06**
MP	0.00ns	0.03**	0.06**	0.07**	0.08**	0.11**	0.09**	0.09**	0.10**	0.07**	0.13**	0.07**	0.12**	0.13**	0.14**	0.15**	0.13**
EK	0.06**	0.08**	0.07**	0.08**	0.12**	0.11**	0.09**	0.14**	0.09**	0.11**	0.17**	0.12**	0.15**	0.17**	0.19**	0.16**	0.16**
DP	0	0.03**	0.04**	0.03**	0.09**	0.09**	0.07**	0.07**	0.08**	0.06**	0.10**	0.04**	0.09**	0.12**	0.13**	0.12**	0.09**
FM	0.04	0	0.07**	0.06**	0.09**	0.10**	0.09**	0.08**	0.08**	0.06**	0.08**	0.05**	0.10**	0.12**	0.13**	0.12**	0.08**
WP	0.07	0.12	0	0.03**	0.02*	0.03**	0.07**	0.02**	0.09**	0.01ns	0.04**	0.02**	0.04**	0.10**	0.08**	0.03**	0.04**
BT	0.05	0.1	0.03	0	0.08**	0.07**	0.06**	0.08**	0.07**	0.05**	0.08**	0.07**	0.10**	0.14**	0.15**	0.12**	0.10**
ST	0.13	0.14	0.01	0.12	0	0.05**	0.05**	0.09**	0.06**	0.05**	0.10**	0.08**	0.10**	0.13**	0.14**	0.10**	0.09**
MC	0.18	0.2	0.03	0.13	0.07	0	0.03**	0.06**	0.05**	0.04**	0.07**	0.07**	0.06**	0.08**	0.10**	0.09**	0.08**
MO	0.11	0.17	0.07	0.1	0.07	0.04	0	0.07**	0.04**	0.04**	0.09**	0.08**	0.09**	0.10**	0.11**	0.10**	0.09**
RP	0.13	0.14	0.02	0.13	0.14	0.1	0.12	0	0.05**	0.02**	0.04**	0.05**	0.06**	0.08**	0.08**	0.07**	0.05**
NB	0.15	0.15	0.13	0.12	0.1	0.08	0.07	0.09	0	0.05**	0.07**	0.08**	0.08**	0.09**	0.12**	0.13**	0.09**
WM	0.1	0.1	0	0.08	0.08	0.06	0.06	0.03	0.08	0	0.02**	0.02**	0.03**	0.06**	0.06**	0.03**	0.02**
MM	0.18	0.14	0.05	0.13	0.14	0.11	0.14	0.06	0.12	0.03	0	0.05**	0.05**	0.12**	0.10**	0.05**	0.04**
SA	0.07	0.08	0.03	0.13	0.12	0.13	0.14	0.08	0.14	0.04	0.09	0	0.04**	0.05**	0.05**	0.05**	0.02*
HC	0.14	0.17	0.05	0.16	0.14	0.1	0.13	0.1	0.14	0.05	0.07	0.07	0	0.05**	0.04**	0.03*	0.01*
GC	0.18	0.18	0.12	0.21	0.18	0.11	0.13	0.11	0.14	0.09	0.16	0.07	0.07	0	0.01**	0.08**	0.06**
SM	0.19	0.19	0.08	0.22	0.19	0.14	0.16	0.11	0.17	0.08	0.13	0.07	0.05	0.01	0	0.07**	0.04**
GM	0.19	0.19	0.02	0.19	0.13	0.14	0.14	0.11	0.2	0.04	0.06	0.07	0.03	0.11	0.08	0	-0.0ns
NW	0.14	0.13	0.02	0.17	0.13	0.12	0.13	0.08	0.15	0.04	0.04	0.03	0.01	0.07	0.05	-0.03	0

Figure A1. Principal coordinate analysis of individuals from all 49 sampling locations, with axis 1 and axis 2 explaining 5.42% and 3.25% of variance, respectively. Legend contains sampling location colour and abbreviations. For full names of sampling locations see Table 2.

Figure A2 Admixture plots produced using no prior location information. Admixture plots for *K*2–*K*8 for all individuals sampled (*K*2–*K*8), and *K*2–*K*4 for the region between the Bow and the Athabasca rivers and the region north of the Athabasca River (*K*2*–*K*4*, each region analysed separately). The clusters represent south of the Bow River (dark orange), between the Bow and the North Saskatchewan rivers (light green), between the North Saskatchewan and the Athabasca rivers (dark blue), between the Athabasca and the Smoky rivers (dark green), and north of the Smoky River (red). Black bars represent the major rivers.
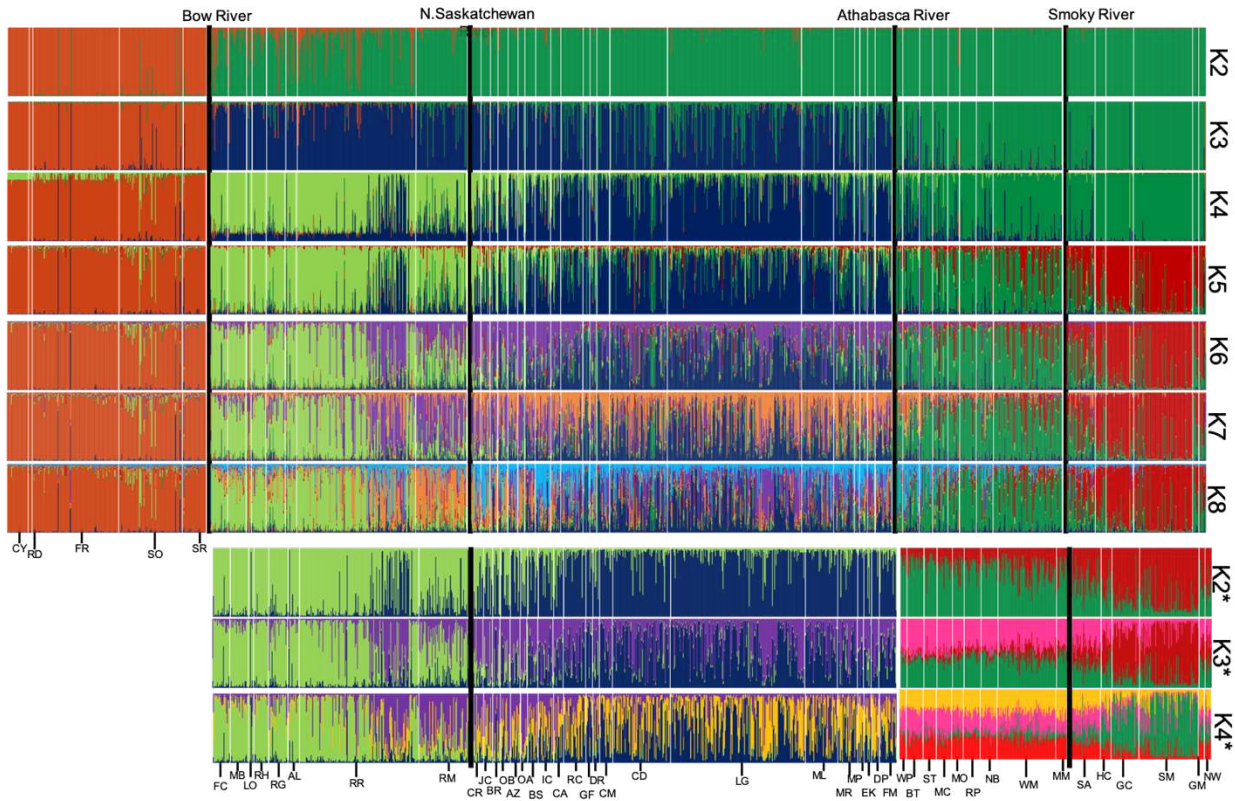
Figure A3 Admixture plots produced using prior location information. Admixture plots for *K*2–*K*8 for all individuals sampled (*K*2–*K*8), and *K*2–*K*4 for the region between the Bow and the Athabasca rivers and the region north of the Athabasca River (*K*2*–*K*4*, each region analysed separately). The clusters represent south of the Bow River (dark orange), between the Bow and the North Saskatchewan rivers (light green), between the North Saskatchewan and the Athabasca rivers (dark blue), between the Athabasca and the Smoky rivers (dark green), and north of the Smoky River (red). Black bars represent the major rivers.
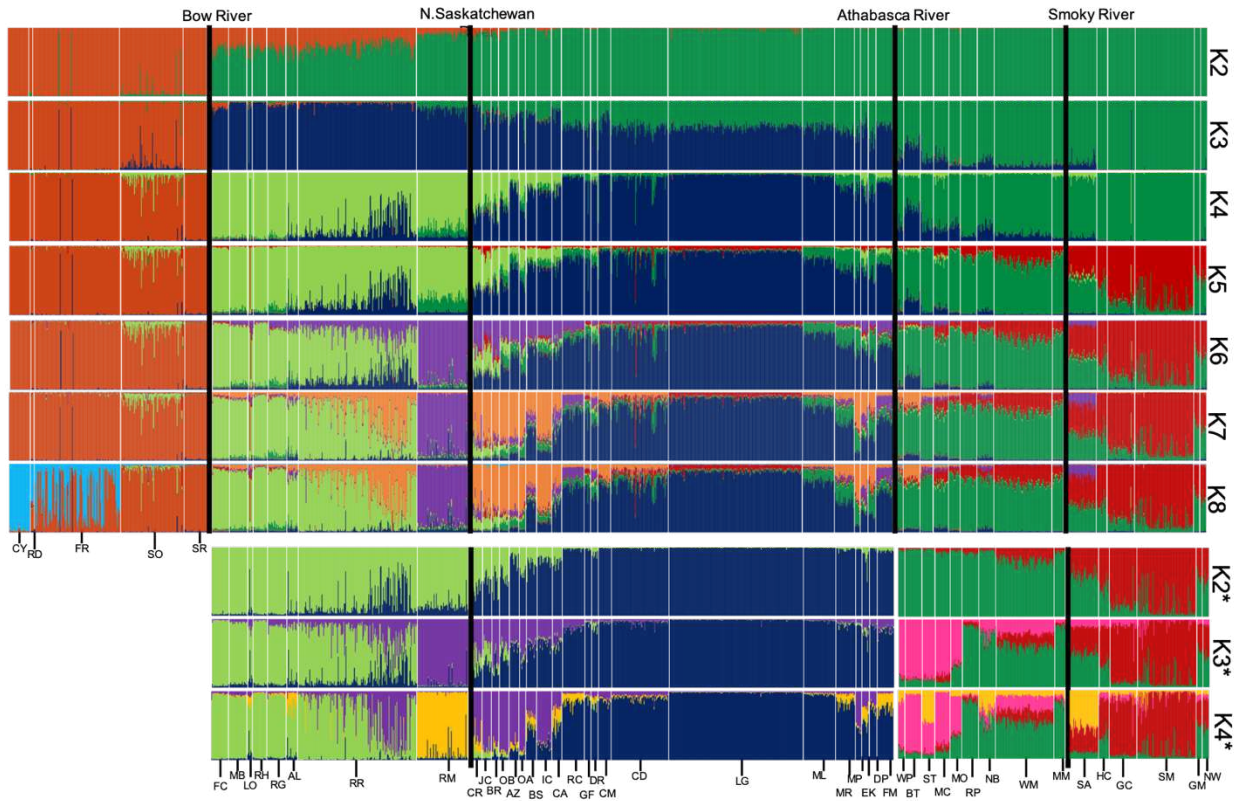
Figure A4 Likelihood plots for all Structure analyses. (a1, a2) Likelihood plots for Structure runs of *K*1–*K*8 performed on all individuals sampled. (b1, b2) Likelihood plots for admixtures of *K*1–*K*4 performed on individuals from sampling locations north of the Athabasca River. (c1, c2) Likelihood plots for admixtures of *K*1–*K*4 performed on individuals from sampling locations between the Bow and the Athabasca rivers. a1, b1, and c1 were performed with prior information about sampling locations, whereas a2, b2, and c2 were performed with no prior information about sampling locations.
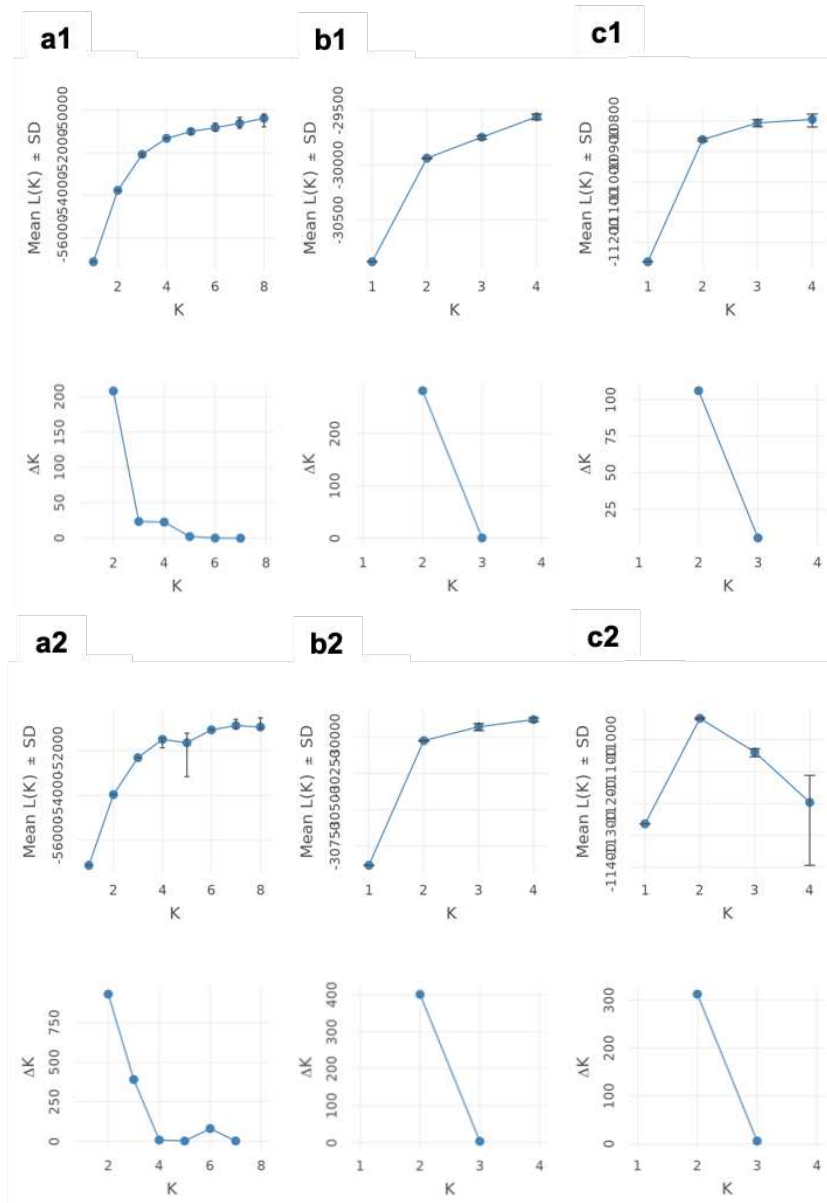
Table A2. Mitochondrial genetic distance matrix. Pairwise F_{ST} distances (upper diagonal) and Nei's standard genetic distances (lower diagonal) between 17 sampling locations, calculated from mtDNA sequence data.

	AZ	BT	CA	CM	CR	CY	FC	JC	LG	MC	ML	NB	NW	RG	RM	SA	SR	ST
AZ	0	0.30	0.30	0.42	0.33	0.23	0.55	0.33	0.46	0.26	0.33	0.37	0.27	0.11	0.05	0.33	0.29	0.28
BT	0.25	0	0.29	0	0	0.05	0.67	0	0	0.18	0	0	0	0.19	0.58	0	0.22	0.18
CA	0.35	0.31	0	0.37	0.29	0.09	0.48	0.29	0.41	0.23	0.29	0.32	0.25	0.26	0.47	0.29	0.27	0.05
CM	0.21	0.15	0.28	0	0	0.13	0.73	0	0	0.27	0	0	0.05	0.33	0.68	0	0.30	0.27
CR	0.21	0.15	0.28	0	0	0.05	0.67	0	0	0.18	0	0	0	0.24	0.61	0	0.22	0.18
CY	0.24	0.19	0.20	0.12	0.12	0	0.54	0.05	0.17	0.09	0.05	0.08	0.01	0.12	0.50	0.05	0.14	-0.11
FC	0.37	0.33	0.41	0.30	0.30	0.32	0	0.67	0.76	0.53	0.67	0.69	0.58	0.56	0.65	0.67	0.20	0.56
JC	0.21	0.15	0.28	0	0	0.12	0.30	0	0	0.18	0	0	0	0.24	0.61	0	0.22	0.18
LG	0.21	0.15	0.28	0	0	0.12	0.30	0	0	0.31	0	0	0.07	0.38	0.71	0	0.34	0.31
MC	0.27	0.22	0.32	0.16	0.16	0.20	0.34	0.16	0.16	0	0.18	0.21	-0.04	0.18	0.50	0.18	0.18	0.16
ML	0.21	0.15	0.28	0	0	0.12	0.30	0	0	0.16	0	0.00	0	0.24	0.61	0	0.22	0.18
NB	0.21	0.15	0.28	0	0	0.12	0.30	0	0	0.16	0	0	0.02	0.27	0.64	0	0.25	0.21
NW	0.24	0.18	0.30	0.10	0.10	0.16	0.32	0.10	0.10	0.06	0.10	0.10	0	0.15	0.54	0	0.17	0.12
RG	0.16	0.16	0.36	0.23	0.23	0.26	0.38	0.23	0.23	0.28	0.23	0.23	0.25	0	0.16	0.24	0.22	0.20
RM	0.09	0.31	0.40	0.29	0.29	0.32	0.42	0.29	0.29	0.33	0.29	0.29	0.31	0.19	0	0.61	0.51	0.53
SA	0.21	0.15	0.28	0	0	0.12	0.30	0	0	0.16	0	0	0.10	0.23	0.29	0	0.22	0.18
SR	0.28	0.23	0.33	0.18	0.18	0.22	0.13	0.18	0.18	0.24	0.18	0.18	0.21	0.29	0.34	0.18	0	0.21
ST	0.30	0.26	0.24	0.21	0.21	0.15	0.37	0.21	0.21	0.27	0.21	0.21	0.24	0.31	0.36	0.21	0.28	0

Appendix B
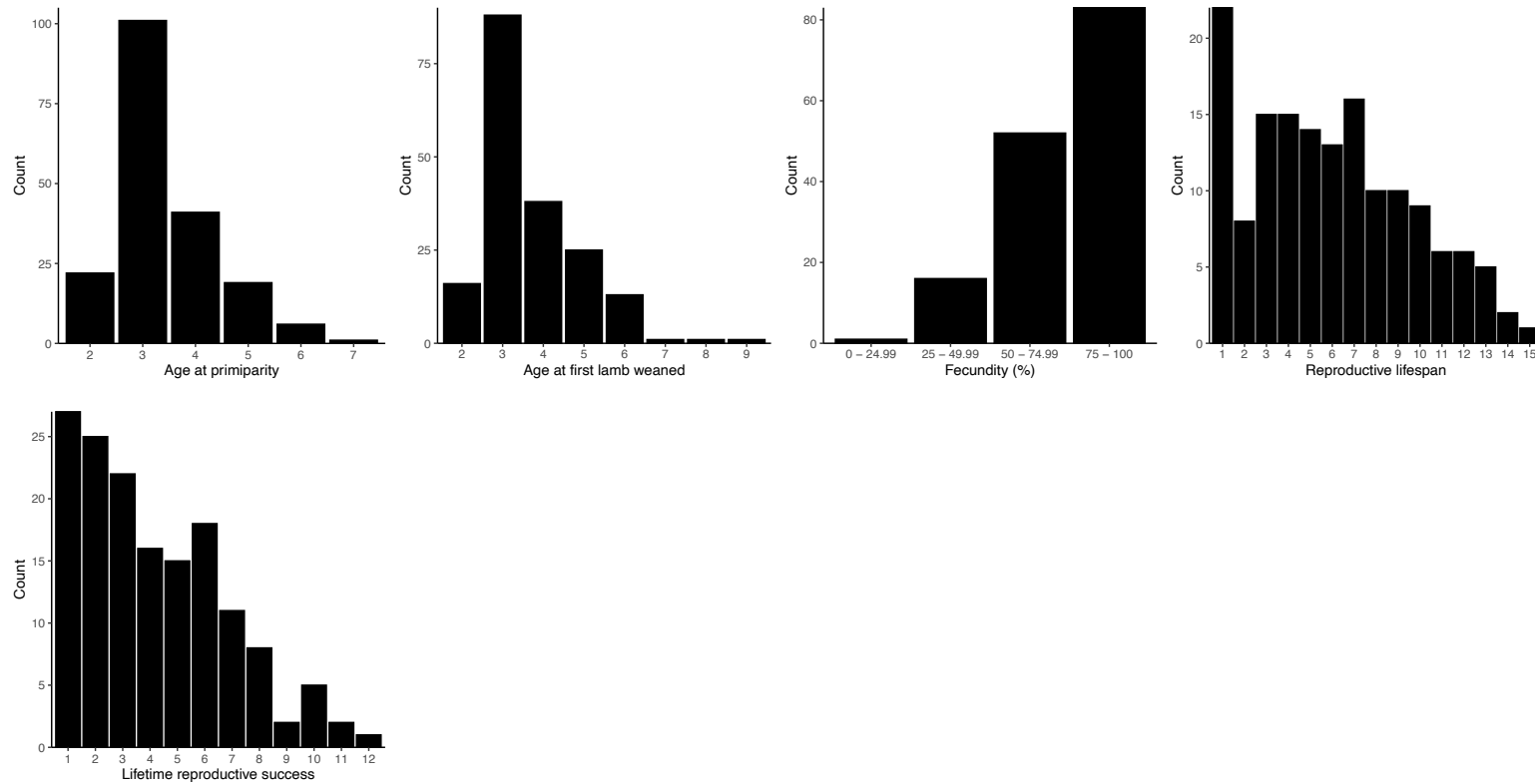
Figure B1. Counts of age at primiparity (only females that experienced primiparity *n* = 189), age at first lamb weaned (Only females that weaned at least one offspring *n* = 182), fecundity, reproductive lifespan (only females who were reproductive for 1 year or more *n* = 143), and lifetime reproductive success for females from the Ram Mountain population from 1973 to 2018.

Table B1. Schoenfeld residuals for each best fitting Cox mixed effect models testing the association of horn length and body mass at two years with age at primiparity, age at first offspring weaned, and reproductive lifespan for female bighorn sheep at Ram Mountain, Alberta between 1973 and 2018.

Model	Age at primiparity			Age at first offspring weaned			Reproductive lifespan		
	Chi^2	df	p	Chi^2	df	p	Chi^2	df	p
Adjusted mass at two years	0.05	1	0.83	0.36	1	0.55			
Adjusted horn length at two years	2.41	1	0.12	1.69	1	0.19			
Density experienced as a lamb	4.17	1	0.04	1.28	1	0.26	0.45	1	0.50
Winter precipitation experienced as a lamb	0.05	1	0.83	0.46	1	0.50	0.10	1	0.75
Winter temperature experienced as a lamb	1.03	1	0.31	0.96	1	0.33	0.26	1	0.61
Yearly density	0.38	1	0.54	0.09	1	0.76	0.07	1	0.78
Yearly winter precipitation	0.02	1	0.90	0.95	1	0.33	0.70	1	0.40
Yearly winter temperature	0.09	1	0.77	0.26	1	0.61	0.53	1	0.46
Global	10.34	8	0.24	8.61	8	0.38	3.38	6	0.76

Table B2. Results of alternate Cox mixed effect models testing the association of female horn length and body mass at two years with age at primiparity. Best model for each trait shaded in bold.

Model	Age at primiparity (birth density removed)			Age at first lamb weaned (birth density interacted with age)		
	df	AICc	ΔAICc	df	AICc	ΔAICc

Environment + horn length + body mass	78.78	1699.07	0	39.38	1557.59	0
Environment + body mass	79.39	1702.31	3.24	38.19	1559.06	1.47
Environment + horn length	81.26	1715.84	16.77	39.04	1559.07	1.48
Environment	80.85	1722.84	23.77	38.61	1565.06	7.47

Table B3. Coefficients, hazard ratios (HR), and p values from alternative Cox mixed effect survival models of associations of environmental variation, body mass and horn length on age at primiparity.

	Age at primiparity (birth density removed)			Age at first lamb weaned (birth density interacted with age)		
Variable	Coefficient (se)	HR	p	Coefficient (se)	HR	p
Fixed effects						
Adjusted mass at two years	0.341 (0.131)	1.407	0.009	0.209 (0.102)	1.233	0.013
Adjusted horn length at two years	0.307 (0.130)	1.360	0.018	0.205 (0.105)	1.227	0.051
Winter precipitation experienced as a lamb	-0.093 (0.325)	0.911	0.770	-0.070 (0.158)	0.933	0.660

	0.465 (0.311)	1.592	0.130	0.082 (0.145)	1.085	0.570
Winter temperature experienced as a lamb	0.465 (0.311)	1.592	0.130	0.082 (0.145)	1.085	0.570
Yearly density	-0.499 (0.402)	0.607	0.210	-0.158 (0.264)	0.854	0.550
Yearly winter precipitation	0.446 (0.271)	1.563	0.100	-0.070 (0.158)	0.933	0.660
Yearly winter temperature	0.110 (0.292)	1.116	0.710	0.082 (0.145)	1.085	0.570
Density experienced as a lamb				-1.392 (0.527)	0.249	0.008
Age * density experienced as a lamb				0.291 (0.128)	1.338	0.023
	Variance			Variance		
Random effects						
Cohort	1.681			0.134		
ID	0.231			<0.001		
Year	1.563			0.487		

Table B4. Estimates from second best generalised mixed effect models examining associations horn length, body mass, and environment with lifetime reproductive success (LRS) for female bighorn sheep at Ram Mountain, Alberta, cohorts 1973 to 2012.

Variable	LRS Coefficient (se)	p	r^2
Fixed effects			
Adjusted body mass at two years	0.068 (0.052)	0.194	0.002
Adjusted horn length at two years	0.104 (0.054)	0.053	0.033
Density experienced as a lamb	-0.013 (0.079)	0.866	0.004
Winter precipitation experienced as a lamb	-0.003 (0.056)	0.956	0.005
Winter temperature experienced as a lamb	-0.056 (0.057)	0.320	0.028
	Variance		
Random effects			
Grouped cohort	0.044		
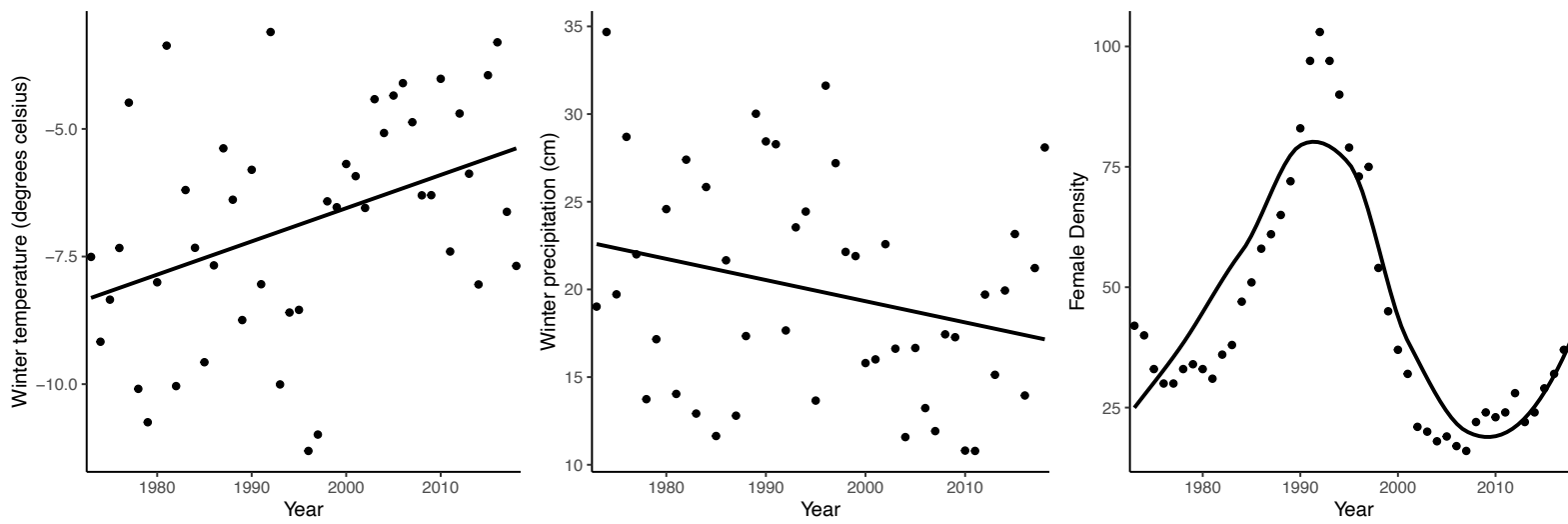
Figure B2. Winter temperature (November-March) (a) winter precipitation (November-March) (b), and female density (c)

Appendix C

<u>TrimGalore commands used to trim raw reads</u>

trim_galore -a2 GAGAGCGATCCTTGC --paired --phred33 --three_prime_clip_R2 5 --three_prime_clip_R1 5 --quality 30 forward_reads.fastq reverse_reads.fastq

Details of TrimGalore commands:

"--paired" instructed the program paired reads were to be used.

"--phred33" indicated to the program a phred33 quality scale were being used.

"--adapter2" followed by adaptor sequence removed matching adaptors from the R2 read.

"--three_prime_clip_R1/R2 5" trimmed five bp from the three-prime end of both the R1 and R2 reads.

<u>Allignment script used for Miller et al. 2013 reads and alligning our reads.</u>

bowtie2-build -f -q ref_seq.fa ref_seq.bwt2ref

samtools faidx ref_seq.fa

bowtie2 --phred33 --sensitive -N 0 -I 0 -X 500 -t -p 16 -x ref_seq.1.bwt2ref -1 trimmed_forward_reads.fq -2 trimmed_reverse_reads.fq -S aligned_reads.sam

samtools view -bt ref_seq.fa.fai aligned_reads.sam > aligned_reads.bam

samtools view -b aligned_reads.bam > aligned_reads.mapped.bam

samtools sort aligned_reads.mapped.bam > aligned_reads.mapped.sorted.bam

Details of bowtie2 alignment commands:

 "--phred33" flag was used to dictate quality was scored on a phred33 scale.

 "--sensitive" set:

"-D" which limits the number of dynamic programming problems (for example seed extensions) allowed to 15

"-R" which sets the maximum number of times bowtie2 will re-seed when attempting to align a read with repetitive seeds to 2

"-N" which sets the number of mismatches allowed during multispeed alignment to 0, "-L" which defines the length of substrings to align during multi-seed alignment to 22

"-I" which dictates a function governing the intervals between substrings in multi-seed alignment to S,1,1.15

"-X" to indicated the maximum distance a forward and reverse pair can span and still be valid (set at 500)

"-I" was set to 0, imposing no minimum length on paired read alignments.

Variant site filtering using vcftools and bcftools

"Thinning" or removing variant sites within a defined proximity to each other. Command: vcftools --vcf input_VCF.vcf --thin 100 --recode --recode-INFO-all --output output_VCF. This command will filter out variant sites within 100 bp of each other and output a file called "output_VCF.recode.vcf".

Insertion/deletion variant were removed using the command: vcftools --vcf input_VCF.vcf --remove-indels --recode --recode-INFO-all --output output_VCF. This command will filter out insertion/deletion variant sites and output a file called "output_VCF.recode.vcf".

Loci were filtered on minor allele frequencies using the command : vcftools --vcf input_VCF.vcf --maf 0.05 --recode --recode-INFO-all --output output_VCF. This command will filter out variant sites with a minor allele frequency of less than 0.05 and output a file called "output_VCF.recode.vcf".

Loci were filtered on missing data using the command : vcftools --vcf input_VCF.vcf --max-missing 0.1 --recode --recode-INFO-all --output output_VCF. This command will filter out variant sites with more than 10% missing data and output a file called "output_VCF.recode.vcf".

Loci were filtered on genotype quality using the command : bcftools filter -e 'QUAL <30' -o output.vcf input.vcf. This command will filter out variant sites with a quality score lower than 30 and output a file called "output.vcf".

Loci were filtered on genotype depth using the command : bcftools filter -e 'INFO/DP <6' -o output.vcf input.vcf. This command will filter out variant sites with a depth lower than 6 and output a file called "output.vcf".

Loci were filtered on allelic depth using the command : bcftools filter -e 'INFO/AC <0' -o output.vcf input.vcf. This command will filter out variant sites with an allelic count 0 or lower and output a file called "output.vcf".

Appendix D
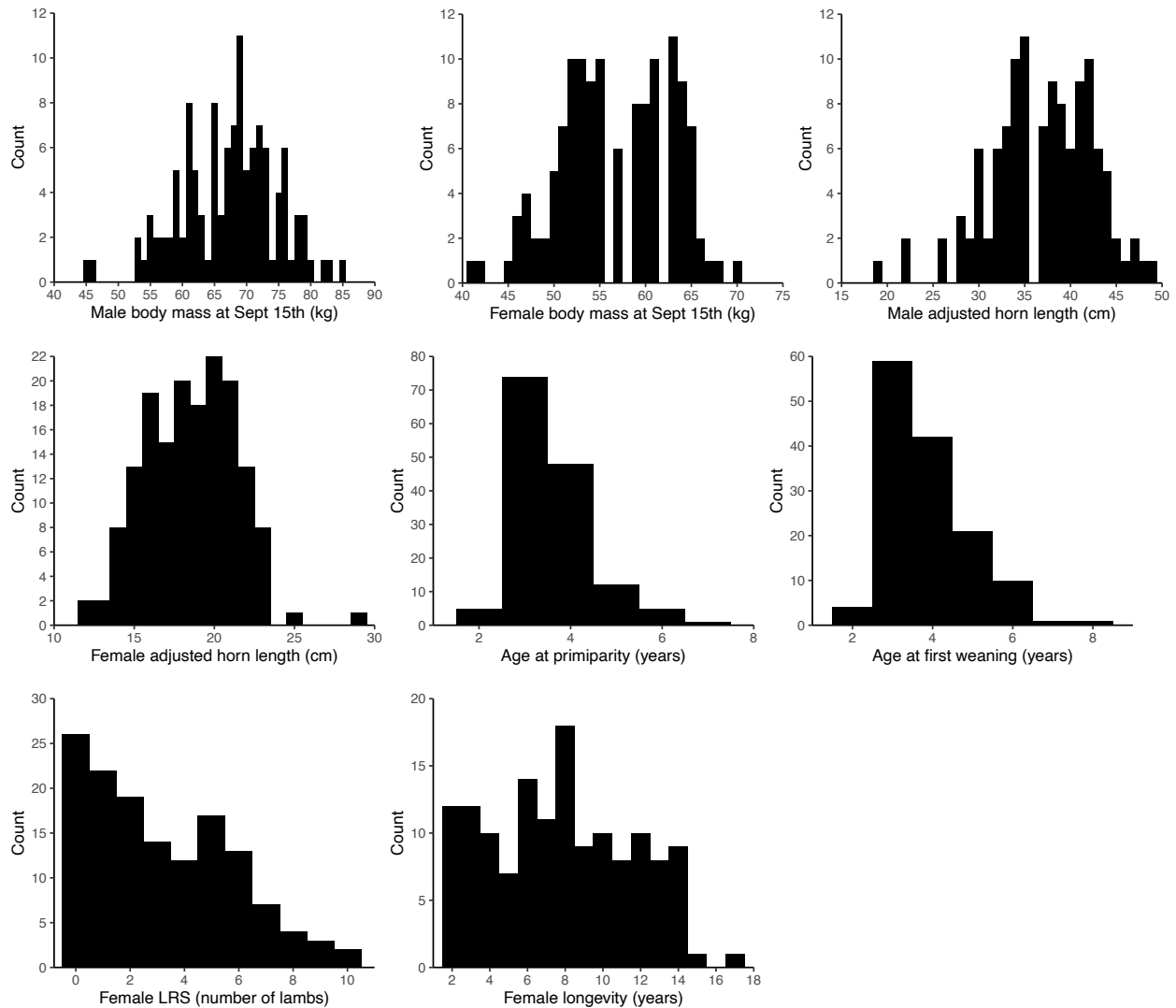
Figure S1 Counts of: male body mass at two years adjusted to September 15th(n= 119), female body mass at two years adjusted to September 15th(n= 170), female body mass at two years adjusted to June 15th(n= 173), male horn length at two years adjusted to September 15th (n= 132), female horn length at two years adjusted to September 15th (n= 162), female age at primiparity (*n* = 145), female age at first lamb weaned (*n* = 138),female lifetime reproductive success (n= 139), and female longevity (n = 140) for individuals from the Ram Mountain population from 1979 to 2022.

Table D1 Information on the 278 loci of significant association identified for all six of our examined traits. Including SNP ID, associated trait, chromosome, base pairs position on chromosome, allele frequency, *p*-value, effect (given in same units as trait), and the value of effects standard error

Trait	SNP ID	Chromosome	Position	Allele frequency	p	Effect	Effect s.e.
LRS	OAR1_11060256	1	11060256	0.2895	0.0014	0.5838	0.1854
Female longevity	OAR1_45335673	1	45335673	0.0746	0.0011	2.9664	0.9242
Male adjusted body mass	OAR1_49138872	1	49138872	0.1581	0.0004	-4.8325	1.3983
Male adjusted horn length	OAR1_49138872	1	49138872	0.1581	0.0001	-3.8276	0.9941
Female age at primiparity	OAR1_54509694	1	54509694	0.4820	0.0005	-0.3327	0.0979
Male adjusted body mass	OAR1_54952090	1	54952090	0.4530	0.0004	-3.3074	0.9478
Male adjusted body mass	OAR1_57180838	1	57180838	0.4829	0.0006	3.6284	1.0781
Male adjusted body mass	OAR1_58851106	1	58851106	0.1581	0.0006	4.2160	1.2490
Male adjusted horn length	OAR1_58851106	1	58851106	0.1581	0.0004	3.0671	0.8859
Female longevity	OAR1_63992816	1	63992816	0.0522	0.0009	3.2344	0.9881
Male adjusted body mass	OAR1_69575994	1	69575994	0.1880	0.0013	-4.1977	1.3264
Male adjusted horn length	OAR1_69575994	1	69575994	0.1880	0.0005	-3.2037	0.9430
Female adjusted horn length	OAR1_71357644	1	71357644	0.2013	0.0005	1.4452	0.4201
Female age at primiparity	OAR1_71817133	1	71817133	0.0971	0.0005	0.5530	0.1622
Female age at first weaning	OAR1_71817133	1	71817133	0.0985	0.0011	0.6518	0.2029
Female age at first weaning	OAR1_77992992	1	77992992	0.1515	0.0015	0.5270	0.1684
Male adjusted horn length	OAR1_110247909	1	110247909	0.3462	0.0003	-3.5581	1.0142
Female longevity	OAR1_118908311	1	118908311	0.3507	0.0002	2.2568	0.6254
Female age at primiparity	OAR1_143270931	1	143270931	0.0612	0.0002	0.7696	0.2123
Female age at first weaning	OAR1_143270931	1	143270931	0.0568	0.0012	0.8866	0.2772
Female adjusted horn length	OAR1_156561646	1	156561646	0.0617	0.0004	2.3979	0.6923

Female adjusted horn length	OAR1_158556873	1	158556873	0.1201	0.0005	-1.9725	0.5766
Female adjusted horn length	OAR1_162014414	1	162014414	0.4610	0.0005	1.1828	0.3449
Female adjusted horn length	OAR1_167704342	1	167704342	0.2435	0.0010	-1.3300	0.4079
Male adjusted horn length	OAR1_168744167	1	168744167	0.1496	0.0011	-3.1225	0.9714
Female adjusted horn length	OAR1_169247378	1	169247378	0.3539	0.0006	1.2566	0.3695
Male adjusted horn length	OAR1_170325566	1	170325566	0.2991	0.0003	-2.8963	0.8217
Male adjusted horn length	OAR1_171245188	1	171245188	0.3291	0.0001	-2.8068	0.7529
Male adjusted horn length	OAR1_177484024	1	177484024	0.2308	0.0008	-2.9634	0.9012
Female adjusted horn length	OAR1_177885814	1	177885814	0.3604	0.0005	1.2538	0.3666
Female adjusted horn length	OAR1_182631664	1	182631664	0.4318	0.0009	-1.2068	0.3690
Female adjusted body mass	OAR1_183871505	1	183871505	0.1515	0.0005	3.0705	0.8990
Female adjusted body mass	OAR1_184102161	1	184102161	0.3030	0.0008	-2.1329	0.6412
LRS	OAR1_192647234	1	192647234	0.4060	0.0018	0.5064	0.1642
Female adjusted body mass	OAR1_205647718	1	205647718	0.0970	0.0001	-4.0175	1.0605
Female adjusted horn length	OAR1_217130138	1	217130138	0.4286	0.0000	-1.4177	0.3507
Female age at primiparity	OAR1_229430493	1	229430493	0.1619	0.0003	0.4304	0.1214
LRS	OAR1_231609918	1	231609918	0.1316	0.0011	0.8556	0.2662
Female adjusted horn length	OAR1_237210818	1	237210818	0.1526	0.0005	-1.7682	0.5173
Male adjusted body mass	OAR1_243481492	1	243481492	0.1496	0.0008	-4.6011	1.4042
Female longevity	OAR1_255752862	1	255752862	0.3507	0.0003	1.6944	0.4755
Male adjusted horn length	OAR1_258490493	1	258490493	0.2692	0.0013	-2.4589	0.7753
Female age at primiparity	OAR1_265603011	1	265603011	0.4856	0.0014	-0.3089	0.0978
Male adjusted body mass	OAR1_268635142	1	268635142	0.1239	0.0012	4.8456	1.5256
Male adjusted horn length	OAR1_268635142	1	268635142	0.1239	0.0015	3.3891	1.0837
Male adjusted body mass	OAR1_269516527	1	269516527	0.2949	0.0012	4.1386	1.2992
Male adjusted body mass	OAR2_28656232	2	28656232	0.1239	0.0007	-5.5969	1.6840
Male adjusted horn length	OAR2_28656232	2	28656232	0.1239	0.0005	-4.0532	1.1954

LRS	OAR2_29085895	2	29085895	0.3421	0.0016	-0.5415	0.1744
Female age at primiparity	OAR2_43529914	2	43529914	0.4460	0.0014	0.4019	0.1273
Female age at primiparity	OAR2_52502605	2	52502605	0.2626	0.0007	-0.3464	0.1034
Female age at first weaning	OAR2_52610666	2	52610666	0.1098	0.0002	-0.4809	0.1340
Female adjusted body mass	OAR2_105508317	2	105508317	0.3394	0.0007	4.3035	1.2931
Female age at first weaning	OAR2_108842429	2	108842429	0.2159	0.0010	0.5951	0.1833
Female age at primiparity	OAR2_118857615	2	118857615	0.2122	0.0012	0.3372	0.1058
LRS	OAR2_120866862	2	120866862	0.1090	0.0017	0.6234	0.2010
Female longevity	OAR2_132198827	2	132198827	0.0634	0.0009	-2.0710	0.6335
Male adjusted body mass	OAR2_135223959	2	135223959	0.2479	0.0009	-4.4739	1.3675
Female adjusted body mass	OAR2_142783504	2	142783504	0.3061	0.0000	-3.3769	0.8497
Female adjusted body mass	OAR2_147158440	2	147158440	0.3848	0.0010	-2.6623	0.8202
Female longevity	OAR2_151245272	2	151245272	0.2127	0.0007	-2.0700	0.6201
Female adjusted horn length	OAR2_155573449	2	155573449	0.4870	0.0008	1.2756	0.3846
Female adjusted horn length	OAR2_160716088	2	160716088	0.0909	0.0009	1.2301	0.3759
Female adjusted body mass	OAR2_162344074	2	162344074	0.0758	0.0000	-4.2580	1.0410
Female longevity	OAR2_198182638	2	198182638	0.1530	0.0008	-2.3874	0.7262
Female longevity	OAR2_198629323	2	198629323	0.1082	0.0010	-1.6033	0.4949
Female longevity	OAR2_199039560	2	199039560	0.3918	0.0002	1.8923	0.5117
Female adjusted horn length	OAR2_237466400	2	237466400	0.1234	0.0009	-1.3618	0.4150
Male adjusted horn length	OAR3_34169839	3	34169839	0.1026	0.0009	-3.1602	0.9667
LRS	OAR3_47827179	3	47827179	0.4211	0.0015	-0.5398	0.1727
Male adjusted body mass	OAR3_53276988	3	53276988	0.2778	0.0006	-7.6659	2.2795
Female adjusted body mass	OAR3_67097685	3	67097685	0.4545	0.0011	-2.7494	0.8508
Male adjusted body mass	OAR3_72852109	3	72852109	0.3846	0.0001	5.5859	1.5044
Male adjusted body mass	OAR3_90237053	3	90237053	0.2949	0.0013	-3.3926	1.0719
Female age at first weaning	OAR3_100498643	3	100498643	0.4583	0.0015	0.4921	0.1574

Female adjusted body mass	OAR3_136938721	3	136938721	0.2970	0.0001	-2.6994	0.6920
Male adjusted body mass	OAR3_146746422	3	146746422	0.4017	0.0006	3.8452	1.1385
Female longevity	OAR3_160198753	3	160198753	0.4776	0.0007	2.8708	0.8562
Female longevity	OAR3_165329068	3	165329068	0.0597	0.0002	1.6457	0.4553
Female adjusted horn length	OAR3_174822003	3	174822003	0.0422	0.0006	1.7706	0.5236
LRS	OAR3_207797112	3	207797112	0.4962	0.0009	0.6881	0.2106
Male adjusted body mass	OAR3_210223058	3	210223058	0.1581	0.0007	7.4380	2.2275
Female adjusted horn length	OAR3_213189925	3	213189925	0.1266	0.0012	1.1353	0.3552
Female longevity	OAR4_3071617	4	3071617	0.0560	0.0010	-1.5359	0.4732
Female age at first weaning	OAR4_17677852	4	17677852	0.3220	0.0015	-0.4262	0.1365
Female longevity	OAR4_26887699	4	26887699	0.1716	0.0010	-2.1758	0.6730
Female longevity	OAR4_29599942	4	29599942	0.2201	0.0007	1.9200	0.5740
Female adjusted body mass	OAR4_30850853	4	30850853	0.4485	0.0009	-3.4245	1.0469
Female adjusted body mass	OAR4_31568859	4	31568859	0.0394	0.0006	-5.1872	1.5395
Male adjusted horn length	OAR4_32271648	4	32271648	0.0684	0.0011	5.5898	1.7410
Female age at first weaning	OAR4_40104650	4	40104650	0.2841	0.0006	-0.4800	0.1421
Female age at first weaning	OAR4_46514323	4	46514323	0.3030	0.0012	-0.7261	0.2269
Female age at primiparity	OAR4_60438913	4	60438913	0.0576	0.0007	0.3303	0.0995
Female age at primiparity	OAR4_66510068	4	66510068	0.0827	0.0005	0.4370	0.1281
Female age at first weaning	OAR4_67307489	4	67307489	0.0833	0.0016	0.4682	0.1502
Female adjusted body mass	OAR4_72040691	4	72040691	0.3242	0.0010	-3.6537	1.1256
Male adjusted horn length	OAR4_76689394	4	76689394	0.1026	0.0004	4.1938	1.2016
Female adjusted body mass	OAR4_89734551	4	89734551	0.2909	0.0008	-2.3591	0.7124
Female adjusted horn length	OAR4_92976068	4	92976068	0.4545	0.0007	1.9374	0.5811
Female adjusted body mass	OAR4_92989940	4	92989940	0.4606	0.0002	4.2635	1.1692
Female longevity	OAR5_3525623	5	3525623	0.1306	0.0010	-1.5692	0.4839
Female longevity	OAR5_6645804	5	6645804	0.3918	0.0004	-2.1727	0.6274

Female adjusted body mass	OAR5_14110658	5	14110658	0.1606	0.0003	-2.6490	0.7471
Male adjusted body mass	OAR5_30398435	5	30398435	0.4573	0.0011	3.2029	0.9998
Male adjusted horn length	OAR5_30398435	5	30398435	0.4573	0.0008	2.3384	0.7094
Female adjusted body mass	OAR5_35193477	5	35193477	0.0606	0.0009	-2.3556	0.7197
Female adjusted body mass	OAR5_36556285	5	36556285	0.1697	0.0005	3.4398	1.0002
Female age at primiparity	OAR5_43459355	5	43459355	0.2554	0.0005	0.3795	0.1102
Female longevity	OAR5_76625371	5	76625371	0.1679	0.0004	-1.7465	0.5038
Female age at first weaning	OAR5_102760318	5	102760318	0.4848	0.0015	-0.5951	0.1901
Female adjusted body mass	OAR5_103203088	5	103203088	0.1152	0.0007	2.3691	0.7054
Male adjusted body mass	OAR6_29594760	6	29594760	0.1282	0.0004	5.2685	1.5318
Male adjusted horn length	OAR6_37318032	6	37318032	0.3718	0.0011	3.8406	1.2009
Male adjusted horn length	OAR6_48120608	6	48120608	0.4915	0.0002	-4.5846	1.2589
Female adjusted horn length	OAR6_66320295	6	66320295	0.3961	0.0005	2.4837	0.7238
LRS	OAR6_66902279	6	66902279	0.3421	0.0004	1.1631	0.3329
LRS	OAR6_71041491	6	71041491	0.4962	0.0010	0.6467	0.1991
Female longevity	OAR6_105349935	6	105349935	0.0933	0.0002	-1.7394	0.4818
Female adjusted body mass	OAR6_110169473	6	110169473	0.2212	0.0001	-3.5363	0.9199
Female age at first weaning	OAR6_116590354	6	116590354	0.3750	0.0002	-0.5112	0.1420
Female age at primiparity	OAR7_17854466	7	17854466	0.0360	0.0000	-0.4102	0.1007
Female age at first weaning	OAR7_17854466	7	17854466	0.0644	0.0010	-0.4115	0.1275
Female adjusted body mass	OAR7_24935220	7	24935220	0.2121	0.0007	-2.8407	0.8513
Female adjusted horn length	OAR7_24935220	7	24935220	0.2045	0.0005	-1.5338	0.4465
Female longevity	OAR7_25168902	7	25168902	0.3657	0.0001	2.4009	0.6309
Female age at primiparity	OAR7_26480397	7	26480397	0.1871	0.0006	-0.3603	0.1069
Female adjusted body mass	OAR7_37277406	7	37277406	0.3455	0.0008	-2.6766	0.8125
LRS	OAR7_38819293	7	38819293	0.2406	0.0015	-0.7534	0.2405
Female age at first weaning	OAR7_47892646	7	47892646	0.1023	0.0011	0.4193	0.1306

Female age at first weaning	OAR7_63301467	7	63301467	0.1023	0.0005	0.4693	0.1375
Male adjusted body mass	OAR7_74911493	7	74911493	0.4188	0.0000	-7.4977	1.7916
Male adjusted horn length	OAR7_74911493	7	74911493	0.4188	0.0011	-4.1015	1.2740
Female age at primiparity	OAR7_87456762	7	87456762	0.1259	0.0003	0.3742	0.1047
Female age at first weaning	OAR7_87456762	7	87456762	0.3674	0.0004	0.4594	0.1333
Female adjusted body mass	OAR7_93300771	7	93300771	0.1121	0.0001	2.4553	0.6279
Female age at primiparity	OAR7_99887079	7	99887079	0.1223	0.0003	0.4454	0.1259
Female age at first weaning	OAR8_15412056	8	15412056	0.2538	0.0011	0.5803	0.1803
LRS	OAR8_17835012	8	17835012	0.4173	0.0003	0.7960	0.2233
Female age at primiparity	OAR8_17864827	8	17864827	0.1007	0.0012	-0.5217	0.1629
LRS	OAR8_18693300	8	18693300	0.0752	0.0013	0.5316	0.1673
Female longevity	OAR8_28485004	8	28485004	0.4104	0.0011	-1.4766	0.4580
Male adjusted body mass	OAR8_40986543	8	40986543	0.2650	0.0006	-4.4914	1.3272
Male adjusted horn length	OAR8_40986543	8	40986543	0.2650	0.0011	-3.0161	0.9434
Female longevity	OAR8_47595171	8	47595171	0.2201	0.0008	-1.3412	0.4057
LRS	OAR8_55486339	8	55486339	0.1955	0.0002	0.7745	0.2155
Female adjusted body mass	OAR8_74260543	8	74260543	0.4545	0.0003	-5.8647	1.6393
Female adjusted body mass	OAR9_4966450	9	4966450	0.0667	0.0009	-2.8220	0.8568
Male adjusted body mass	OAR9_17490961	9	17490961	0.2692	0.0013	3.5814	1.1326
Female adjusted body mass	OAR9_37886047	9	37886047	0.4545	0.0011	-2.6430	0.8177
Female adjusted horn length	OAR9_81655908	9	81655908	0.2175	0.0007	-1.4718	0.4403
LRS	OAR9_86380240	9	86380240	0.4699	0.0007	0.5537	0.1665
LRS	OAR9_88446389	9	88446389	0.1654	0.0007	-0.6613	0.1983
Female age at first weaning	OAR10_2343885	10	2343885	0.3902	0.0010	0.4686	0.1442
Female age at first weaning	OAR10_6600641	10	6600641	0.4848	0.0007	0.4335	0.1293
LRS	OAR10_18325789	10	18325789	0.1128	0.0012	0.5738	0.1796
Female adjusted horn length	OAR10_25327228	10	25327228	0.4123	0.0001	1.7522	0.4514

Female age at first weaning	OAR10_27502130	10	27502130	0.4583	0.0005	-0.7000	0.2040
Female age at primiparity	OAR10_49642699	10	49642699	0.0971	0.0007	0.4690	0.1406
Female longevity	OAR10_71463644	10	71463644	0.5000	0.0006	1.6934	0.5034
Female longevity	OAR10_72689443	10	72689443	0.4328	0.0001	2.6745	0.6883
Female age at first weaning	OAR10_82331867	10	82331867	0.2273	0.0016	-0.4612	0.1482
Male adjusted body mass	OAR10_85454908	10	85454908	0.3846	0.0001	-4.5121	1.1555
Male adjusted horn length	OAR10_85454908	10	85454908	0.3846	0.0001	-3.0542	0.8207
Male adjusted body mass	OAR10_85695769	10	85695769	0.2094	0.0010	-3.2229	0.9942
Female adjusted horn length	OAR11_13624061	11	13624061	0.3539	0.0010	1.2342	0.3796
Male adjusted body mass	OAR11_36562764	11	36562764	0.3077	0.0006	4.3980	1.3097
Female longevity	OAR12_18568991	12	18568991	0.2239	0.0013	1.9945	0.6275
Male adjusted body mass	OAR12_25413868	12	25413868	0.2265	0.0004	4.2131	1.2160
LRS	OAR12_26727032	12	26727032	0.3158	0.0010	0.6885	0.2129
Male adjusted horn length	OAR12_28767510	12	28767510	0.3504	0.0010	3.3849	1.0434
Female longevity	OAR12_35846569	12	35846569	0.4590	0.0010	1.4420	0.4437
Female longevity	OAR12_39364746	12	39364746	0.2612	0.0004	1.8784	0.5364
Female adjusted body mass	OAR12_58215003	12	58215003	0.4030	0.0001	-3.1218	0.8321
Female age at primiparity	OAR12_71606247	12	71606247	0.4496	0.0010	-0.3337	0.1029
LRS	OAR14_8154337	14	8154337	0.3346	0.0006	-0.5589	0.1667
Female age at first weaning	OAR14_29295269	14	29295269	0.2462	0.0016	0.6047	0.1938
Female age at primiparity	OAR14_41565785	14	41565785	0.2914	0.0011	0.6338	0.1971
Female age at first weaning	OAR14_41565785	14	41565785	0.4053	0.0008	0.8049	0.2437
Female adjusted body mass	OAR14_42114145	14	42114145	0.2758	0.0002	-4.6443	1.2572
LRS	OAR14_43260094	14	43260094	0.3195	0.0013	-0.5938	0.1877
Female adjusted body mass	OAR14_44253325	14	44253325	0.3485	0.0002	2.4356	0.6719
Female adjusted body mass	OAR14_49165647	14	49165647	0.3909	0.0007	2.4661	0.7370
LRS	OAR15_3326525	15	3326525	0.4737	0.0006	0.8940	0.2659

Trait	SNP	Chr	Position				
LRS	OAR15_4253038	15	4253038	0.2256	0.0019	0.5724	0.1863
Female age at primiparity	OAR15_21442584	15	21442584	0.0719	0.0015	0.2873	0.0916
Female adjusted body mass	OAR15_45493057	15	45493057	0.3242	0.0002	-2.2934	0.6176
Female age at primiparity	OAR15_55012476	15	55012476	0.0827	0.0005	0.3220	0.0937
Female adjusted horn length	OAR15_68713127	15	68713127	0.2760	0.0013	-1.0915	0.3439
Female adjusted horn length	OAR15_76762627	15	76762627	0.3312	0.0009	-1.4410	0.4417
Female adjusted horn length	OAR16_3815901	16	3815901	0.1818	0.0011	-1.3083	0.4058
Female adjusted body mass	OAR16_6316056	16	6316056	0.1152	0.0002	-3.0169	0.8206
Female adjusted horn length	OAR16_6316056	16	6316056	0.4318	0.0005	-1.5534	0.4506
Male adjusted body mass	OAR16_11845792	16	11845792	0.3974	0.0005	5.7275	1.6849
Male adjusted horn length	OAR16_30657682	16	30657682	0.1624	0.0007	3.2181	0.9713
Male adjusted body mass	OAR16_32743302	16	32743302	0.1368	0.0006	5.2848	1.5754
Male adjusted body mass	OAR16_36923016	16	36923016	0.0769	0.0006	-3.4492	1.0229
Female adjusted horn length	OAR16_40416848	16	40416848	0.1883	0.0011	-1.4894	0.4619
Male adjusted body mass	OAR16_54629740	16	54629740	0.3162	0.0002	-8.0914	2.1945
Male adjusted horn length	OAR16_54629740	16	54629740	0.3162	0.0002	-5.6977	1.5560
Female adjusted body mass	OAR16_62691230	16	62691230	0.4273	0.0002	2.1813	0.6004
LRS	OAR16_67565174	16	67565174	0.0865	0.0009	-0.8145	0.2493
Male adjusted body mass	OAR17_29580001	17	29580001	0.3205	0.0006	4.3220	1.2853
Female age at primiparity	OAR17_42308088	17	42308088	0.1655	0.0008	-0.4281	0.1295
Female age at primiparity	OAR17_46581232	17	46581232	0.1259	0.0006	-0.3564	0.1053
Female age at first weaning	OAR17_46581232	17	46581232	0.1212	0.0003	-0.4591	0.1299
Female age at primiparity	OAR17_47425703	17	47425703	0.2158	0.0004	0.3412	0.0982
Female longevity	OAR17_55255290	17	55255290	0.4030	0.0000	-2.1214	0.5179
Female longevity	OAR17_59028275	17	59028275	0.4403	0.0011	-1.5953	0.4954
Female longevity	OAR17_63826616	17	63826616	0.1493	0.0012	-1.3675	0.4274
Female age at first weaning	OAR17_68562729	17	68562729	0.4167	0.0001	0.6923	0.1852

Male adjusted horn length	OAR18_1339099	18	1339099	0.4060	0.0006	2.7233	0.8111
Female longevity	OAR18_2911846	18	2911846	0.2313	0.0006	-3.1503	0.9385
Female adjusted horn length	OAR18_10184590	18	10184590	0.0519	0.0002	1.2263	0.3366
Female age at primiparity	OAR18_16920384	18	16920384	0.0935	0.0013	-0.3346	0.1055
Female age at first weaning	OAR18_16920384	18	16920384	0.1212	0.0004	-0.4602	0.1322
Female adjusted horn length	OAR18_29887685	18	29887685	0.2565	0.0011	1.8570	0.5772
Male adjusted horn length	OAR18_31495406	18	31495406	0.4573	0.0014	-4.2506	1.3509
Female longevity	OAR18_43389828	18	43389828	0.2799	0.0005	-1.4736	0.4337
Female adjusted horn length	OAR18_46027604	18	46027604	0.2630	0.0008	-1.1817	0.3577
Female age at primiparity	OAR19_17691776	19	17691776	0.2374	0.0004	0.6955	0.2016
Female age at first weaning	OAR19_17691776	19	17691776	0.4621	0.0004	0.8856	0.2536
Female adjusted horn length	OAR19_26247528	19	26247528	0.2695	0.0004	-1.2940	0.3687
LRS	OAR19_27234301	19	27234301	0.0639	0.0009	-1.1322	0.3459
Male adjusted horn length	OAR19_35471004	19	35471004	0.1581	0.0003	3.8748	1.1066
Female longevity	OAR19_39850616	19	39850616	0.1791	0.0002	-4.4880	1.2453
Female longevity	OAR19_43672689	19	43672689	0.2164	0.0013	-2.4007	0.7579
Female adjusted body mass	OAR19_45737580	19	45737580	0.1030	0.0004	-2.0520	0.5926
Female age at first weaning	OAR19_46331439	19	46331439	0.1174	0.0014	0.3955	0.1254
Female longevity	OAR19_56902887	19	56902887	0.1828	0.0004	2.1172	0.6137
LRS	OAR19_59040766	19	59040766	0.3459	0.0018	-0.5637	0.1830
Female adjusted body mass	OAR20_8921150	20	8921150	0.3848	0.0002	-2.6687	0.7192
Male adjusted horn length	OAR20_12854347	20	12854347	0.2778	0.0001	-8.0501	2.1386
Male adjusted horn length	OAR20_18446009	20	18446009	0.4872	0.0015	-2.2863	0.7317
Female age at first weaning	OAR20_24908528	20	24908528	0.4432	0.0011	-0.5768	0.1791
Male adjusted horn length	OAR20_25218223	20	25218223	0.4444	0.0009	2.8875	0.8863
Male adjusted body mass	OAR20_25796186	20	25796186	0.4402	0.0006	3.8506	1.1436
Male adjusted body mass	OAR20_25831346	20	25831346	0.4530	0.0003	4.5260	1.2663

Male adjusted body mass	OAR20_26795935	20	26795935	0.0812	0.0000	5.6814	1.3813
Male adjusted horn length	OAR20_26795935	20	26795935	0.0812	0.0000	3.8707	0.9787
Female adjusted body mass	OAR20_35404398	20	35404398	0.0697	0.0003	2.4533	0.6937
Female adjusted horn length	OAR20_35404398	20	35404398	0.4318	0.0004	1.3447	0.3834
Female adjusted horn length	OAR20_40624051	20	40624051	0.1818	0.0009	-1.1481	0.3498
Female adjusted body mass	OAR20_41231599	20	41231599	0.1879	0.0009	-2.2121	0.6731
Male adjusted horn length	OAR20_43535105	20	43535105	0.0684	0.0009	2.9203	0.8961
Female adjusted horn length	OAR20_45780133	20	45780133	0.1201	0.0008	-1.5598	0.4698
Male adjusted horn length	OAR21_1500121	21	1500121	0.3504	0.0010	2.6248	0.8118
Female adjusted body mass	OAR21_20835472	21	20835472	0.0545	0.0008	-2.6899	0.8108
Female adjusted horn length	OAR21_27963370	21	27963370	0.2110	0.0006	-1.6074	0.4755
Female longevity	OAR21_44995452	21	44995452	0.0448	0.0013	-1.4085	0.4430
LRS	OAR22_10440006	22	10440006	0.1391	0.0006	-0.5909	0.1758
Female adjusted body mass	OAR22_30297480	22	30297480	0.4273	0.0005	-3.3660	0.9848
Female adjusted body mass	OAR23_24238883	23	24238883	0.2879	0.0005	-4.2904	1.2531
Male adjusted horn length	OAR23_38912014	23	38912014	0.4701	0.0005	-4.5731	1.3328
Male adjusted body mass	OAR23_41417635	23	41417635	0.3291	0.0014	3.9800	1.2639
Female adjusted horn length	OAR24_9650351	24	9650351	0.2338	0.0008	-1.1571	0.3488
Female age at primiparity	OAR24_14867988	24	14867988	0.3453	0.0015	-0.3840	0.1224
Female adjusted body mass	OAR24_22600888	24	22600888	0.3212	0.0000	2.8365	0.7013
Male adjusted body mass	OAR24_38913627	24	38913627	0.3462	0.0003	-3.8453	1.0948
Female age at primiparity	OAR25_7843678	25	7843678	0.1079	0.0012	0.4755	0.1492
Male adjusted body mass	OAR25_22969112	25	22969112	0.4188	0.0001	3.7717	1.0111
Female age at primiparity	OAR25_24773806	25	24773806	0.1475	0.0004	-0.3636	0.1038
Female age at primiparity	OAR25_30217488	25	30217488	0.0612	0.0001	0.6791	0.1794
Female longevity	OAR26_19079146	26	19079146	0.2463	0.0006	-2.1759	0.6460
Female age at first weaning	OAR26_20933251	26	20933251	0.2008	0.0008	-0.7645	0.2325

Female age at primiparity	OAR26_23881373	26	23881373	0.2878	0.0009	-0.3788	0.1156
Female age at first weaning	OAR26_25158608	26	25158608	0.2235	0.0000	-0.5446	0.1343
Female longevity	OAR26_25948723	26	25948723	0.3918	0.0002	-2.0948	0.5808
Female adjusted horn length	OAR26_35176677	26	35176677	0.3506	0.0009	1.2318	0.3759
LRS	OAR26_42554917	26	42554917	0.1955	0.0011	-1.0926	0.3402
Female adjusted body mass	OARX_9530888	27	9530888	0.2394	0.0004	2.6411	0.7612
Female adjusted body mass	OARX_10706559	27	10706559	0.3030	0.0009	-2.2821	0.6950
Female adjusted horn length	OARX_25240414	27	25240414	0.2695	0.0008	1.3947	0.4200
Female adjusted body mass	OARX_27629558	27	27629558	0.1576	0.0004	-3.0911	0.8856
Female age at primiparity	OARX_102009771	27	102009771	0.2518	0.0004	0.4859	0.1390
Female longevity	OARX_118818627	27	118818627	0.3619	0.0007	-1.6983	0.5077
Female adjusted body mass	OARX_124393512	27	124393512	0.2576	0.0002	3.3143	0.8905
Female adjusted body mass	OARX_124761764	27	124761764	0.4030	0.0009	2.3813	0.7270
Female adjusted body mass	OARX_134638480	27	134638480	0.1394	0.0009	3.3371	1.0218

Table D2 Details of linear regressions run on loci which did not fit piecewise regression examining the frequencies of alleles positively associated with traits in cohorts from 1979 – 2017 from the Ram Mountain, Alberta population.

Trait	Locus	Intercept	Coefficient (se)	p-value	r^2
Female adjusted body mass	OAR2_162344074	4.706	-0.00193 (0.003)	0.506	0.012
Male adjusted horn length	OAR1_171245188	-4.515	0.00259 (0.002)	0.300	0.030
Female longevity	OAR17_55255290	6.022	-0.00272 (0.002)	0.266	0.034