BIOLOGICAL CONSEQUENCES OF RAPID ENVIRONMENTAL CHANGE IN THE AMERICAN PIKA OCHOTONA PRINCEPS

by

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Abstract

Species are often confronted with rapid environmental change that require an adaptive response to maintain viability. The source of this environmental change can be natural, but is frequently anthropogenic in nature. In this thesis, I document the biological consequences of rapid environmental change in a sensitive mammal, the American pika (*Ochotona princeps*). In Chapter 2, I used microsatellite genetic makers to investigate the consequences of landscape modifications for pika populations relative to two major developments in British Columbia, Canada: a large open-pit copper mine (Highland Valley Copper) under partial reclamation and a bisecting major highway (97C). I found evidence for restricted movement associated with human-modified landscapes and showed evidence for fragmentation potentially resulting from the highway. Rapid environmental change often elicits a stress response in animals involving elevated glucocorticoids. In Chapter 3, I applied a novel technique to measure chronic stress in pikas by measuring extracted corticosterone from hair samples. Applying this method along two elevational transects of populations in North Cascades National Park, WA, allowed me to assess individual factors influencing stress in the American pika. My results showed elevated stress levels associated with smaller body sizes, female pikas, and with low early-spring ambient temperatures. This study provided direct physiological evidence for thermal stress in a climate-sensitive mammal and provides a useful tool for assessing climate stress in the American pika. Selective pressure from such thermal stress can result in local adaptation via natural selection. In Chapter 4, I used genotyping-by-sequencing to assess genotype-environment associations along these same elevational transects. The results showed a consistent genomic response to climate conditions across both transects and provide preliminary evidence for cold stress and hypoxia in the
American pika. Additionally, I resolved consistent evidence for a downslope bias in gene flow, which may interfere with the upslope movement of individuals that is predicted with ongoing climate change. Taken together, these chapters show consistent patterns of restricted gene flow and thermal stress in the American pika and show the potential that climate-mediated natural selection is resulting in the development of local climate adaptations.
 Preface

Chapters 2, 3, and 4 of this thesis are or will be published with the contributions of several co-authors.

Chapter 2 is based on work conducted in collaboration with Cheryl Blair and Karl Larsen at Thompson Rivers University. Cheryl Blair collected all samples and Karl Larsen was responsible for developing the Highland Valley Copper mine as a study site. Michael Russello and I designed the study. I was responsible for genotyping all samples, performing the statistical analysis, and writing the manuscript. A version of Chapter 2 has been published:


Michael Russello and I designed the study for Chapter 3. Chris Ray and Jenifer Wilkening contributed data and reference samples for this project. Liesl Erb helped develop study sites in the North Cascades National Park. I collected samples, Bryson Sjodin extracted and quantified corticosterone samples, I conducted all statistical analysis, and wrote the manuscript. A version of Chapter 3 has been published:


Michael Russello, Liesl Erb, Erik Beever, and I designed the study for Chapter 4. I collected all samples with the help of field technicians, I conducted all statistical analysis and wrote the chapter. A version of this chapter has been submitted to *Molecular Ecology*. 
Table of Contents

Abstract ................................................................................................................................. iii
Preface .................................................................................................................................... v
Table of Contents .................................................................................................................. vi
List of Tables ........................................................................................................................ ix
List of Figures ....................................................................................................................... x
Acknowledgements ............................................................................................................. xi

Chapter 1: Introduction ........................................................................................................ 1
1.1 Climate change ................................................................................................................. 1
1.2 Local adaptation .............................................................................................................. 3
1.3 Tests for selection ........................................................................................................... 4
1.4 Study system ................................................................................................................... 6
1.5 Thesis objectives ............................................................................................................ 7

Chapter 2: Genetic variation and fine-scale population structure in American pikas across a human-modified landscape ............................................................................. 10
2.1 Background ..................................................................................................................... 10
2.2 Methods ........................................................................................................................ 12
  2.2.1 Study sites ............................................................................................................... 12
  2.2.2 Sampling ............................................................................................................... 13
  2.2.3 Genetic data collection ............................................................................................ 14
  2.2.4 Site-level analysis .................................................................................................. 15
  2.2.5 Landscape-level analysis ...................................................................................... 16
2.3 Results ........................................................................................................................... 18
  2.3.1 Data quality ............................................................................................................ 18
  2.3.2 Site-level genetic analysis ...................................................................................... 18
  2.3.3 Landscape-level genetic analysis .......................................................................... 19
2.4 Discussion ....................................................................................................................... 20

Chapter 3: Individual-based analysis of hair corticosterone reveals factors influencing chronic stress in the American pika .................................................................................. 32
3.1 Background........................................................................................................................................32
3.2 Methods...............................................................................................................................................35
  3.2.1 Sample site and sample collection ............................................................................................35
  3.2.2 Microclimate measurements ....................................................................................................36
  3.2.3 Molecular sexing .......................................................................................................................36
  3.2.4 Extraction and immunoassay of corticosterone .........................................................................37
3.3 Results..................................................................................................................................................40
  3.3.1 Laboratory validation ..................................................................................................................40
  3.3.2 Equivalence of corticosterone estimates from different sample sources ..............................40
  3.3.3 Pika stress analysis along elevational gradients ......................................................................41
3.4 Discussion............................................................................................................................................42
  3.4.1 Laboratory validation ..................................................................................................................42
  3.4.2 Field study ..................................................................................................................................45

Chapter 4: Adaptation to climate warming from environmentally-mediated selection
may be impeded due to directional gene flow in a thermal-sensitive mammal........... 58
4.1 Background.........................................................................................................................................58
4.2 Materials and methods ......................................................................................................................61
  4.2.1 Sample site and sample collection ............................................................................................61
  4.2.2 Climate measurement................................................................................................................63
  4.2.3 DNA extraction and RADseq genotyping .................................................................................64
  4.2.4 Reference assembly and SNP discovery ....................................................................................65
  4.2.5 Outlier detection and annotation ..............................................................................................66
  4.2.6 Population genetic analysis ........................................................................................................69
  4.2.7 Gene flow analysis ......................................................................................................................70
  4.2.8 Stress hormone analysis .............................................................................................................71
4.3 Results................................................................................................................................................71
  4.3.1 Sample and environmental data collection ...............................................................................71
  4.3.2 Reference assembly and SNP discovery ....................................................................................72
  4.3.3 Outlier detection ........................................................................................................................73
  4.3.4 Population genetic analysis ........................................................................................................75
  4.3.5 Gene flow analysis ......................................................................................................................76
  4.3.6 Stress hormone analysis .............................................................................................................76
4.4 Discussion ................................................................................................................................. 77
  4.4.1 Outlier detection .................................................................................................................. 78
  4.4.2 Outlier annotation .............................................................................................................. 80
  4.4.3 Population genetic analysis ............................................................................................... 81
  4.4.4 Directional migration ......................................................................................................... 83
  4.4.5 Stress hormone analysis .................................................................................................... 84
  4.4.6 Summary ............................................................................................................................ 85

Chapter 5: Conclusion ...................................................................................................................... 102
  5.1 Research findings .................................................................................................................... 102
    5.1.1 Evidence for restricted dispersal ...................................................................................... 102
    5.1.2 Evidence for cold stress .................................................................................................. 104
    5.1.3 Metabolic implications .................................................................................................... 105
  5.2 Limitations ............................................................................................................................... 106
    5.2.1 Sampling limitations ........................................................................................................ 106
    5.2.2 Genetic limitations .......................................................................................................... 107
    5.2.3 Geographic limitations .................................................................................................... 108
  5.3 Management implications ........................................................................................................ 109
  5.4 Significance and future directions .......................................................................................... 110

References ..................................................................................................................................... 112
List of Tables

Table 2.1. Sample locations, approximate patch size (m$^2$), sample sizes ($n$), and genetic diversity metrics for the 15 sampling sites ................................................................. 26

Table 2.2. AMOVA results showing the distribution of genetic variation explained by different hierarchical classification schemes ................................................................. 27

Table 2.3. Pairwise site comparisons of genetic differentiation ($\theta$) for American pika within and around Highland Valley Copper and highway 97C. ........................................ 28

Table 3.1. Site description of the Pyramid Peak (PP) and Thornton Lakes (TL) sampling transects in North Cascades National Park, WA ................................................................. 50

Table 3.2. Correlation matrix of independent variables ................................................................. 51

Table 3.3. Information-theoretic analysis of mixed-effects models explaining variation in corticosterone estimates of American pika samples ................................................................. 52

Table 4.1. Summary of sample sizes ($n$), observed heterozygosity ($H_o$), total corrected heterozygosity ($H_e$), effective number of alleles ($A_e$), and inbreeding estimates ($F_is$) for sample sites along the Pyramid Peak (PP) and Thornton Lakes (TL) transects in the North Cascades National Park, WA................................................................. 87

Table 4.2. Number of SNPs retained after various filtering procedures ........................................ 88

Table 4.3. Summary of outlier detection analyses ........................................................................... 89

Table 4.4. Summary of top BLASTN search results for outlier loci detected by BAYESCAN (BS), LFMM (LF), and BAYPASS (BP) ........................................................................... 90

Table 4.5. Pairwise comparisons of $F_{st}$ (Top diagonal; Weir & Cockerham 1984) and associated p-value from a permutation analysis ................................................................. 91

Table 4.6. Directional migration analysis for pairwise movement between sites along the PP and TL transects using either the neutral or outlier datasets ........................................... 92

Table S4.1. Climate variables from i-Button data obtained along the elevational transects established in the North Cascades National Park, WA................................................................. 97

Table S4.2. ClimateWNA data for each site along the elevational transects established in the North Cascades National Park, WA................................................................. 98

Table S4.3. Gene Ontology terms associated with significant outlier loci Blast hits................. 99
**List of Figures**

Figure 2.1. Map of study area indicating sampling locations within and around the Highland Valley Copper mine in south central British Columbia................................................. 29

Figure 2.2. Photograph depicting typical artificial habitat (a) contrasted with natural habitat (b) in the Highland Valley Copper mine......................................................... 30

Figure 2.3. STRUCTURE bar plots averaged over 25 iterations showing the genetic division (a) between sites north and south of highway 97C ($\Delta K = 507.9$) and (b) in the south only when analyzed independently ($\Delta K = 73.7$)................................................................. 31

Figure 3.1. Photograph of an American pika and reference hair sample (inset) weighing approximately 10 mg .............................................................................................................. 53

Figure 3.2. Sample sites in North Cascades National Park, Washington, USA. Thornton Lake (TL) and Pyramid Peak (PP) sampling sites shown as circles........................................ 54

Figure 3.3. Top: Parallelism between the standard curve (solid line with circles) and serial dilutions of one sample (squares, no line) ............................................................. 55

Figure 3.4. Relationship between sample mass and estimated corticosterone concentration using NOCA samples (squares), hair samples from paired plasma and fecal samples (triangles), and differing masses from PP04T08 (circles).............................................. 56

Figure 3.5. Box and whisker plot showing average corticosterone per site after correcting for extraction efficiency........................................................................................................... 57

Figure 4.1. Map of Pyramid Peak (PP) and Thornton Lakes (TL) transects in the North Cascades National Park, Washington, USA ................................................................. 94

Figure 4.2. Summary plots for genotype-environment associations in American pika........ 95

Figure 4.3. Admixture proportions for American pika along Pyramid Peak (panels A and C) and Thornton Lakes (panels B and D) transects using only neutral (panels A and B) or outlier loci (panels C and D) with inset cross-entropy (CE) estimations for each value of K ................................................................................................................................. 96

Figure S4.1. Climate variables and loadings for the first (PC1) and second principal component (PC2) of the environmental PCA for the Pyramid Peak and Thornton Lakes transects .............................................................................................................. 100

Figure S4.2. Admixture plot for all the sites sampled in NOCA......................................... 101
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Chapter 1: Introduction

1.1 Climate change

Anthropogenic climate change is causing profound and ubiquitous alterations to natural environments (Stocker et al. 2013). An estimated 350 billion tons of carbon dioxide have been emitted by human activities since 1959 (Ballantyne et al. 2012), resulting in a 0.72°C increase in global temperature (Stocker et al. 2013). Long-term climate modeling indicates that the past three decades were the warmest of the past millennium and that the current rate of warming is far greater than previous natural rates (Jones et al. 2001; Pacifici et al. 2017). While some action has been taken, the moral complexity of the problem (Gardiner 2012) and necessity of a global consensus on action makes abatement of climate-change unlikely.

Furthermore, the repercussions of additional carbon already added to the atmosphere will last for at least a millennium (Solomon et al. 2009). The resulting environmental perturbations from climate change are extensive and include sea-level rise, ocean acidification, increased ocean and land surface temperatures, changes in precipitation, and an increase in extreme weather events (Stocker et al. 2013).

These rapid alterations to the environment are resulting in dramatic ecological and physiological stresses on natural populations. The direct ecological impacts of climate change have been documented on every continent, in every ocean, and in most major taxonomic groups (Parmesan 2006). One of the major influences of climate change will be the direct physiological stress of rising temperature (Somero 2010; but see Cahill et al 2013). Many species are adapted to a narrow range of temperatures (stenothermy), and exposure to elevated temperatures can reduce metabolic efficiency or, in extreme cases, mortality can result from acute heat shock. To maintain viability, impacted species must either disperse,
adapt, or acclimate (Lovejoy & Hannah 2005; Beever et al. 2017).

Species are expected to undergo poleward or altitudinal range shifts in the face of climate change to maintain optimal thermal conditions (Root et al. 2003). Parmesan & Yohe (2003) documented this expected range shift in 80% of the 434 species surveyed in a meta-analysis, with an average poleward range shift of 6.1 km per decade or an increase in elevation of 6.1 m per decade. A more recent meta-analysis (Chen et al. 2011), reported median shifts nearly 2 to 3 times greater than these estimates, indicating species responses to climate change are much greater than previously expected. Additionally, the focus on unidirectional movement ignores complex interactions among temperature, precipitation and species-specific tolerances, and therefore may underestimate the influence of climate change (VanDerWal et al. 2012). Consequently, some species are disproportionately affected, such as European butterflies, which showed a 200 km northward range shift (Parmesan et al. 1999). The majority of these range shifts likely result from the extirpation of low-elevation or equatorward populations and expansion of poleward or high-elevation populations (Davis et al. 2005).

The ability of a species’ range to shift in response to climate change may be significantly impaired by biotic and abiotic factors. Interactions with native species may limit the ability of dispersing species to occupy new niches (Urban et al. 2012). Natural geographic boundaries can limit the poleward or altitudinal dispersal of many species, especially alpine specialists. Anthropogenic impediments to dispersal may impose the most serious threat to the range shift of species in response to climate change (Hoffmann & Sgro 2011). Habitat fragmentation, in particular, is a significant threat to biodiversity (Wilcox & Murphy 1985; Opdam & Wascher 2004), and can reduce dispersal, depending on species-
specific behavior (Baguette et al. 2003; van Dyck & Baguette 2005). These natural and anthropogenic limitations imposed on dispersal and colonization may severely limit range shifts in response to climate change, making local adaptation increasingly important.

1.2 Local adaptation

Biotic response to the local environment can come from either phenotypic plasticity or local adaptation. Phenotypic plasticity is the non-genetic alteration of an organism’s behavior, morphology, physiology, or development in response to environmental change. Some studies suggest that phenotypic plasticity could play an important role in population acclimatization to elevated temperatures (Hoffmann & Sgrò 2011). Phenotypic change can happen rapidly in natural populations, especially as a result of anthropogenic disturbances (Hendry et al. 2008). For example, warmer temperatures have led to earlier spring emergence in yellow-bellied marmots, Marmota flaviventris, and earlier weaning of young (Ozgul et al. 2010). The resulting lengthening of the growing season for the marmots led to increased body masses and a decline in adult mortality. This type of phenotypic plasticity may provide a short-term mechanism to cope with climate change; however, this response is unlikely to provide long-term solutions to populations experiencing continued directional selection from climate change (Gienapp et al. 2008).

Climate change is likely to increase selective pressures on populations, which may lead to a genetic response in impacted species (Hoffmann & Sgrò 2011). Directional selection from increased drought or thermal stress may drive adaptation in affected populations. Selection experiments have provided evidence for the rapid evolution of climate-related characteristics (Reusch & Wood 2007). For example, Drosophila
melanogaster evolves increased heat shock tolerance when subjected to artificially elevated temperatures (Cavicchi et al. 1995). Equally compelling, heritable morphological adaptations induced in D. melanogaster afforded a twofold increase in desiccation resistance after selective pressures were applied to 26 generations (Telonis-Scott et al. 2006). While these studies provide examples of artificially induced climate adaptations, documentation of genetic adaptations to climate change in natural settings has been more difficult (Merilä & Hendry 2014). Rigorous observations have revealed that the pitcher-plant mosquito, Wyeomyia smithii, has undergone genetic adaptation to alter its phenology with altered seasonality in response to recent climate change (Bradshaw & Holzapfel 2001). However, other studies show either a lack of genetic response to climate change (Gienapp et al. 2008) or the lack of adaptability of the species. For example, populations of Drosophila birchii appear incapable of adapting to drier conditions due to low levels of genetic variability for desiccation resistance and will likely go extinct in the drier conditions predicted to occur with climate change (Hoffmann et al. 2003). This lack of adaptability in D. birchii indicates that impacted species will likely require preexisting genetic variation for evolutionary responses to match climate change (Davis et al. 2005; Hoffmann & Sgrò 2011).

1.3 Tests for selection

Current genomic technologies allow for unparalleled insight into evolutionary processes occurring in natural populations (Ellegren 2014). The application of next-generation sequencing to non-model organisms continues to shed light on the inner workings of adaptive evolution. Although the precipitous drop of sequencing costs has facilitated the application of direct sequencing to conservation-related questions, whole genome sequencing is still
prohibitively expensive and generally superfluous for addressing ecological questions. Reduced-representation techniques have facilitated the application of next-generation sequencing technology for ecological applications by providing high-quality genomic datasets that are still computationally feasible to analyze. One such technique, restriction-site associated DNA sequencing (RADseq), has become increasingly popular due to the ease of application and cost effectiveness for identifying and genotyping of thousands of single nucleotide polymorphisms (SNPs) randomly distributed throughout the genome of organisms with or without a reference genome (Baird et al. 2008).

Analytical methods to detect candidate loci under selection from this type of genomic data generally scan for excessive divergence ($F_{ST}$-based analysis) at specific loci or attempt to resolve genomic correlations to environmental parameters (spatially explicit analysis; Rellstab et al. 2015). $F_{ST}$-based analyses scan for allelic frequencies that diverge from neutral patterns as a potential result of natural selection (Beaumont & Balding 2004; Excoffier & Lischer 2010; Helyar et al. 2011; Günther & Coop 2013). Spatially explicit analysis methods can identify informative correlations between allele frequency and environmental data (Joost et al. 2007; Frichot et al. 2013; Jones et al. 2013). The resulting “outlier” SNPs from either of these two methods may not be directly under selection but instead may be linked to adaptive loci that are providing a selective advantage (i.e., genetic hitch-hiking; Smith and Haigh 1974). These approaches are powerful for teasing apart locus-specific and genome-wide patterns in natural populations (Luikart et al. 2003). Furthermore, combining these two independent analytical approaches has the benefit of reducing the rate of false positives when tests occur over thousands of loci (Rellstab et al. 2015).
1.4 Study system

The family Ochotonidae contains 30 species of pikas, two of which are native to North America: the Collared pika, *Ochotona collaris*, and the American pika, *Ochotona princeps* (Hoffman and Smith 2005). The American pika is an alpine specialist distributed from central British Columbia, Canada south to the Sierra Nevada Mountains in California, USA and east to the eastern edge of the Rocky Mountains from New Mexico to Alberta (Smith & Weston 1990). Five discrete lineages of American pikas associated with separate mountain ranges were reconstructed using mitochondrial DNA from the cytochrome b/D-loop (Galbreath *et al.* 2009). The Cascade lineage was likely the first to diverge and extends along the Cascade Mountain range from southern British Columbia to northern California.

The geographic range and habitat requirements of pikas are influenced by the species’ thermal sensitivity (MacArthur & Wang 1974; Smith 1974a). Pikas inhabit talus slopes ranging in elevation from sea level to 3,000 m in the northern range and are rarely found below 2,500 m near the southern limits of their distribution (Smith and Weston 1990; but see Millar and Westfall 2010 and Varner and Dearing 2014). Talus provides a cool, moist microclimate that acts as a refuge from the summer heat (Beever *et al.* 2008); for example, temperatures can be as much as 32°C cooler than ambient temperatures just 1 m into the talus in the Columbia River Gorge, OR (Varner & Dearing 2014). Winter temperatures are also moderated by the insulation of talus and snowpack (Simpson 2009). American pikas use these microclimates to thermoregulate behaviorally (Jeffress *et al.* 2013; Rodhouse *et al.* 2017). MacArthur & Wang (1974) found that pika strictly regulate the intensity and duration of activity to maintain their body temperatures just 2-3°C below upper lethal temperatures, albeit this study may have suffered from a low sample size (6 pikas).
Although the direct physiological evidence for thermal sensitivity in pikas is limited (Smith 1974), several lines of evidence indicate that climate change has already had a negative impact on American pika populations. Paleontological analysis of pikas in the Great Basin shows that the minimum inhabitable elevation has risen 150 m over the past century (Grayson 2005; Beever et al. 2011). Furthermore, recent extirpations of pika populations in the Great Basin (Beever et al. 2010) and marked declines in abundance (Beever et al. 2013) have been associated with regional climate change. Likewise, Galbreath et al. (2009) found genetic evidence of recent demographic declines in all five lineages of the American pika. Niche modeling projects that American pikas in the USA could disappear from 55% of their current range even under low-carbon emission scenarios (Ray et al. 2012). Concerns over the impacts of climate change on pika populations have spurred consideration of the species for listing as threatened or endangered at both federal and state levels (USFWS 2010; Osborn and Applebee 2011). Moreover, the thermal sensitivity of *O. princeps* makes it a useful mammalian model for testing the ecological and genetic impacts of climate change. In this light, the America pika may serve as an early warning sign of climate change (Ray et al. 2012) and may inform conservation efforts seeking to abate the loss of biodiversity in changing environments.

1.5 Thesis objectives

This thesis consists of three complementary approaches to assessing the biotic consequences of rapid environmental change. In Chapter 2, I apply population-genetic analyses to investigate the response of pikas to major anthropogenic habitat alterations in the form of open-pit mining and potential fragmentation induced from road construction. I demonstrate
that the resulting genetic structure of pikas colonizing the newly created habitat from mining activity is likely influenced by human-made impediments to gene flow across the landscape. I show several lines of evidence that a major highway (97C) bisecting the sample site has likely reduced connectivity to the point where populations delineated by the highway represent distinct genetic units.

In Chapter 3, I validate and apply a technique for measuring corticosterone from pika hair samples and show the ecological application of this method by conducting an individual-based analysis of chronic stress. This chapter represents the first application of this technique to pika and potentially the first direct physiological evidence of climate-induced physiological stress in this species. I show how smaller body size increases stress in pikas likely resulting from an increase in the mass-specific metabolic rate of smaller individuals. Additionally, I show higher stress levels in female pikas and demonstrate the potential for cold stress during the early spring molting period by establishing a relationship between increased stress levels and lower ambient temperatures.

Chapter 4 focuses on the robust identification of genomic correlates to environmental parameters along elevational gradients. In this chapter, I use three distinct analyses for detecting outlier loci along two independent elevational transects to identify potential targets of climate-induced natural selection in the American pika. The results of these analyses provided consistent evidence for numerous loci that have been influenced by climatic patterns. Furthermore, I highlight several potential genomic regions and functional mechanisms that may be a source of thermal adaptation in the American pika.

These findings highlight several important biotic responses to rapid environmental change in a species of conservation interest. More specifically, I show that anthropogenic
habitat modification can alter the connectivity of American pika populations, potentially limiting the species’ range shift in response to climate change and increasing the importance of local adaptation to climate. Next, I show that climate can have measurable impacts on physiological stress in the American pika highlighting the role climate could be playing as a selective force in pikas. Finally, I reveal direct genomic correlates to microclimate conditions, which may represent thermal adaptations resulting from climate-induced natural selection. This body of research presents evidence for restricted gene flow, environmental stressors, natural selection and adaptive genetic diversity in the American pika. Taken together, these patterns represent the necessary conditions for local adaptation to occur, which may be especially critical during the current period of rapid environmental change.
Chapter 2: Genetic variation and fine-scale population structure in American pikas across a human-modified landscape

2.1 Background

There is a diverse array of research and knowledge on how habitat alterations influence wildlife populations (Saunders et al. 1991; Keyghobadi 2007). Fragmentation of habitat impedes dispersal (Baguette et al. 2003; Buchmann et al. 2013), having disproportionate effects on some taxa, such as small mammals (Sauvajot et al. 1998). Reduced dispersal can hinder metapopulation dynamics (Fischer & Lindenmayer 2007), leading to reduced resilience in the face of ecological stress. Furthermore, barriers to dispersal may limit the potential of a species to shift its range in response to climate change (Parmesan & Yohe 2003). Habitat modifications leading to reductions in population size can also negatively impact genetic diversity (Frankham 1996), which may influence a species’ ability to adapt to changing environments (Sgrò et al. 2011).

Reclamation activities are increasingly applied to disturbed landscapes in an attempt to improve the suitability of the land for wildlife, with or without a specific target species in mind (Ruiz-Jaen & Aide 2005). For example, many mining operations are now heavily involved in reclamation, often with the end goal of mitigating the initial disturbance and improving habitat recolonization by plants and animals (Eaton et al. 2014). Such activities could represent an important mechanism for reducing impacts to biodiversity on multiple scales. However, we have little direct evidence of the potential consequences colonizing artificial habitat may have on the resulting demographic structure of wildlife populations on these landscapes. In particular, the juxtaposition of local, artificial habitat and neighboring
natural habitat may influence the extent and direction of gene flow. Moreover, anthropogenic landscape features (e.g., roadways) commonly associated with these areas may lead to further habitat fragmentation. Understanding how ecological processes act within reclaimed landscapes is an important step forward in our efforts to improve the resiliency of wildlife populations.

Species with narrow habitat requirements that are able to colonize human-modified environments may provide good opportunities for investigating the relationship between demography, population genetics, and landscape modifications. The American pika (*Ochotona princeps*) is a small lagomorph that has shown a narrow tolerance range for ambient temperature (Smith 1974b; Hafner & Sullivan 1995; Beever *et al*. 2010; Stewart *et al*. 2015). This species is patchily distributed in rocky, talus-type habitats across mountainous areas throughout western North America from central British Columbia and Alberta, Canada, south to the Sierra Nevada in California and east to New Mexico, USA. In some instances, American pikas have colonized reclaimed mining landscapes, most notably in Bodie, California, USA (Peacock and Smith 1997a). The fragmented nature of their habitat and limited dispersal ability (Henry *et al*. 2012; Castillo *et al*. 2014; Robson *et al*. 2016) has increased focus on the American pika for studies of metapopulation dynamics, island biogeography, and source-sink dynamics (Peacock & Smith 1997a; Moilanen *et al*. 1998; Kreuzer & Huntly 2003; Beever *et al*. 2013).

In this study, we investigated the genetic consequences of landscape modifications on the American pika relative to two major developments in British Columbia, Canada, a large open-pit copper mine under partial reclamation and a bisecting major highway. We collected microsatellite genotypic data for individuals sampled at sites within and adjacent to the mine.
both north and south of the highway, and employed site- and landscape-level analyses to quantify levels of variation and connectivity across this human-modified landscape.

2.2 Methods

2.2.1 Study sites

Highland Valley Copper (HVC) is located approximately 54 km southwest of Kamloops, BC (Figure 2.1a). The original mine was commissioned in 1962, although mineral explorations in the area date back to 1954. Originally, three mines operated in the Highland Valley. In 1986, they were amalgamated into one mine, which is now one of the world’s largest open-pit copper mines. Currently, mining operations occupy approximately 6,200 ha (Freberg & Gizikoff 1999). Surface developments from the operation of the mine include open pits, waste rock dumps, tailings, infrastructure, water diversions, and roads. Several natural and anthropogenic features punctuate the landscape. The Highland Valley runs east to west through the study site representing an approximate 300 m change in elevation with a seasonal stream (Witches Brook) at the bottom. Additionally, Highway 97C was completed in 1990, and runs along the bottom of this valley and bisects the HVC mine. Approximately 1,320 vehicles use this highway daily, with peak hours between 5-8 am and 4-8 pm (British Columbia Ministry of Transportation and Highways 2009).

Major mining operations in the section of HVC north of the highway ceased in 1982 while the southern section remains active. Extensive reclamation activities at HVC were initiated in the late 1980s (Freberg & Gizikoff 1999) and have largely been structured around revegetation and lake remediation. Revegetation goals include: the establishment of forage for cattle, native shrubs and trees for wildlife browse, and conifers for wildlife corridors (Bloodgood et al. 1998). More recently, reclamation plans have been modified to increase
biodiversity of the mine (Teck Resources Limited 2012). These plans have never specifically identified American pikas as a target species, but the presence of pikas in the reclaimed landscape was first observed by mine workers around 2005, and then formally documented by Howie (2007). The closest natural population of American pikas is adjacent to the mine property within 0.5 km. The elevation of pika-occupied sites in the mine (1350-1550 MSL) is comparable to occupied surrounding sites (1350-1850 MSL).

2.2.2 Sampling

This study is part of a larger project investigating the population ecology of pikas both within and near the HVC operating area (Blair & Larsen unpubl.). Sampling locations were selected on both natural (n = 7) and artificial (n = 8) habitat north and south of Highway 97C based on site occupancy. Pika-occupied sites were initially located by using aerial mapping and local knowledge to identify rocky areas of both natural and artificial habitat. Intense searching on the ground then was conducted to identify individual pika territories through direct observation of the animals and/or their hay piles. Pika territories were considered to be in artificial habitat when in waste rock dumps or riprap from road construction (Figure 2.2a). All other sites were considered ‘natural’ and represented talus patches in a relatively undisturbed state (Figure 2.2b). Sites were exhaustively trapped between April and October 2012 and between April and November 2013, allowing for the identification of individual pika territories. Pikas were captured using Tomahawk model 202 (Hazelhurst, WI) collapsible live traps in accordance with BC Ministry of Forests, Lands and Natural Resource Operations wildlife permits KA12-78714 and KA13-86652, and Animal Ethics Protocol #100102 (Thompson Rivers University, BC). All habitat patches within a 500 m radius were
considered part of the sample site and were measured using a range finder and tape measure to approximate the total area of habitat for each site. A GPS coordinate was taken at each individual territory and all GPS points within the 500 m radius were averaged to obtain site coordinates.

To determine the age class of captured animals, we combined observational data with estimates of mass using a spring scale and cranial diameter using calipers. Individuals with a mass under 150 g and cranial diameter under 2.5 cm were categorized as juveniles (informed by Smith and Weston 1990); all such individuals were generally trapped emerging from their natal nest and were considered young of the year. To eliminate resampling individuals, each pika was marked with two Monel #1 ear tags (unique number combinations) and one unique color tag. A small tuft of hair was plucked and stored in a coin envelope for subsequent genetic analysis.

2.2.3 Genetic data collection

DNA was extracted using the Macherey-Nagel NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co. KG, Duren, Germany) and manufacturer’s protocols. Eleven polymorphic microsatellite loci were used to genotype each sample (Supplemental Table 2.1; Peacock et al. 2002; Peacock and Kirchoff, unpublished report). Conditions for PCR amplification followed Henry et al. (2012) including one additional locus (Ocp 10) that was not previously used, but was amplified under the same touchdown PCR protocol. PCR products were co-loaded and run on an ABI 3130XL DNA automated sequencer (Applied Biosystems, Foster City, CA) with GENESCAN™ 500 LIZ® size standard. Genotype calls were made using GENEmapper 4.0 (Applied Biosystems, Foster City, CA). To assess allele-scoring error, 40%
of the samples were re-amplified and re-genotyped independently and compared to the original scores.

Sex was determined for each sample by the selective co-amplification of an allosomal-linked locus (SRY) and an autosomal control locus (Ocp 10) as described by Lamb et al. (2013). Scoring was conducted by running the PCR product on a 1.5% agarose gel containing 2.5% SYBR Safe (Invitrogen, Carlsbad, California).

The genotypic data were examined for evidence of large allele dropout and null alleles using MICROCHECKER (van Oosterhout et al. 2004). All loci were tested for deviations from Hardy-Weinberg expectations (HWE) in each sample site using an exact test implemented in GENEPOP 4.0 (Raymond and Rousset 1995; Rousset 2008). Linkage disequilibrium was tested between all pairs of loci in each site using the exact test of Guo and Thompson (1992) as implemented in GENEPOP 4.0. Type I error rates for tests of linkage disequilibrium and departure from HWE were corrected for multiple comparisons using the sequential Bonferroni method (Rice 1989).

2.2.4 Site-level analysis
Sex ratios (M:F) were calculated for each site based on the molecular sexing data. We tested for even sex ratios using a chi-squared ($\chi^2$) goodness-of-fit test and the chisq.test function in R version 3.3.1 (R Core Team 2015) between the number of males and females for all artificial and natural sites, respectively. Unbiased expected heterozygosity ($H_e$) was calculated for each population using ARLEQUIN 3.5 (Excoffier & Lischer 2010). Allelic richness ($A_r$) was estimated using a rarefaction method described by Leberg (2002) to account for biases caused by unequal sample sizes as implemented in HP-RARE v1.0.
(Kalinowski 2005). The inbreeding coefficient ($F_{is}$) was calculated for each site and tested for statistical deviations from zero using 10,000 permutations in GENETIX (Belkhir et al. 2004). Pairwise relatedness was calculated between all samples at each site using the estimator developed by Queller and Goodnight (1989) and tested for significance using a permutation test with 1,000 replicates in GENALEX (Peakall & Smouse 2006). Heterozygosity, rarefied allelic richness, site-level relatedness, and $F_{is}$ were compared between natural and artificial sites using a two-tailed $t$-test assuming unequal variances using the $t.test$ function in R.

2.2.5 Landscape-level analysis

The presence of discrete genetic units was assessed using a Bayesian clustering method implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). An admixture model with correlated allele frequencies was used with a run length of 1,000,000 MCMC replicates after a burn-in period of 500,000. The most likely number of clusters ($K$) was determined by varying $K$ from 1 to 17 with 25 iterations per value of $K$ and implementing the $\Delta K$ method (Evanno et al. 2005) using STRUCTURE HARVESTER (Earl & VonHoldt 2011). Additional population structure was assessed by re-running the STRUCTURE analysis for each resolved genetic unit separately using the same parameters but varying $K$ from 1 to the number of sampling sites in the resolved genetic unit plus 2. An Analysis of Molecular Variance (AMOVA) was performed in GENALEX using the resolved genetic groups from the STRUCTURE analysis in addition to the natural/artificial classification to determine the degree of genetic differentiation explained by these groupings and tested for significance using a permutation test with 1,000 replicates.

The level of genetic differentiation between pairwise comparisons of sites was
estimated using $\theta$ (Weir & Cockerham 1984) and tested for significance using 10,000 permutations as calculated in GENETIX (Belkhir et al. 2004) and corrected for multiple comparisons using the false discovery rate correction (Benjamini and Hochberg 1995). To test for a pattern of isolation-by-distance (IBD), a matrix of genetic distances ($\theta$) was compared to a matrix of Euclidean distances using a Mantel test implemented in the ISOLATION BY DISTANCE WEB SERVICE (Jensen et al. 2005) with default parameters and tested for significance using 1,000 permutations. This analysis was repeated using the same parameters, but using only sites north or south of highway 97C, respectively. Additionally, to examine the possibility of spatially variable IBD patterns, we first constructed a genetic similarity matrix of $(1 - \theta)$ between all pairwise comparisons of sites. We then used this genetic similarity matrix to construct a non-stationary genetic friction map displaying the relative genetic divergence per unit of geographic distance as implemented in LOCALDIFF (Dufrenot-Frebourg & Blum 2014) using 4 simulated neighbors at a distance of 0.1 and 100 posterior replicates.

The direction and magnitude of contemporary gene flow were assessed using a non-equilibrium Bayesian method implemented in BAYESASS v. 3 (Wilson & Rannala 2003). A run length of 10,000,000 MCMC replicates with a burn-in period of 1,000,000 replicates was used, sampling the chain every 100 iterations. To evaluate consistency, the program was run five times with a different random seed. Given the recovered structure in the dataset (see Results below), we grouped sites for this analysis as follows: north natural, north artificial, south natural, and south artificial. Significance was assumed when the 95% credibility set [mean ± 1.96 × standard deviation (sd)] did not encompass zero, as recommended by the developers (Wilson & Rannala 2003).
2.3 Results
2.3.1 Data quality

A total of 109 pikas were sampled from 15 sites, 8 artificial and 7 natural, with an average sample size of 7.3 animals per site (Table 2.1). Our habitat assessment showed an average of 4,117 m² of habitat per site with a mean pika territory of approximately 590 m². These parameters agree with previous reports (Smith and Weston 1990) indicating our sampling was likely representative of the total population at these sites. Of these individuals, 37 were unambiguously assigned as juveniles, 66 were assigned as adults, and the remaining 6 were indeterminate. The overall dataset contained 1.0% missing genotypic data, with no sample having missing data at more than 3 loci. Independently genotyping 47 random samples showed a 1.4% allelic scoring error rate, which is within reasonable expectations for the use of hair as a genetic source material and is not expected to skew population genetic analyses (Smith & Wang 2014). There was no evidence for large allele dropout or null alleles at any sampling sites with the exception of Ocp 15, which showed evidence of null alleles at four sites (HFG, Relic_1, SGS, and FHF). Following sequential Bonferroni corrections, no locus deviated from HWE or showed evidence of linkage disequilibrium. Given the lack of a consistent trend in the evidence for null alleles across sites, all loci were retained for further analysis.

2.3.2 Site-level genetic analysis

The average sex ratio (M:F) was 1.9 (sd = 1.5) for artificial sites and 1.3 (sd = 1.0) for natural sites (Table 2.1). There was no significant difference between the number of males and females on either natural sites ($\chi^2 = 5.43, df = 7, p = 0.61$) or on artificial sites ($\chi^2 = 5.00, df = 6, p = 0.54$). Site-level heterozygosity of pikas ranged from 0.530 (EC) to 0.707 (BRCH),
while $A_R$ ranged from 2.14 (SGS) to 2.58 (BRCH). Site-level relatedness ranged from 0.028 to 0.394 and was significantly greater than expected under random mating for 11 of 15 sites (Table 2.1). Inbreeding estimates ranged from -0.179 to 0.270; 2 of the 15 sites exhibited inbreeding estimates significantly greater than 0. There was no difference in mean genetic diversity estimates between natural and artificial sites for $H_e$ ($t = 0.965$, df = 7.694, $p = 0.82$), $A_R$ ($t = 1.816$, df = 9.996, $p = 0.95$), or $F_{is}$ ($t = 1.034$, df = 11.628, $p = 0.84$). Relatedness of pikas was significantly higher at artificial sites (mean = 0.251, sd = 0.098) than natural sites (mean = 0.146, sd = 0.071; $t = -2.396$, df = 12.580, $p = 0.016$).

### 2.3.3 Landscape-level genetic analysis

The **STRUCTURE** analysis revealed evidence for $K = 2$, corresponding to clusters of sites north and south of highway 97C ($\Delta K = 507.9$; Figure 2.3a). Further analysis did not reveal additional genetic units in the north, but resolved three southern genetic units ($\Delta K = 73.7$; Figure 2.3b). The two natural sites (Relic_1 and Relic_2) grouped together with an artificial, admixed site (SGE). Two additional artificial sites (HNR and HFGR) grouped together, while one artificial site (SGS) formed a largely distinct cluster from the other southern sites. The **AMOVA** results showed the north/south divide of highway 97C consistently explained the greatest amount of genetic variation, and the natural/artificial demarcation explained the least (Table 2.2). While all grouping scenarios were significant, the four genetic clusters resolved by the **STRUCTURE** analysis were the most explanatory followed by the natural/artificial grouping when the north/south divide was added to the hierarchical structure ($K = 4$).

Significant genetic differentiation was found for 35 of 105 of the pairwise site comparisons of $\theta$ (Table 2.3). A weak but significant pattern of IBD was also detected ($r^2 =$
0.080, \( p = 0.009 \) where genetic distance increased with geographic distance when considering all sites. This pattern did not hold when only considering sites north (\( r^2 = 0.003, p = 0.402 \)) or south (\( r^2 = 0.087, p = 0.122 \)) of highway 97C. The genetic friction map resolved a localized area of disproportionately high genetic differentiation in the central region of the study area largely corresponding with landscape modification associated with HVC and highway 97C (indicated in red; Figure 2.1b). There was no genetic evidence of migration between the northern and southern genetic units. Within these genetic units, 7.6\% (sd = 3.8\%) and 24.8\% (sd = 6.7) of pikas residing on natural sites in the north and south, respectively, were estimated to be recent migrants from adjacent artificial sites. No significant migration from natural to artificial sites was detected in either region.

2.4 Discussion

In this study, we investigated genetic variation and connectivity within and among sites occupied by American pikas across a human-modified landscape. We detected evidence that American pikas are influenced by habitat modification at both site- and landscape-level spatial scales, the nature of which may have implications for metapopulation dynamics of wildlife populations inhabiting such landscapes.

Previous research of American pikas inhabiting mining locations have shown that these populations can be highly variable and smaller than expected given habitat availability (Smith 1980; Moilanen et al. 1998), which may have potential genetic consequences. For example, in ore dumps in Bodie, California, American pika averaged 2.49 alleles per locus (Klinger and Peacock, in prep), similar to the levels reported for artificial sites here (\( A_r = 2.33 \)). These values are substantially lower than those reported by studies that used partially
overlapping loci within natural habitat in the range core in Nevada ($A_r = 4.4$; Meredith 2002), and Oregon ($A_r = 5.7$; Castillo et al. 2014). It is important to note, however, that our study occurred towards the northern range margin of the American pika (Smith & Weston 1990); theory predicts that levels of within-population genetic diversity declines towards range peripheries (Lesica & Allendorf 1995; Durka 1999; Eckert et al. 2008). As a case in point, levels of allelic richness and heterozygosity detected in the current study were similar to those reported at natural sites at the northern range margin in Tweedsmuir South Provincial Park in British Columbia ($A_r = 2.8$, $H_e = 0.62$; Henry et al. 2012). Consequently, we cannot disentangle the relative impacts of fine-scale landscape modification from broader-scale range-wide patterns in interpreting the low levels of within-site genetic variation in and around HVC.

Additionally, we saw no difference in either heterozygosity or allelic richness between pika inhabiting artificial and natural sites possibly owing to the limited sample sizes associated with such a fine-scale assessment or a lack of significant demographic perturbation associated with development. There was, however, a significant increase in relatedness of pika on artificial sites. Artificial sites were originally formed by mining activities (1962 or newer). Given their relatively contemporary origin, these sites were likely colonized much more recently than surrounding natural sites, and are therefore potentially subject to founder effects (Mayr 1963; Nei et al. 1975). Moreover, the artificial sites show some evidence of isolation, exhibiting both detectable levels of genetic divergence from and unidirectional migration towards natural sites, which may have contributed to the elevated levels of relatedness.

The evidence of directional migration from artificial to natural sites also has
implications for metapopulation dynamics in this system. Peacock (1997) found that dispersal in American pikas is resource dependent, where the primary resource is available habitat, and dispersing individuals generally settle on the first available territory. Moreover, immigration patterns in American pikas are largely a function of local demographic processes of birth rates and habitat saturation (Kreuzer & Huntly 2003). Habitat saturation can be highly variable, but can occur even in artificial habitat in a mine setting (Smith 1980). In this context, the artificial sites studied here may have a lower carrying capacity, spurring directional movement towards more natural settings. As a case in point, preliminary analyses indicate significant differences of both thermal and vegetative characteristics between our artificial and natural sites. Both surface and subsurface temperatures at American pika territories on artificial habitat were significantly more variable than their natural counterparts, and ambient temperatures tended to be higher on artificial habitat (Spilker, unpublished data). Additionally, there were marked differences in the plant (forage) communities between the artificial and natural territories, due in part to the types of species used in the reclamation process. However, nutritional (i.e., nitrogen) composition did not notably differ in the plants appearing in haypiles at the two territory types (Leung, unpublished data). These thermal and vegetative differences could alter habitat quality for American pikas on artificial sites and, in turn, influence local metapopulation dynamics and patterns of gene flow as has been found in other well-studied pika populations (Moilanen et al. 1998). Ongoing ecological assessment of American pikas at our study site could further elucidate metapopulation dynamics in the region and help determine the degree to which variable habitat quality may play a role.

On a broader scale, we resolved extensive genetic structure associated with landscape features. We found evidence for a significant genetic break in this system,
corresponding to north and south of the highway, respectively (Figure 2.3). Moreover, the central region of the study system bisected by the highway also constitutes an area of high genetic friction (Figure 2.1). An increase in genetic structure from reduced connectivity is a central prediction of the genetic effects of roads on wildlife (Balkenhol & Waits 2009), and can occur over relatively short timespans (Martínez-Cruz et al. 2007). However, the degree of genetic impact is species- and context-specific as exemplified by the lack of genetic structure detected in the pygmy rabbit (*Brachylagus idahoensis*), another small bodied lagomorph with a presumed limited dispersal ability (Estes-Zumpf *et al.* 2010). While this study was conducted over a similar geographic scale and across comparable landscape impediments such as highways, creeks, and reservoirs, the study area contained no mining activity or associated reclaimed habitat.

At a finer level, three genetic units were detected south of the highway, one of which was comprised of a single site, SGS, that formed a unique genetic unit despite close proximity to SGE (550 m). This distance is well within the American pika dispersal capacity reported elsewhere across the range (maximum distances between 2 km-10 km; Hafner and Sullivan 1995; Peacock 1997). Interestingly, SGS is the only site completely surrounded by intense mining activity (Cheryl Blair, personal observation). Although direct mining activities may have contributed to the isolation of SGS, the additional structure detected south of the highway may be the result of differing sources or timing of colonization of newly created habitat with landscape modifications subsequently limiting gene flow. This hypothesis could be tested in the future with broader sampling of potential source populations to the south.

There are several natural geographic barriers to gene flow that may account, in part,
for the genetic structure observed around the mining site. The highway lies at the bottom of a valley representing an approximate 300 m change in elevation, with a small seasonal creek at the bottom. Previous research indicates that both topographic relief (Henry et al. 2012) and water bodies (Castillo et al. 2014) can significantly inhibit pika movement, making natural geographic boundaries a possible alternative explanation for the north/south genetic division.

However, natural geographic barriers would only account for the north-south genetic division and not the degree of genetic structure observed in the south nor the pattern of genetic friction across the landscape since no other natural barriers to gene flow were observed. Additionally, the degree of topographic relief previously shown to inhibit American pika dispersal was far more extreme than anything found around HVC (Henry et al. 2012; Robson et al. 2016). Future research could potentially disentangle the influence of natural and anthropogenic barriers to gene flow by using a larger sampling of the American pika genome and coalescence-based genetic analyses to determine if the development of observed genetic structure was concurrent with human modifications of the landscape.

In summary, we found evidence that landscape modifications have likely influenced the distribution of genetic variation within this study system, documenting several of the expected patterns of fragmentation on small mammals (Gaines et al. 1997). Specifically, we detected site-level changes in genetic characteristics of pika, a slight but significant degree of genetic differentiation of American pikas inhabiting artificial sites, and significant impacts on genetic structuring and migration that were likely associated with landscape modifications. These alterations could influence metapopulation dynamics, including responses to future environmental stressors. In general, it appears that inhabiting artificial habitat might predispose some species to develop fine-scale genetic structure due, in part, to the
colonization patterns of the newly available area. Additionally, by its nature, artificial habitat is generally in close proximity to other landscape modifications; in this study, the artificial habitat sites were bisected by a major highway. These additional landscape modifications could act to further reinforce the development of fine-scale genetic structure.

Overall, this area of reclamation appears successful in promoting occupancy for American pikas within HVC, even though the species was not specifically targeted; however, barriers to gene flow likely associated with resource extraction and road construction may limit connectivity across the landscape. Mitigation strategies for promoting connectivity may be limited for American pikas given their thermal sensitivity and habitat requirements. However, American pikas have been documented inhabiting riprap around a small bridge (Henry et al. 2012), indicating habitat corridors and highway bypasses may be effective in this species, but additional study is required. Furthermore, awareness of the potential demographic and genetic consequences of similar landscape alterations may help encourage the integration of mitigation promoting connectivity directly into management planning in order to benefit other wildlife species in the affected areas. Additionally, this study may serve as a reference point for fine-scale genetic analysis across a human-modified landscape enabling contrast between natural and anthropogenically-induced genetic structure in the American pika.
Table 2.1 Sample locations, approximate patch size (m$^2$), sample sizes ($n$), and genetic diversity metrics for the 15 sampling sites. Sex ratios (males per female; $M:F$), unbiased expected heterozygosity ($H_e$), rarified allelic richness ($A_R$), inbreeding coefficient ($F_{is}$), and relatedness ($r_{xy}$) are shown for each site, type indicates either natural talus or artificial habitat. Significance ($\alpha < 0.05$) is shown by an asterisk for $F_{is}$ and $r_{xy}$.

<table>
<thead>
<tr>
<th>Site</th>
<th>Area</th>
<th>$n$</th>
<th>$M:F$</th>
<th>Type</th>
<th>$H_e$</th>
<th>$A_R$</th>
<th>$F_{is}$</th>
<th>$r_{xy}$</th>
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Table 2.2 AMOVA results showing the distribution of genetic variation explained by different hierarchical classification schemes. Numbers represent percentages of genetic variation explained by each grouping. The STRUCTURE clusters ($K = 2$) represent the north/south divide of highway 97C, while the STRUCTURE clusters ($K = 4$) takes into account the observed substructure in the south. Natural/artificial ($K = 2$) groups sites by habitat type, while natural/artificial ($K = 4$) includes groupings by habitat type divided into north and south of highway 97C.

<table>
<thead>
<tr>
<th></th>
<th>Among groups</th>
<th>Among sites within groups</th>
<th>Within sites</th>
<th>Significance</th>
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</thead>
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<td>STRUCTURE clusters ($K = 2$)</td>
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<tr>
<td>Natural/artificial ($K = 4$)</td>
<td>6.0</td>
<td>9.0</td>
<td>85.0</td>
<td>&lt;0.001</td>
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</table>
Table 2.3. Pairwise site comparisons of genetic differentiation ($\theta$) for American pika within and around Highland Valley Copper and Highway 97C.

<table>
<thead>
<tr>
<th></th>
<th>BLD</th>
<th>BRCC</th>
<th>BRCH</th>
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<th>BSDG</th>
<th>EC</th>
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<th>HNR</th>
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* indicates values that are statistically significant after correction for false discovery rate, $P_{\text{critical}} < 0.015$
Figure 2.1. Map of study area indicating sampling locations within and around the Highland Valley Copper mine in south central British Columbia (a). See Table 2.1 for site descriptions. Green diamonds represent sites with natural habitat while red squares represent sites with artificial habitat. Topographic lines show 100 m changes in elevation and highway 97C is shown. Gridlines show 0.05° changes in latitude and longitude. Two large open pit mines are visible at (-121.04, 50.59) and (-121.04, 50.45). Darker areas represent wooded areas whereas lighter areas represent cleared areas from mining or other human activities; talus is not visible at this scale. Aerial photograph obtained from Google™Earth. Genetic friction map computed across American pika sampling locations (b). The degree of genetic friction is indicated by the inset contour lines and color (red indicates a relative increase in genetic differentiation per unit of geographic distance). Points indicate relative locations of sampling sites as shown on the site map.
Figure 2.2. Photograph depicting typical artificial habitat (a) contrasted with natural habitat (b) in the Highland Valley Copper mine. Artificial habitat consisted of rock dumps generated from mining activities or road construction. Relatively undisturbed talus patches were considered natural habitat.
Figure 2.3. STRUCTURE bar plots averaged over 25 iterations showing the genetic division (a) of pikas between sites north (BBB through TG) and south of highway 97C (HFGR through SGS; ΔK = 507.9) and (b) in the south only when analyzed independently (ΔK = 73.7). No further genetic subdivisions were resolved within the northern genetic unit.
Chapter 3: Individual-based analysis of hair corticosterone reveals factors influencing chronic stress in the American pika

3.1 Background

Rapid environmental change represents a potential stressor and selective force on wildlife populations (Reeder & Kramer 2005; Wingfield & Romero 2011). The main physiological response to long-term environmental stress is the activation of the hypothalamic-pituitary-adrenal axis (HPA) resulting in the release of glucocorticoids (GC), in the form of corticosterone or cortisol, into the bloodstream (Sapolsky et al. 2000; Sheriff et al. 2011). This increase in GC facilitates a suite of adaptive responses to stressful stimuli, such as behavioral changes and energy mobilization via gluconeogenesis, which can enhance short-term survival (Wingfield & Romero 2011). However, long-term activation of the HPA signifies chronic stress and can have detrimental physiological consequences including: suppressed immune response and growth, severe protein loss, fat deposition and hypertension, as well as undesirable behavioral changes including decreased cognitive functioning, inhibition of reproductive behavior, and depression (Wingfield et al. 1998; Sapolsky et al. 2000). For these reasons, the relative levels of GC often reflect overall health and fitness (Blas et al. 2007; Bonier et al. 2009), and measurement of GC is increasingly incorporated into ecological and conservation studies (Busch & Hayward 2009). The relative strengths and feasibility of methodologies to assess stress in wildlife has therefore been a major recent focus (Sheriff et al. 2011).

Several techniques have been developed for measuring stress in wildlife, including measuring GC levels in hair, blood, or saliva, and measuring glucocorticoid metabolites
(GCM) in fecal samples. Levels of GC in both saliva and blood respond rapidly to stress and therefore require capture techniques that allow sampling before the activation of the HPA in response to capture stress (generally 2-5 minutes; Sheriff et al. 2011). Where rapid sampling has been possible, this technique has revealed fundamental insights into factors governing GC levels within and across mammalian species. For instance, in a recent meta-analysis, Haase et al. (2016) found a surprisingly strong connection between plasma cortisol levels and mass-specific metabolic rate across a wide variety of taxa, providing a predictive framework for GC levels within and among species. However, obtaining timely blood samples may not be feasible for all species. In such cases, measuring fecal GCM offers a less invasive technique for assessing stress in wildlife (Touma & Palme 2005). Fecal samples accumulate the metabolic byproducts of stress hormones only during gut passage, and therefore primarily reflect chronic stress experienced over a number of hours or days. Additionally, most species exhibit diurnal and seasonal shifts in GC (Reeder & Kramer 2005; Sheriff et al. 2012), making it necessary to obtain a time-series of samples to effectively assess long-term chronic stress using blood, saliva or fecal samples.

The measurement of GC incorporated into hair is a relatively new approach to assess stress in wildlife (Koren et al. 2002). While the direct mechanism by which GC is incorporated into hair is still unknown (Gow et al. 2010), this sample source offers the potential to measure relative levels of GC over the duration of time the hair was grown, which typically encompasses several weeks or months. This longer-term record makes hair analysis a powerful approach for assessing long-term chronic stress (Russell et al. 2012). Despite this major advantage, only a limited number of wildlife studies have used hair as opposed to more established alternatives (Sheriff et al. 2011). While it is likely that using
hair samples for stress analysis would provide deeper insights into climate-induced stress in wildlife, another study cautioned that this approach may be more appropriate for detecting population rather than individual stress responses (Mastromonaco et al. 2014). This suggestion came after analyzing long-term trends in fecal GCM and hair GC in eastern chipmunks, *Tamias striatus*, there was a significant increase in GCM associated with logging but no change in hair GC. The authors concluded the time period measured by hair samples was too long to reflect individual differences in stress. However, this critique would depend on the research objective and species examined. If this methodology can detect individual responses to long-term chronic stress, then it could afford important insights into physiological responses to environmental stressors in climate-sensitive species.

The American pika, *Ochotona princeps*, is a small lagomorph generally considered to be a thermally sensitive, cold-adapted specialist (Figure 3.1; MacArthur & Wang 1974; Smith 1974). Pikas have an exceptionally high metabolic rate (Lovegrove 2003) and low thermal conductance (MacArthur & Wang 1973), which allows them to survive in an alpine climate without hibernating. However, these features also result in the pika having a resting body temperature only a few degrees below its lethal threshold (MacArthur & Wang 1974; Smith 1974). Pikas require access to cool microclimates to behaviorally thermoregulate (MacArthur & Wang 1974; Hafner 1993). It is thought that this thermal sensitivity may predispose the American pika to the negative ramifications of climate change, casting them as a sentinel species for detecting the ecological consequences of climate change (Beever et al. 2003; Wilkening et al. 2011; Jeffress et al. 2013; Schwalm et al. 2016). Recent analysis of pika populations in the Great Basin supports this view; the minimum elevation inhabited by pikas in the region has risen by 150 m in the past century (Grayson 2005), and climate has
been implicated in local extirpations (Beever et al. 2011). Therefore, assessing the biotic response to rapid environmental change in the American pika has become increasingly important as an early warning sign.

Here, we evaluated the utility of hair samples for measuring long-term chronic stress in the American pika. First, we demonstrate the sensitivity of the assay protocol through validation, and we compare estimates of GC from hair with estimates of plasma GC and fecal GCM from the same individuals. Next, we apply this method to investigate relationships between hair GC, microclimate, body size and sex over two elevational gradients to assess whether hair samples can provide direct insights related to climate-mediated stress responses.

3.2 Methods

3.2.1 Sample site and sample collection

Sample sites were located along two previously established transects representing elevational gradients in North Cascades National Park, WA (NOCA; Russello et al. 2015). Four sites were located along each transect, spanning ~1,000 m (Figure 3.2, Table 3.1). The two transects, Thornton Lakes (TL) and Pyramid Peak (PP), ran roughly southwest and northeast, respectively. Each transect included the highest (subalpine) and lowest occupied sites identified in its respective region.

Transects were sampled between July 20th and August 29th of 2014; pikas were live-trapped using Tomahawk (Hazelhurst, WI) model 202 collapsible traps following University of British Columbia Animal Care Protocol # A11-0371-006 and U.S. National Park Service Permit # NOCA-2014-SCI-0022. Trapping was generally conducted between 0700 and 1100 or 1600 and 2000 when temperatures were between 5°C and 18°C. After capture, each
animal was transferred to a handling bag. A small tuft of fur (about 5 mm²; Figure 3.1 inset) was plucked from a hind limb, as shaving was not practical in this species. Hair samples were stored in individually labeled coin envelopes within a container of silica desiccant. Two small samples of ear tissue (3 mm in diameter) were collected and stored in ethanol for molecular sexing. Cranial diameter was measured with digital calipers to the nearest millimeter, and body mass was measured to the nearest 5 grams using a Pesola scale.

3.2.2 Microclimate measurements

Microclimate measurements were taken at each site by deploying four temperature sensors (DS1921G Thermochron i-Button, Maxim Integrated Products, Sunnyvale, CA). Following sampling, sensors were deployed in weather-proof housing; two “ambient sensors” were placed 1.5 m above the talus, each under a white plastic shade in neighboring trees, while two “talus sensors” were deployed in a central location at each site approximately 0.8 m below the talus surface. Temperature was recorded every four hours (starting at 0200) during August 24-31, 2014 and June 1-August 15, 2015. To represent summer microclimatic differences among sites, mean daily maximum and mean daytime (1000 to 1800) temperatures were averaged for the two talus (Tal_max and Tal_day, respectively) and the two ambient sensors at each site (Amb_max and Amb_day, respectively). Additionally, mean nighttime (1800 to 1000) talus temperatures were calculated for each site (Tal_night).

3.2.3 Molecular sexing

Morphological differences between male and female genitalia are poorly defined in pika (Duke 1951); therefore, sex was determined using the molecular protocol described by Lamb
et al. (2013). DNA was extracted from tissue samples using the Macherey-Nagel NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co. KG, Duren, Germany) following the manufacturer’s protocols. Sex was determined by the selective co-amplification of an allosomal-linked locus (SRY) and an autosomal control locus (Ocp 10). Scoring was conducted by running the PCR product on a 1.5% agarose gel containing 2.5% SYBR Safe (Invitrogen, Carlsbad, California). To ensure accuracy, 50% of the samples were sexed independently a second time and assigned sexes were compared.

3.2.4 Extraction and immunoassay of corticosterone

Corticosterone extraction from hair samples followed Meyer et al. (2014) using the DetectX® Corticosterone Enzyme Immunoassay (EIA) kit (Arbor Assays Design, Inc., catalogue no. K014-H1). All hair follicles were removed with a razor blade to avoid the addition of skin tissue (Gow et al. 2010). The remaining hair was added to a 15 mL tube, washed twice with 3 mL 99.7% high-performance liquid chromatography (HPLC) grade isopropanol by rotating for 3 minutes, then decanted to remove external contaminants, and dried under a fume hood. Dried samples were weighed to the nearest 0.1 mg and transferred to a reinforced 2.0 mL tube with three 3.2 mm chrome-steel beads. Samples were then pulverized in 3-minute intervals for 3-18 minutes at 30 hertz on a MM301 Mixer Mill (Retsch®, Newtown, PA). Once samples were uniformly pulverized, 1.5 mL of HPLC-grade methanol was added then samples were rotated for 24 hours at room temperature. Samples were then centrifuged at 12,000 rpm for 10 minutes and 1 mL of the supernatant was transferred to a 1.5 mL microcentrifuge tube without disturbing the hair pellet. This extract was dried under a gentle stream of air in a fume hood for approximately 1-3 days until all methanol had evaporated.
The extract was reconstituted using 200 µL of the EIA buffer supplied with the kit, vortexed vigorously, and then immediately frozen at -20°C until analyzed.

Each sample was run in duplicate along with six standard concentrations of corticosterone and two nonspecific binding (NSB) and two maximum binding (B₀) wells for each plate. Absorption values at 450 nm were recorded using a Synergy HT microplate reader (Biotek, Winooski, VT). Final concentrations of GC were expressed as picogram (pg) of corticosterone per milligram (mg) of washed, dried hair.

Methods used to measure hair corticosterone concentrations were validated by: 1) demonstrating parallelism between the standard curve and serial dilutions of hair extract; and 2) determining the recoverability of exogenous corticosterone added to hair extracts prior to analysis. For the addition of exogenous corticosterone, six samples were diluted 1:1 each with one of the standard curve solutions. Extraction efficiency likely varies with initial sample mass yielding proportionally higher estimates of glucocorticoids in smaller samples (Millspaugh & Washburn 2004; Tempel & Gutierrez 2004). All corticosterone estimates were plotted against sample mass to identify potential relationships, and, if present, a nonlinear model was fitted using the \textit{nls} function and used to account for the influence of extraction efficiency. For further comparison, nine additional hair samples were obtained from pikas previously analyzed for plasma corticosterone (Wilkening & Ray 2016) and baseline fecal GCM (i.e., stress levels before capture; Wilkening et al. 2013). Plasma samples were not collected within 3 minutes of capture and thus measured an acute stress response, while fecal samples were collected prior to the stress signature documented for pikas (GCM increases 11-15 hours after capture, Wilkening et al. 2013) and represented a chronic stress response. Relationships between hair, plasma, and fecal GCM were assessed with a linear
regression using the \textit{lm} function. All analyses (unless otherwise noted) were conducted using the \textit{stats} package in R version 3.1.3 (R Core Team 2015).

3.2.5 Data analysis

The distribution of each independent variable was assessed for normality according to the Shapiro Wilk test using the \textit{shapiro.test} function, and by inspecting a normal probability plot using the \textit{qqnorm} function. Any variable that deviated from normality was transformed using a natural log transformation and retested for normality. Since elevation can have an overriding influence on most environmental parameters, we next tested for collinearity among all independent variables by using the Pearson correlation coefficient and \textit{corr.test} function in R. To reduce collinearity, where significant (alpha = 0.05) and strong ($|r| \geq 0.80$) correlations were found, the more physiologically relevant variable was retained based on previous studies of pika ecology (see Results). Mixed-effects models were used to compare corticosterone estimates to all combinations of remaining independent variables using the \textit{lmer} function of the \textit{lme4} package (Bates \textit{et al.} 2015), after setting REML = FALSE to allow for model selection via AIC$_c$. Model fit was assessed by calculating the marginal $R^2$ as suggested by Nakagawa & Schielzeth (2013) using the \textit{sem.model.fits} function of the \textit{piecewiseSEM} package (Lefcheck, \textit{in press}). The top model was tested for all the basic assumptions of linear regression including: linearity, homoscedasticity, and normality of residuals. Additionally, excessively influential data points were assessed using the \textit{influence.ME} package (Nieuwenhuis \textit{et al.} 2012).
3.3 Results

3.3.1 Laboratory validation

Serial dilutions showed a parallel response of samples across the entire standard curve, but the GC concentration from the highest dilution (1:24) was lower than expected relative to the standard curve (Figure 3.3). Due to the lower than expected GC concentrations, all remaining samples were analyzed undiluted. The addition curve showed that 99.8% of exogenous corticosterone was recoverable in the sample matrix. The mean intra-assay variation was 2.01 and 1.88%, and the inter-assay variation was 2.02 and 4.58% for the B₀ and NSB standards, respectively. There was a negative nonlinear relationship between sample mass and corticosterone concentration, indicating a decrease in extraction efficiency with increasing sample mass (Figure 3.4). To verify this trend, varying initial quantities of hair (1.2, 3.0, 5.2, 10.2, and 18.3 mg) were extracted and analyzed from one sample (PP04T08) resulting in the same nonlinear relationship. A power model was fitted to the data and all subsequent analyses were conducted on the residuals from this model to correct for extraction efficiency. The residuals of this model showed heteroscedasticity, indicating sample masses less than approximately 2.3 mg could lead to inaccurate estimates of corticosterone.

3.3.2 Equivalence of corticosterone estimates from different sample sources

The sample masses for the nine hair samples used to contrast hair GC, plasma GC, and fecal GCM were low (5.8 mg ± 4.7 SD) and the mean coefficient of variation (CV) of corticosterone estimates was high (29.85%) between replicates. There was a substantial skew in plasma GC estimates; to facilitate comparison, plasma GC estimates were natural log transformed. There was a non-significant positive relationship between hair and plasma GC
(F = 3.57, df = 7, R² = 0.338, P = 0.101) and no relationship between hair GC and fecal GCM (F = 1.09, df = 7, R² = 0.134, P = 0.332). Additionally, there was no relationship between plasma GC and fecal GCM (F = 0.830, df = 7, R² = 0.106, P = 0.393).

3.3.3 Pika stress analysis along elevational gradients

Hair samples were obtained from a total of 49 pikas (23 females and 26 males; Table 3.1). All pikas were unambiguously sexed with no replicate returning a different sex. A mean of 19.4 mg (±7.3 SD) of washed, trimmed hair was obtained from each sample, and the minimum sample weight was 6.5 mg. Resulting corticosterone estimates had a mean CV of 7.19% between replicates. A one-way ANOVA showed significant deviation in corticosterone levels among the sample sites (Figure 3.5).

Only cranial diameter and body mass were non-normally distributed (respectively: W = 0.948, P = 0.032; W = 0.911, P = 0.001). A natural log transformation did not establish normality nor approximate a normal distribution; therefore, non-transformed data were used in subsequent analysis. Due to significant collinearity among all temperature metrics and elevation (Table 3.2), elevation was eliminated in favor of a more direct assessment of microclimate variation. Similarly, mean ambient daily temperature was eliminated in favor of mean maximum daily temperature (Amb_max), which may be a better metric of thermal stress (Beever et al. 2011). All talus temperature metrics were collinear with Amb_max; however, we included Tal_night as this metric represented the mean nighttime temperature pika were likely subjected to, in contrast to Amb_max, which represented the mean maximum daytime temperature. Finally, body mass was eliminated in favor of cranial diameter since body mass is likely to fluctuate on a seasonal basis and cranial diameter was more accurately
measured in the field (personal observation).

A total of 13 mixed-effects models were assessed using Amb_max, Tal_night, sex, and cranial diameter as independent variables, including all possible models except those based on the highly collinear variables Amb_max and Tal_night. The top model incorporated all of these variables except Tal_night (Table 3.3), with lower corticosterone estimates at sites with higher ambient temperatures and for larger, male pika. The marginal $R^2$ for this model showed these variables explained about 36.8% of the variation in corticosterone estimates. The residuals of this model were normally distributed ($W = 0.9628$, $P = 0.1231$), showed no signs of heteroscedasticity, nor a linear relationship with the fitted value ($F = 0.251$, $df = 47$, $R^2 = 0.005$, $P = 0.617$) or with the main predictor (cranial estimates; $F = 1.411E^{-26}$, $df = 47$, $R^2 = 3.00E^{-28}$, $P = 1$). No excessively influential data points were identified (Cook’s distance < 0.85 for all sites and < 0.5 for all samples).

3.4 Discussion

3.4.1 Laboratory validation

While we agree with other authors regarding the power of biological validation of GC measurements (Touma & Palme 2005; Sheriff et al. 2011), the limited feasibility of validation must also be acknowledged for some species. The logistical difficulties of a long-term stress trial in wild animals can lead to mixed results when conducting a biological validation of hair-based stress analyses (Koren et al. 2002; Mastromonaco et al. 2014). For example, Mastromonaco et al. (2014) was only able to resample 12 of the original 23 eastern chipmunks used in a biological validation of hair samples (ACTH challenge), and only 3 of the 5 samples in their experimental group exhibited elevated glucocorticoid levels.
Additionally, some species of conservation interest, such as the American pika, would likely exhibit high mortality during a rigorous stress trial (MacArthur & Wang 1973). However, hair-based measurements of GC have been validated in other lagomorph species; for example, Peric et al. (2017) documented a significant increase in cortisol incorporated into hair samples from New Zealand white rabbits after stressful events.

Using individual-based comparisons, we documented a limited connection between plasma- and hair-based estimates and no connection between hair GC and fecal GCM. This lack of correspondence may be attributable to the different time periods over which these sample sources are sensitive. Levels of GC in the bloodstream can be significantly elevated in just a few minutes after a stressful stimulus (Sheriff et al. 2011), and plasma measurements in our study reflect an acute stress response. GCM measurements reflect GC levels on a time scale of several hours or days prior to collection; however, levels of GC in hair represent the accumulation of GC during the relatively long period of hair growth (Yang et al. 1998; Koren et al. 2002). Accordingly, our hair samples likely measured long-term chronic stress following the summer molt, whereas plasma or fecal samples reflected chronic stress experienced during the few hours or days before the time of capture. Additionally, plasma GC, fecal GCM and hair GC measure slightly different hormonal signatures, and other lagomorph studies have documented a lack of correlation among alternative stress metrics (Monclús et al. 2006; Cabezas et al. 2007). For example, only free GCs (those not bound to the carrier protein, corticosterone-binding globulin) circulating in the bloodstream are metabolized by the liver and converted into GCMs; thus, fecal GCM levels mirror free GC levels, but not total GC levels in plasma (Sheriff et al. 2010). The manner in which GCs are incorporated into hair is largely unknown, so questions remain about whether circulating...
free GC concentration in the blood is proportionately reflected in hair and the influence of confounding factors such as GC contributions from saliva or scents (Sheriff et al. 2011). These temporal and measurement differences are likely responsible for the weak correlations previously observed when hair hormone levels have been compared to those of plasma (Yang et al. 1998) and fecal samples (Mastromonaco et al. 2014).

One of the potential difficulties of using hair is the apparent decrease in extraction efficiency with higher sample masses. Interestingly, this same pattern was reported in fecal samples for both mourning doves, *Zenaida macroura* (Millspaugh & Washburn 2004) and California spotted owls, *Strix occidentalis* (Tempel & Gutierrez 2004), reinforcing the need to correct for extraction efficiency. Our approach was to establish a nonlinear relationship to account for this influence. This approach may be preferable when the mass or number of samples is low, as it obviates the need to standardize sample sizes by eliminating smaller samples or truncating larger ones. Of course, this nonlinear relationship suggests that estimates based on low sample masses are less precise (another reason not to standardize samples to the lowest sample mass). Of particular note, the nine hair samples used here to contrast with estimates of fecal GCM and plasma GC were generally low in mass, potentially contributing to the weak relationship observed. We agree with Macbeth et al. (2010) who recommended a minimum sample weight of 5 g when analyzing GC from hair. In our analysis, the relationship between sample mass and GC estimates was approximately linear for samples larger than 5 g and the residuals of our model explaining extraction efficiency were disproportionally high for the low sample masses. We further recommend researchers consider the influence of sample mass in GC extraction efficiencies even for larger samples; our data suggests such effects continue even at higher sample masses.
To summarize, we recommend researchers employ a traditional validation technique if possible when applying a novel stress analysis protocol. When traditional validation is not feasible, as in our case, we suggest a cautious approach to cross-validation as each sample source may measure unique temporal and physiological elements of stress. Additionally, we emphasize the importance of considering and correcting for the influence of extraction efficiency. Sample mass was demonstrated here and elsewhere to have a strong influence on estimated corticosterone concentrations. We recommend standardizing sample masses, when possible, above 5 mg; however, the relationship between extraction efficiency and sample mass may be species-specific, so this cutoff may need to be reassessed, especially in species with hair that is coarser. When logistical considerations make standardizing sample mass impractical, we recommend correcting for extraction efficiency by assessing for a nonlinear correlation between sample mass and GC estimates. The resulting equation can then be used to correct for the influence of sample mass.

3.4.2 Field study

We demonstrated the utility of hair samples by directly investigating factors influencing long-term chronic stress at the individual level. The sensitivity of this analysis allowed us to evaluate the American pika for several patterns of stress hormone activity well-documented in other mammals. Our results showed that hair GC was mainly influenced by body size, a pattern perhaps mediated by individual differences in mass-specific metabolic rates. In mammals, there is a negative relationship between body mass and GC concentration, underpinned by a relative increase in mass-specific metabolic rate with decreasing body mass (Haase et al. 2016). Since the production but not the degradation of GC is a metabolic
function, smaller pikas with higher metabolic rates would be more prone to accumulate GC. Furthermore, smaller animals generally lose heat faster due to their higher surface area to volume ratios, and would need to elevate their baseline metabolism disproportionally to compensate. Additionally, it may be possible that larger pikas would have longer hair, better insulating them from cold stress or influencing the incorporation of GC into the hair. However, an analysis of New Zealand White rabbits found no influence of hair length or body location on GC estimates using a similar protocol (Comin et al. 2012). To our knowledge, this is the first time that a relationship between GC and body size has been reported within a single species; however, pikas may be exceptional given their relatively high metabolic rate (Lovegrove 2003), and further investigation is needed to determine whether this pattern is prevalent within other mammals.

Here, we report perhaps the first direct connection between chronic stress and microclimate variation in the American pika, a species with a reputation for narrow thermal tolerance (Smith 1974; Moyer-Horner et al. 2015). Our results further support the potential for the negative effects of chronic cold stress in this species (Beever et al. 2010, 2011; Ray et al. 2012; Jeffress et al. 2013; Schwalm et al. 2016). The increase in GC observed at colder sites could be a function of when our hair samples were grown. The American pika molts twice each year, during summer and fall (Smith & Weston 1990). While we cannot determine the exact time period over which stress was measured, our samples likely captured the GC profile of pikas just after the summer molt, which typically occurs around June to mid-July (Krear 1965). An increase in GC associated with lower ambient temperatures may indicate the necessity of a higher metabolic rate to maintain homeostasis in colder conditions (Lovegrove 2003), particularly during a molt. Being a small alpine mammal that does not
hibernate, the American pika may be especially dependent on a fine-tuned metabolic rate, given that smaller animals would be disproportionately affected by low temperatures (Moyer-Horner et al. 2015). As a case in point, Boratyński et al. (2016) found that both the basal metabolic rate and non-shivering thermogenesis in Siberian hamsters, *Phodopus sungorus*, was highly plastic during the summer months to meet local thermal conditions. If such patterns generalize to the current study, the elevational pattern of GC reported here may represent the metabolic plasticity of pikas to local thermal conditions. We should note that these data do not refute the potential for heat stress in pikas, as the record of GC in our hair samples would likely have been from early summer when the risk of heat stress was minimal. Additionally, it was the mid-elevational sites that had the lowest stress levels along each of the respective transects, a pattern indicating these sites may have been thermally optimal for pikas, with the potential for stress at lower or higher temperatures.

The lower stress levels reported here for male pikas match the general pattern observed in most mammalian species (Reeder & Kramer 2005). As both male and female pikas are highly territorial (Smith & Weston 1990), this aspect of behaviour is unlikely to contribute to sex-specific differences in stress. Our samples likely represented the post-breeding period and thus would not capture the increase in stress associated with mating found in other small mammals (Koren et al. 2008). However, our sampling period coincided with gestation and lactation. The costly metabolic demands of rearing offspring may be responsible for elevated stress hormone levels in female mammals (Gittleman & Thompson 1988; Wade & Schneider 1992). Interestingly, female pikas possess a larger adrenal gland than males (Smith & Weston 1990), potentially to meet these physiological demands. However, the relative stress level of each sex may fluctuate seasonally as males and females
perform differing tasks, which could decouple acute and long-term stress measurements. For instance, Wilkening et al. (2013) reported higher GCM levels in male pikas, but a longer duration of GCM response to an acute stressor in females.

One of the known limitations of the elevational transect experimental design is the high degree of covariation among microclimate variables typically observed, which can preclude the identification of specific climate influences (Sundqvist et al. 2013). The high degree of covariation within our microclimate estimates was indicative of the overarching influence of elevation on climate within our sample area. As such, our measurements likely represent relative microclimate differences between our sites, independent of time period. Fittingly, our direct measurement of $Amb_{max}$ was highly related to mean annual temperature at our sites for 2014 ($R^2 = 0.878$, $F = 43.2$, df = 6, $P < 0.001$) using downscaled weather station data from the CLIMATEWNA model (Wang et al. 2012). While this addresses our concern over using microclimate measurements taken subsequent to our hair samples and over a short period of time, it does render identifying more specific climate influences challenging with this dataset. This limitation could potentially be addressed by careful selection of additional elevational transects in the future.

In conclusion, we suggest a cautionary approach when attempting GC measurements in a species without the ability to validate the methodology. Identifying biologically relevant and well-supported relationships such as GC covariance with body size can assist in the development of novel measurement protocols. In addition, cross-referencing GC metrics among analysis methods may support novel applications in some cases. However, we urge careful consideration of sample type in addressing physiological stress in wildlife, as sample sources vary in the time periods over which they actively measure stress. Finally, we report
the only known correlation between directly measured physiological stress and climate variation in the American pika. Our results add to the recent evidence of cold stress in pikas (Beever et al. 2010, 2011; Ray et al. 2012; Jeffress et al. 2013; Schwalm et al. 2016). We suspect that the elevated metabolic rate needed to endure colder ambient conditions as a small bodied, non-hibernating mammal may be responsible for the elevated GC levels reported here. Further research assessing physiological stress in the American pika may assist in conservation and monitoring efforts as we enter a period of rapid environmental change.
Table 3.1. Site description of the Pyramid Peak (PP) and Thornton Lakes (TL) sampling transects in North Cascades National Park, WA. Mean daily temperatures for ambient maximum ($Amb_{max}$), ambient daytime ($Amb_{day}$), talus maximum ($Tal_{max}$), talus daytime ($Tal_{day}$), and talus nighttime temperatures ($Tal_{night}$) are reported in °C along with elevation (m), sample size ($n$), mean cranial diameter (mm), mean weight (g), and sex ratio ($M/F$) of pikas at each site.

<table>
<thead>
<tr>
<th>Site</th>
<th>Elevation</th>
<th>$Amb_{max}$</th>
<th>$Amb_{day}$</th>
<th>$Tal_{max}$</th>
<th>$Tal_{day}$</th>
<th>$Tal_{night}$</th>
<th>$n$</th>
<th>Cranial</th>
<th>Weight</th>
<th>$M/F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP01</td>
<td>480</td>
<td>25.2</td>
<td>22.8</td>
<td>16.6</td>
<td>16.2</td>
<td>15.8</td>
<td>5</td>
<td>24.5</td>
<td>153.0</td>
<td>0.67</td>
</tr>
<tr>
<td>PP02</td>
<td>810</td>
<td>23.8</td>
<td>20.3</td>
<td>14.2</td>
<td>13.7</td>
<td>13.7</td>
<td>6</td>
<td>25.6</td>
<td>138.3</td>
<td>1.00</td>
</tr>
<tr>
<td>PP03</td>
<td>1327</td>
<td>20.0</td>
<td>17.7</td>
<td>14.7</td>
<td>13.9</td>
<td>12.6</td>
<td>3</td>
<td>23.5</td>
<td>123.3</td>
<td>0.50</td>
</tr>
<tr>
<td>PP04</td>
<td>1550</td>
<td>17.8</td>
<td>15.6</td>
<td>13.1</td>
<td>12.4</td>
<td>11.4</td>
<td>9</td>
<td>23.9</td>
<td>152.2</td>
<td>2.00</td>
</tr>
<tr>
<td>TL01</td>
<td>504</td>
<td>23.6</td>
<td>21.2</td>
<td>19.2</td>
<td>18.3</td>
<td>17.9</td>
<td>7</td>
<td>27.7</td>
<td>182.1</td>
<td>0.75</td>
</tr>
<tr>
<td>TL02</td>
<td>760</td>
<td>23.3</td>
<td>20.6</td>
<td>16.3</td>
<td>15.8</td>
<td>14.6</td>
<td>8</td>
<td>29.2</td>
<td>182.5</td>
<td>1.67</td>
</tr>
<tr>
<td>TL03</td>
<td>1409</td>
<td>17.6</td>
<td>15.7</td>
<td>11.0</td>
<td>10.4</td>
<td>10.4</td>
<td>7</td>
<td>26.9</td>
<td>162.6</td>
<td>1.33</td>
</tr>
<tr>
<td>TL04</td>
<td>1665</td>
<td>15.4</td>
<td>14.2</td>
<td>12.4</td>
<td>11.6</td>
<td>10.9</td>
<td>4</td>
<td>23.8</td>
<td>131.3</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Table 3.2. Correlation matrix of independent variables including pika morphological variables and site microclimate variables (see Table 3.1 for definitions).

<table>
<thead>
<tr>
<th></th>
<th>Cranial</th>
<th>Weight</th>
<th>Elevation</th>
<th>Amb_max</th>
<th>Amb_day</th>
<th>Tal_max</th>
<th>Tal_day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td></td>
<td>0.78*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elevation</td>
<td>-0.35</td>
<td>-0.23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amb_max</td>
<td>0.29</td>
<td>0.17</td>
<td>-0.97*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amb_day</td>
<td>0.29</td>
<td>0.19</td>
<td>-0.98*</td>
<td>0.99*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tal_max</td>
<td>0.28</td>
<td>0.24</td>
<td>-0.86*</td>
<td>0.80*</td>
<td>0.84*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tal_day</td>
<td>0.29</td>
<td>0.24</td>
<td>-0.88*</td>
<td>0.83*</td>
<td>0.86*</td>
<td>1.00*</td>
<td></td>
</tr>
<tr>
<td>Tal_night</td>
<td>0.30</td>
<td>0.24</td>
<td>-0.93*</td>
<td>0.86*</td>
<td>0.89*</td>
<td>0.98*</td>
<td>0.98*</td>
</tr>
</tbody>
</table>

* Significance after sequential Bonferroni correction (p ≤ 0.007)
Table 3.3. Information-theoretic analysis of mixed-effects models explaining variation in corticosterone estimates of American pika samples (see Table 3.1 for definitions and sample sizes). All variables demonstrated negative relationships with corticosterone estimates. The negative slope for sex indicates males had lower corticosterone estimates. Site was used as a random effect in all models and the null model included only the random effect.

<table>
<thead>
<tr>
<th>Model</th>
<th>K</th>
<th>AICc</th>
<th>ΔAICc</th>
<th>Weight</th>
<th>Evidence Ratio</th>
<th>Marginal R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Cranial - Sex - Amb_max</td>
<td>6</td>
<td>264.60</td>
<td>-</td>
<td>0.248</td>
<td>1.00</td>
<td>0.368</td>
</tr>
<tr>
<td>- Amb_max - Cranial</td>
<td>5</td>
<td>264.80</td>
<td>0.20</td>
<td>0.225</td>
<td>1.10</td>
<td>0.326</td>
</tr>
<tr>
<td>- Cranial</td>
<td>4</td>
<td>265.31</td>
<td>0.71</td>
<td>0.174</td>
<td>1.43</td>
<td>0.208</td>
</tr>
<tr>
<td>- Sex - Cranial</td>
<td>5</td>
<td>265.60</td>
<td>1.00</td>
<td>0.151</td>
<td>1.64</td>
<td>0.239</td>
</tr>
<tr>
<td>- Tal_night - Cranial</td>
<td>5</td>
<td>266.80</td>
<td>2.20</td>
<td>0.083</td>
<td>3.00</td>
<td>0.261</td>
</tr>
<tr>
<td>- Cranial - Sex - Tal_night</td>
<td>6</td>
<td>266.90</td>
<td>2.30</td>
<td>0.079</td>
<td>3.16</td>
<td>0.299</td>
</tr>
<tr>
<td>- Amb_max</td>
<td>4</td>
<td>271.01</td>
<td>6.41</td>
<td>0.010</td>
<td>24.64</td>
<td>0.152</td>
</tr>
<tr>
<td>- Amb_max - Sex</td>
<td>5</td>
<td>271.10</td>
<td>6.50</td>
<td>0.010</td>
<td>25.73</td>
<td>0.186</td>
</tr>
<tr>
<td>Null</td>
<td>3</td>
<td>271.83</td>
<td>7.23</td>
<td>0.007</td>
<td>37.21</td>
<td>0.000</td>
</tr>
<tr>
<td>- Sex</td>
<td>4</td>
<td>272.21</td>
<td>7.61</td>
<td>0.006</td>
<td>44.90</td>
<td>0.027</td>
</tr>
<tr>
<td>- Tal_night</td>
<td>4</td>
<td>272.61</td>
<td>8.01</td>
<td>0.005</td>
<td>54.85</td>
<td>0.091</td>
</tr>
<tr>
<td>- Tal_night - Sex</td>
<td>5</td>
<td>272.80</td>
<td>8.20</td>
<td>0.004</td>
<td>60.20</td>
<td>0.122</td>
</tr>
</tbody>
</table>
Figure 3.1. Photograph of an American pika and reference hair sample (inset) weighing approximately 10 mg. Photo courtesy of Andrew Veale.
Figure 3.2. Sample sites in North Cascades National Park, Washington, USA. Thornton Lake (TL) and Pyramid Peak (PP) sampling sites shown as circles. Inset map shows approximate location in Washington state. Topographic lines represent 100-m intervals of elevation.
Figure 3.3. Top: Parallelism between the standard curve (solid line with circles) and serial dilutions of one sample (squares, no line). Bottom: Addition curve showing a linear relationship ($P < 0.001$) between observed and expected GC when samples were mixed 1:1 with standard concentrations of corticosterone from the standard curve.

$$y = 1.009x - 29.53$$

$R^2 = 0.998$
Figure 3.4. Relationship between sample mass and estimated corticosterone concentration using NOCA samples (squares), hair samples from paired plasma and fecal samples (triangles), and differing masses from PP04T08 (circles). Inset exponential relationship (solid line) was developed using all samples. Dashed line shows our suggested 5-mg minimum sample weight cutoff.
Figure 3.5. Box and whisker plot showing average corticosterone per site after correcting for extraction efficiency (see Table 3.1 and Figure 3.2 for site descriptions). Boxes represent medians, 25% and 75% quartiles while whiskers extend through 95% interquartile range. A one-way ANOVA showed significant deviation among sites (F = 5.028, df = 7, P < 0.001). Sites are numbered to reflect relative elevation, where 01 = lowest.
Chapter 4: Adaptation to climate warming from environmentally-mediated selection may be impeded due to directional gene flow in a thermal-sensitive mammal

4.1 Background

Global climate change has profound biological ramifications for nearly all species, many of which have already responded through alterations in morphology, phenology, behavior, abundance, and/or range shifts (Walther et al. 2002; Parmesan & Yohe 2003; Brown et al. 2016; Pacifici et al. 2017). However, in many cases these ecological responses are constrained by species interactions (Van der Putten et al. 2010; Urban et al. 2012), natural limits to dispersal (Pearson & Dawson 2003), or anthropogenic barriers (Opdam & Wascher 2004). As an alternative or complementary outcome, many species have been able to respond to climate change due to phenotypic plasticity (Merilä and Hendry 2014), but this mechanism alone is unlikely to provide a long-term solution when populations are subjected to consistent directional selection (Visser 2008). Adaptation to new climate conditions offers a potential long-term mechanism for impacted species to maintain viability in rapidly changing environments (Savolainen et al. 2013). Therefore, the identification of adaptations to climate and thermal stress has become an important focus in conservation biology (Reusch & Wood 2007; Merilä & Hendry 2014).

Identifying genetic adaptations requires the initial detection of genetic changes in a population over time in response to a selective pressure and the subsequent demonstration that this genetic change leads to increased fitness. Documenting evolutionary responses to climatic pressure in wild populations at this level has presented many methodological difficulties. Although induced thermal tolerance has been documented in laboratory settings
few studies have attempted to document longitudinal genetic changes in natural populations associated with climate pressure (Merilä 2012). The high degree of phenotypic plasticity in natural populations and difficulty of longitudinal genetic analysis has largely confounded the direct identification of genetic adaptations. Gienapp et al. (2007) concluded that the direct evidence for evolutionary responses to climate change is limited and insufficient to draw inferences on the adaptability of species to climate change. Even given these difficulties, several studies have documented local adaptation in response to climate change, albeit using common-garden experiments to indirectly assess genetic changes (Bradshaw & Holzapfel 2001; Franks et al. 2007). For example, an investigation of pitcher plant mosquitoes (Wyeomyia smithii) revealed genetic shifts in critical photoperiod related to seasonal shifts induced by climate change (Bradshaw & Holzapfel 2001).

Additionally, an experiment that contrasted flowering phenology in Brassica rapa before and after a severe drought showed a rapid genetic response to climate-induced stress (Franks et al. 2007). Still, major challenges exist in identifying specific genomic responses to climate change in a broader array of taxa, including highlighting gene regions that may be influenced by natural selection and determining the pace at which impacted species will respond to environmental change.

Population genomics provides a framework for detecting signatures of natural selection associated with local adaptation (Luikart et al. 2003) and for predicting the long-term viability of species impacted by climate change (Visser 2008; Gienapp & Lof 2013; Beever et al. 2016). In particular, genotype-environment association (GEA) analyses provide a powerful approach for differentiating the locus-specific effects of natural selection from genome-wide patterns of evolution caused by processes such as gene flow, genetic drift, and
inbreeding (Balding & Nichols 1995; Vitalis et al. 2001; Oleksyk et al. 2010; Vitti et al. 2013; Rellstab et al. 2015). GEA methods seek to detect natural selection by developing functional association between allelic diversity and environmental pressures. For example, Guo et al. (2016) found strong genomic evidence for adaptive divergence in Andrew’s toads (Bufo andrewsi) by demonstrating elevational patterns in genes associated with binding and metabolic processes while controlling for neutral divergence. Such applications of GEA analyses to identify potential climate adaptations are still very limited in mammals. A recent review of phenotypic and evolutionary responses in mammals found no evidence for adaptation in any of the reviewed studies, concluding that it was unclear whether this lack of evidence indicates a lack of adaptability or simply a paucity of data (Boutin & Lane 2014).

The American pika, Ochotona princeps, has been identified as a sentinel mammalian species for the ecological impacts of climate change (Beever et al. 2003; Wilkening & Ray 2016). Recent population extirpations (Grayson 2005; Beever et al. 2011), genetic evidence of range-wide declines (Galbreath et al. 2009), and direct physiological evidence (Waterhouse et al. 2017) all suggest that American pikas are thermally sensitive. Due to this sensitivity, numerous niche models predict precipitous declines in the range of the America pika in response to climate change (Galbreath et al. 2009; Wilkening et al. 2011; Jeffress et al. 2013; Stewart et al. 2015). A draft reference genome and relatively close phylogenetic relationship with a model organism, the European rabbit (Oryctolagus cuniculus; Lanier and Olson 2009), make the American pika a useful mammalian system to assess genomic responses to climate change (Lemay et al. 2013; Henry & Russello 2013; Russello et al. 2015; Robson et al. 2016). For instance, Henry and Russello (2013) identified genomic signatures of environmentally-induced natural selection using anonymous AFLP markers.
Similarly, Lemay et al. (2013) identified fixed differences in transcriptome sequences between populations living in different climate zones. Moreover, several elevationally-associated loci were identified in the first proof-of-concept studies to pair non-invasive sampling and genotyping-by-sequencing (Russello et al. 2015). These studies were useful for providing preliminary genomic insights and resources for the American pika. However, the characteristics of the employed markers and limitations in sampling populations or the genome precluded a thorough investigation of climate adaptation in these previous studies.

In this study, we used a space-for-time design and restriction site-associated DNA sequencing (RADseq; Baird et al. 2008; Etter et al. 2011) to genotype tissue samples of American pika in order to: (1) investigate evidence for local adaptation across two elevational transects established in North Cascades National Park, Washington, USA; (2) annotate candidate gene regions exhibiting robust signatures of selection and explore biological implications relative to previously published results; (3) assess elevational patterns of genomic diversity and gene flow to infer both the extent and directionality of pika movement across a sharp climate gradient; and (4) investigate if individual levels of inbreeding have a functional impact on stress hormone levels associated with climate stress.

4.2 Materials and methods

4.2.1 Sample site and sample collection

Two previously established elevational transects in North Cascades National Park (NOCA), WA, USA were resampled for this study (Waterhouse et al. 2017; Russello et al. 2015). Four sites were located along each transect, representing an approximately span of 1,000 m in elevation, an expected 6.5°C change in mean annual temperature (Briggs 1997), and an
approximate 10% reduction in available oxygen (Peacock 1998). The Thornton Lakes transect (TL) spanned 6.1 km from the low (TL01) to highest site (TL04) and all sites were approximately linear along this south- to southeast-facing transect (Figure 4.1). The Pyramid Peak transect (PP) was 18.8 km east (from TL01 to PP01), spanned 5.4 km, and exhibited a largely northeast aspect; the limited availability of low-elevation pika populations in the area necessitated the low site be slightly offset from the general aspect of this transect. Both transects started at the lowest elevation at which we found pika occupied sites large enough for our sampling requirements and terminated in the subalpine region. Additionally, we sampled pikas at supplemental low-elevation sites where one or several pikas were identified.

Pikas were live-trapped between July 20th and August 29th of 2014 using Tomahawk model 202 (Hazelhurst, WI) collapsible traps and following University of British Columbia Animal Care Protocol #A11-0371-006 and U.S. National Park Service Permit # NOCA-2014-SCI-0022. Between 15 and 22 traps were set at each main sampling site around signs of pika activity (e.g., hay piles and latrine sites) and approximately 50 m apart to avoid re-trapping the same individual. Traps were set one week in advance and baited with alfalfa cubes every other day to acclimate the animals to the presence of the traps. Trapping was generally conducted between 0700 and 1100 or 1600 and 2000 when temperatures were between 5°C and 18°C and traps were checked every three hours to avoid heat stress. After capture, each pika was transferred to a handling bag and two small samples of ear tissue (each 3 mm diameter) were collected and stored in ethanol for subsequent DNA extraction. Cranial diameter (zygomatic width) was measured with digital calipers to the nearest millimeter and body mass was measured to the nearest 5 grams using a Pesola scale. A small tuft of fur was removed from each individual for corticosterone analysis (Waterhouse et al.)
2017). We sampled each of the 8 main sites until a target of 8 pikas per site were captured, or until three consecutive trap sessions failed to capture any new pikas.

4.2.2 Climate measurement

We obtained climate data from each site using two methods: first, by using an elevational model to downscale existing climate measurements; and second, by obtaining direct thermal measurements at each site using temperature sensors. The ClimateWNA model uses partially derived elevational models to downscale climate data from PRISM (Daly et al. 2002) and ANUSPLIN (Hutchinson 1989) to a high-resolution raster providing both downscaled direct climate measurements and derived variables of biological relevance (Wang et al. 2012). Long-term averages (1981-2010) for the directly calculated annual (n = 8) and seasonal (n = 20) variables provided by the ClimateWNA model were calculated based on the longitude, latitude, and elevation of each site (Table S4.1).

Since local climatic variations driven by small-scale topographic elements and talus specific properties are not captured by ClimateWNA, we also took direct measurements of ambient and below-talus temperatures using four Thermochron iButton temperature sensors (model DS1921G, Maxim Integrated Products, Sunnyvale, CA) deployed in weather-proof housings at each of the eight sites. Two “ambient sensors” were deployed 1.5 m above the talus, each under a separate white plastic shade in neighboring trees, and two “talus sensors” were placed in a central location at each site approximately 0.8 m below the talus surface. Each sensor collected temperature readings every 4 hours starting at 0200 from September 8, 2014 through August 15, 2015. To better approximate site-wide conditions, temperature data were averaged between paired sensors (i.e., between the 2 ambient and between the 2
interstitial sensors) at each site. To represent the fine-scale annual climate conditions at each site, we calculated the following biologically relevant summary variables for ambient and talus sensors at each site (Beever et al. 2010, 2011, 2013; Wilkening et al. 2011): number of days with a daily minimum below -10° C, number of days with a daily maximum above 28° C, mean annual temperature, mean summer temperature (June – August), and mean winter temperature (December – February).

We expected a high degree of collinearity among climate variables due to the influence of elevation on climate patterns (Sundqvist et al. 2013). To reduce the dimensionality of the original climate dataset, a principal component analysis (PCA) was conducted using all directly calculated annual and seasonal variables from ClimateWNA (n = 28) and summary variables from iButton measurements (n = 8). We ran a PCA for each transect using prcomp in R v.3.3.2 (R Core Team 2015) on normalized climate variables (mean set to zero and standard deviation set to 1). Subsequently, we used the PCA eigenvectors that collectively accounted for >95% of the variation in the climate data as synthetic climate variables.

4.2.3 DNA extraction and RADseq genotyping
DNA was extracted from one tissue sample per individual using the NucleoSpin Tissue Kit (Macherey-Nagel) following the manufacturers’ suggestions with the addition of RNase A (Qiagen). The extracted concentration of genomic DNA was measured using a Quant-iT™ PicoGreen® dsDNA quantification assay kit (Thermo Fisher Scientific) following the manufacturers’ recommendations and using a ViiA 7 real-time PCR machine (Thermo Fisher Scientific).
We constructed two RADseq libraries using a protocol described by Baird et al. (2008) as modified in Lemay & Russello (2015). Briefly, 500 ng of DNA was digested with the Sbf I restriction enzyme for each individual (New England Biolabs). P1 adaptors were ligated, each containing a unique barcode for each sample in the library. Barcodes were six nucleotides in length and differed by at least two bases (Hohenlohe et al. 2010; Narum et al. 2013). Once pooled, samples were sheared to a mean length of approximately 500 bps using a Bioruptor® (Diagenode) and all fragments between 400-600 base pairs in length were separated using a Pippin Prep™ (Sage Science) by following the manufacturers’ recommendations. Library amplification was carried out following the addition of the P2 adaptor to pooled samples. A second round of size selection was conducted targeting fragments between 450 and 650 bps to account for the addition of the P2 adaptor. Libraries were sequenced using one full lane of Illumina HiSeq 2000 per library and targeting 100-bp single-end reads.

4.2.4 Reference assembly and SNP discovery

Libraries were demultiplexed and trimmed to 94-bp using the process_radtags program in STACKS v.1.30 (Catchen et al. 2011, 2013). We used BWA v.0.7.12 (Li & Durbin 2009) to index the pika reference genome (Ensembl, release 88, Ochotona_princeps.pika.dna_sm.toplevel.fa) and masked repeated gene scaffolds in order to make a reference for the subsequent assembly of RADseq reads. Each read was aligned to this reference using the aln algorithm and the resulting SAM files were indexed and converted into BAM files using SAMTOOLS v.1.2 and piped into BCFTOOLS v.1.3.1 for SNP discovery using default parameters (Li et al. 2009a). The resulting VCF file was filtered in
VCFTOOLS v.1.12b (Danecck et al. 2011) to ensure: a minimum depth of 6, a maximum depth of 100, no greater than 30% missing data, a minor allele frequency greater than 0.05, only one SNP retained per RADtag, presence of a maximum of 2 alleles, and minimum phred-scale quality score of 20, which corresponds to 99% confidence in genotypes. Additionally, we removed any indels and sequences that mapped to multiple locations of the reference genome. We used the –hardy option in VCFTOOLS to check for Hardy-Weinberg Equilibrium (HWE) at each site with greater than 3 individuals. We excluded any locus that was out of HWE ($\alpha = 0.05$) at greater than 50% of these sites (minimum of 2). The resulting data were split into PP- and TL-specific datasets, which only contained polymorphic loci present in 70% of the individuals along their respective transects.

4.2.5 Outlier detection and annotation
The vast number of genomic markers needed to conduct genomic scans makes controlling for false positives in outlier locus detection a major analytical hurdle (François et al. 2016). One method for overcoming this limitation is to combine multiple independent statistical analyses. In a simulation study, De Villemereuil et al. (2014) found this approach greatly reduces error rates when identifying outlier loci in large genomic datasets. Here, we used three outlier detection methods conducted independently for each transect. First, we used the $F_{ST}$-outlier method of Beaumont and Balding (2004) as implemented in BAYESCAN 2.1 (Foll & Gaggiotti 2008) with a prior odds value of 10, using 100,000 iterations and a burn-in of 50,000 iterations. This analysis was conducted 5 independent times for each transect using a different random seed each time and median scores were calculated for all test statistics. Outliers were identified using a q-value of 0.2 to test for statistical significance.
We next used latent factor mixed models (LFMM) to identify specific genomic correlates to environmental parameters using the R package LEA v.1.4.0 (Frichot & François 2015). This method introduces hidden latent factors to account for the influence of genetic structure and spatial autocorrelation of genetic data and then tests for nonrandom associations between environmental parameters and allele frequencies (Frichot et al. 2013). The number of latent factors introduced is determined by the genetic structure of the data. Each transect was first analyzed for the presence of genetic subdivisions (K) using the pca and the snmf functions. We inferred the appropriate number of latent factors by assessing the number of significant axes in the PCA; significance was assessed by comparing eigenvalues to a Tracy-Windom distribution using the tracy.windom function (Patterson et al. 2006). The snmf function uses a Bayesian clustering algorithm to estimate individual admixture coefficients similar to the program STRUCTURE (Pritchard et al. 2000). We identified the K value which returned the minimum cross-entropy in the snmf analysis with 10 repetition for each K value between 1 and 6 for each transect. Next, we used this K value to inform the LFMM analysis assessing if allele frequencies were associated with any of the climate variables. We used the lfmm function with 100,000 iterations, 50,000 burin-in cycles, and 10 replicates. The resulting z-scores were combined using the Stouffer method (Whitlock 2005) recommended by the authors of the software and we applied the genomic inflation factor recommended by Devlin & Roeder (1999). A Bonferroni correction was used to control for multiple comparisons (Rice 1989).

Lastly, we used BAYPASS v.2.1.0 as a third independent method for identifying loci under divergent selection (Gautier 2015). BAYPASS employs an updated version of the Bayesian hierarchical model proposed by Coop et al. (2010). This analysis first constructs a
population covariance matrix to estimate neutral genetic divergence, then identifies outliers by checking for nonrandom associations between locus-specific divergence ($F_{st}$) and environmental parameters using a Bayesian framework. BAYPASS introduces modeling modifications to improve the accuracy of the estimated population covariance matrix and a complete reprogramming of the core Markov Chain Monte Carlo (MCMC) algorithm from BAYENV2 (Günther & Coop 2013), which allows for more efficient analysis of genomic datasets. We conducted five independent runs of the standard covariate model with 20 pilot runs of 1,000 iterations and confirmed the proposal distributions for Metropolis and Metropolis-Hastings updates were within an acceptance range between 0.2 and 0.4 to ensure proper convergence (Gilk et al. 1996). A run length of 25,000 iterations was used, sampling every 25 iterations (thinning procedure) after a burn-in of 5,000 iterations. We selected the run with the value of the lowest deviance information criterion and assessed evidence of natural selection at each locus using a cutoff Bayes Factor of 10 decibans as evidence for ‘strong support’ (following Jeffreys 1961).

We constructed Venn diagrams of overlapping outliers that were significant between transects and methods (BAYSCAN, LFMM, and BAYPASS) using an online tool available at http://bioinformatics.psb.ugent.be/webtools/Venn/. All outliers that were either identified in both transects or in multiple methods were separated into an ‘outlier dataset’. We assessed linkage disequilibrium between all pairs of outlier loci in all populations using the exact test of Guo and Thompson (1992) with 10,000 dememorization steps, 100 batches, and 10,000 iterations per batch implemented in GENEPOP 4.3 (Rayman & Rousset 1995; Rousset 2008) and used a Benjamini-Hochberg correction procedure to assess significance (Benjamini & Hochberg 1995). Additionally, the reference DNA sequence from a 200-bp window around
each outlier SNP was subjected to a BLASTN search of all sequences in the NCBI non-redundant database (Altschul et al. 1990). We used a word size of 11, mismatch score of 2, -3, and maximum e-value of $10^{-10}$.

4.2.6 Population genetic analysis

Natural selection can bias estimates of genetic diversity and divergence (Beaumont & Nichols 1996; Luikart et al. 2003). We form a ‘neutral dataset’ by eliminating all loci that were identified as outliers by any analysis in either transect. This dataset was used to estimate observed and expected heterozygosity, effective number of alleles, and $F_{is}$ inbreeding (Nei 1978) at each site. We tested for differences in diversity metrics between transects using a two-tailed $t$-test implemented in R v.3.1.3 (R Core Team 2015). Next, the hierarchical portioning of genomic diversity was estimated by conducting an analysis of molecular variance (AMOVA) and levels of genetic differentiation among sites were assessed using pairwise comparisons of $\theta$ (Weir & Cockerham 1984). All genetic diversity tests were performed in GENODIVE v.2.0b27 (Meirmans & Van Tienderen 2004) with a permutation test of 1,000 replicates to test $F_{is}$, $\theta$, and AMOVA results for statistical significance.

We investigated the genetic structure among our main sites using a Bayesian clustering method implemented in the program ADMIXTURE v1.3 (Alexander et al. 2009). Overall genetic structure was inferred from the neutral dataset using all the sites in both transects and by running the program for each value of $K$ between 1 and the number of sites plus 2. The optimal number of genetic units was assessed by inspecting the ten-fold cross-validation statistic (CV) and selecting the $K$ with the lowest CV. Strong local genetic structure can mask further subdivision; therefore, we repeated the ADMIXTURE analyses for
each transect individually using the same parameters as above. We investigated if outlier loci followed a similar spatial structure by generating transect-specific datasets of loci identified either in both transects or by two outlier detection methods within their respective transects, and reran admixture again using the same parameters as above.

4.2.7 Gene flow analysis
Evidence of recent migration (i.e., during the past few generations) along each transect was assessed using the Bayesian method implemented in BAYESASS (Wilson & Rannala 2003). This analysis detects migration by extracting information from transient disequilibria observed at individual multilocus genotypes. Importantly, this method makes relatively few assumptions, allowing it to be applied to nonstationary populations and to genotype proportions out of Hardy-Weinberg equilibrium. We estimated migration rates separately for neutral and outlier loci along each transect using 10,000,000,000 iterations after a burn-in of 1,000,000 steps and sampled every 100th iteration. The inbreeding coefficient, allele frequency, and migration rate mixing parameters were adjusted for each dataset to ensure acceptance rates between 0.2 and 0.6 for each variable. We conducted 5 independent runs each using a separate random number and compared results using the mean coefficient of variance for migration estimates between runs to ensure proper convergence. We used the median migration rates from these runs and constructed 95% credible sets by multiplying the mean standard deviation for each migration rate by 1.96 as suggested by Wilson & Rannala (2003). Significance was indicated when the credible set did not encompass zero.

The supplemental sites we sampled along each transect likely represent recently dispersed pikas since no established population was identified. To further elucidate migration in this system, we conducted an assignment test for each individual at these supplemental
sites using 5,000 randomly selected neutral SNPs and the estimator of Rannala & Mountain (1997) in GENECLASS version 2.0.b (Piry et al. 2004).

4.2.8 Stress hormone analysis
To investigate the potential role of inbreeding in the climate response of the American pika, we first calculated individual estimates of $F_{is}$. To do this, we used a method of moments using the –het function in VCFtools (Danecek et al. 2011). Since this estimate requires population-wide gene frequencies we calculated this estimate separately for each transect using neutral loci (See Admixture results below). We used the hair corticosterone estimates of 49 pikas from Waterhouse et al. (2017) as estimates of long-term chronic stress. This study found that body size, as measured by cranial diameter, sex and maximum summer ambient temperature were all important predictors of individual stress levels. We re-ran the linear mixed-effects models from Waterhouse et al. (2017) by using all combinations of these variables with the addition of individual $F_{is}$ as fixed effects and used site as a random effect and selected the best mixed effect model via $AIC_c$.

4.3 Results
4.3.1 Sample and environmental data collection
We sampled 59 pikas during the summer of 2014 (Table 4.1). Our main sites had an average sample size of 6.6 pikas, ranging from 3-11 per site. Additionally, three supplemental sites along the PP transect and two along the TL transect were sampled. One pika was captured from each supplemental site with the exception of TL0.5 where two pikas were captured.

All 32 iButton sensors captured temperature data from all sites from September 8, 2014 through August 15, 2015. Despite the 1.5 m-high placement, one pair of ambient
sensors (PP04) appeared to be insulated by snow cover, leading to an overestimation of ambient temperatures at that site between January 15 and May 15, 2015. Despite this additional insulation at PP04, there was high correspondence between climate metrics obtained from ClimateWNA and iButtons (Tables S4.1 and S4.2). A Pearson correlation showed these two methods of assessing climate produced highly comparable estimates of mean annual temperature ($r = 0.992, n = 8, p < 0.001$).

The PCA of ClimateWNA and iButton variables showed the overriding influence of elevation on site-level climate. The first principal component (PC1) explained 90.3% and 94.9% of the variance in climate in the PP and TL transects, respectively. Adding the second principal component (PC2) increased the amount of variation explained to 98.6% and 99.1% for the PP and TL transects, respectively. PC1 had nearly equal loadings of all the climate variables along each transect (Figure S4.1) and was positively correlated to elevation in both the PP transect ($r = 0.999, n = 4, p = 0.001$) and TL transect ($r = 0.999, n = 4, p = 0.001$).

Along the PP transect, the second principal component primarily reflected high solar radiation, high below-talus winter temperature and low ambient temperature. Along the TL transect, the second principal component primarily reflected low estimated solar radiation, high temperature differential between summer and winter, high talus temperature, and low ambient temperature.

4.3.2 Reference assembly and SNP discovery
DNA sequencing resulted in ~317.8 million reads; after demultiplexing and filtering reads with ambiguous barcodes, low quality scores (phred score < 10), and ambiguous RAD-tags, we obtained a mean of 4.1 (SD = 1.2) million reads per sample. Approximately 76.1% (SD =
0.44%) of these reads aligned to the reference genome scaffolding during assembly. After various filtering methods, we obtained a final dataset of 30,763 high-quality SNPs for the 59 samples with an average depth of 25.6 reads (SD = 7.5; Table 4.2). Samples had a mean of 6.4% missing genotypes (SD = 4.8%) per individual. There were 3,634 and 3,784 loci that were monomorphic along the PP and TL transects, respectively.

4.3.3 Outlier detection

The BAYESCAN analysis identified 67 and 52 outlier loci along the PP and TL transects, respectively; among these, one locus was shared between the two transects (Table 4.3). Outlier loci had high $F_{st}$ values, with a mean of 0.226 (SD = 0.052) and 0.225 (SD = 0.044) as compared to putatively neutral loci with mean $F_{st}$ values of 0.056 (SD = 0.005) and 0.070 (SD = 0.006), along the PP and TL transects, respectively. These results were highly consistent; the mean coefficient of variation for $q$ values between runs was 1.01% and 0.95% for PP and TL transects, respectively.

To conduct the LFMM analysis, we first determined the appropriate number of latent factors by summarizing our genomic data along each transect using a PCA and snmf analysis. The PCA indicated no axes were significant for the PP transect ($p > 0.20$), whereas the first two axes explained 18.7% of the genomic variation along the TL transect ($p < 0.01$). The snmf analysis for the PP transect returned a minimum cross-entropy for $K = 1$ of 0.879 with $K = 2$ being the next lowest of 0.885. The snmf analysis supported $K = 2$ for the TL transect returning a cross-entropy of 0.763 while the next lowest was $K = 4$ with a minimum cross-entropy of 0.765. We therefore used one latent factor along the PP transect and two latent factors along the TL transect to account for neutral genetic structure. The LFMM analysis
highlighted 991 loci along the PP transect as being correlated with either PC1 or PC2, with 23 being correlated to both principal components. There were 445 loci along the TL transect correlated with either PC1 or PC2, with 21 being correlated to both principal components. Of these, 54 loci were highlighted independently in both transects by the LFMM analysis (Figure 4.2a and 4.2b).

**BAYPass** highlighted 292 loci associated with either PC1 or PC2 along the PP transect, with one locus being associated with both environmental principal components. Along the TL transect, 354 loci were associated with either PC1 or PC2, with one locus being associated with both environmental principal components. There were seven outlier loci that were highlighted independently in both transects by the BAYPass analysis (Figure 4.2c and 4.2d).

To control for type 1 error, we considered only loci highlighted by multiple methods or in both transects for further analysis. This method indicated robust evidence for 173 outlier loci (Table 4.3). There were 70 loci highlighted by multiple methods along the PP transect and 55 along the TL transect. A total of 79 loci were highlighted in both transects as outliers, 7 of which were highlighted by multiple methods in one of the transects as well. There were no indications of linkage between any pairwise comparisons of outlier loci in any sites after applying a Benjamini-Hochberg correction procedure.

Our BLastN results returned 44 matches to the NCBI nr database, 11 of which were to genes of known function including those with metabolic processes involving acyl-coenzyme A, oxygen transportation, cellular structure and division, and immune function (Table 4.4). Several gene ontology (GO) terms were common: iron ion binding, oxidoreductase activity, metal ion binding, protein binding, and hydrolase activity were all...
associated with at least two of these genes (Table S4.3).

4.3.4 Population genetic analysis

Population genetic analyses based on the neutral dataset (e.g. outliers removed; n = 28,750 loci; Table 2) revealed higher genetic diversity along the PP transect compared to the TL transect across most genetic diversity metrics (Table 1). Both observed (T = 2.63, df = 5.15, p = 0.05) and expected heterozygosity (T = 5.93, df = 5.90, p < 0.01) were significantly higher along the PP transect than along the TL transect. There tended to be a relatively higher effective number of alleles along the PP transect compared to along the TL transect (T = 2.33, df = 4.76, p = 0.07). Both PP01 and TL03 exhibited significant inbreeding rates, while all other sites showed some indications of outbreeding.

Our results revealed significant genetic divergence between transects and among sites within transects. All θ values between pairwise comparisons of sites between transects were significant, ranging from 0.143 to 0.201 (Table 4.5). Pairwise comparisons within transects showed lower θ values ranging from 0.020 to 0.107, apart from PP01–PP03 and PP02–PP03 all comparisons were significant. Likewise, AMOVA results indicated approximately 11.1% divergence between transects (p = 0.029) and 4.7% divergence among sites within transects (p = 0.001), with the remaining genetic diversity being housed within sites.

The ADMIXTURE results clearly signaled that each transect comprised a unique genetic unit with little genetic mixture between transects (CV = 0.524 for K = 2, next closest CV = 0.526 for K = 3; Figure S4.2). We resolved no additional substructure when analyzing the PP transect-specific neutral data and identified weak evidence for two genetic units along the TL transect (Figure 4.3). There was support for further subdivisions when assessing the
outlier datasets along each transect. For the PP transect, two genetic units were resolved from outlier loci, while there was evidence to support $K = 3$ among outliers along the TL transect. In both cases, there was clear evidence of a unique low-elevation genetic unit.

4.3.5 Gene flow analysis

All runs of BAYESASS returned optimal acceptance rates after adjusting mixing rates except for the allele frequency parameter (-a), which returned acceptance rates higher than our maximum target of 0.6 in all analyses. Elevated acceptance rates can occur when the likelihood surface is relatively flat, as is likely in biallelic data (Wilson & Rannala 2003). However, independent runs of these analyses returned a low coefficient of variation for all analyses (<1%), indicating consistent model convergence. The only evidence for migration along the PP transect was from PP04 to PP02 and this migration was only apparent when analyzing the neutral dataset (Table 4.6). The TL transect revealed more significant dispersal from TL03 to all other sites along the transect in the neutral dataset and from TL03 to TL02 in the outlier dataset. The 95% credibility intervals of all other migration rates encompassed zero and were therefore taken to be non-significant. The quality index of self-assignment was high along both transects (82.6% and 76.7% long PP and TL, respectively) indicating relatively high assignment power. All three pikas captured from the PP supplemental sites assigned to PP04 with 100% probability. Both pikas from TL0.5 assigned to TL03 while the one pika from TL07 assigned to TL02 again with 100% probability in all cases.

4.3.6 Stress hormone analysis

Individual inbreeding levels were relatively low, averaging 0.016 but highly variable among
individuals (SD = 0.097). The best fit mixed-effects model incorporated cranial diameter, ambient maximum temperature, and individual $F_{is}$ (Table 4.7). This model indicated that smaller pikas in colder climates with higher inbreeding levels had elevated stress levels and explained 37.6% of the variance in chronic long-term stress. A similar model but without the individual $F_{is}$ explained 32.6% of the variance, which indicated the incorporation of $F_{is}$ increased the explanatory value of the model by approximately 5%.

4.4 Discussion

Here we investigated evidence for climate-mediated natural selection by identifying genotype-environment associations (GEA) across two independent elevational transects of American pika populations. We employed RADseq genotyping-by-sequencing within a space-for-time design, which enabled us to circumvent the difficulties associated with long-term genetic monitoring and allowed us to highlight several environmental axes potentially driving natural selection (Lotterhos & Whitlock 2015). Additionally, GEA methods have relatively high power to resolve loci under natural selection (De Mita et al. 2013) and do not require specific candidate loci (Hoffmann & Willi 2008). In our case, these were major advantages given the relative paucity of specific candidate loci from other mammalian systems under similar climatic conditions (Boutin & Lane 2014).

While GEA methods have the power to highlight specific environmental drivers to natural selection, it is common for many biotic and abiotic parameters to be geographically autocorrelated, especially along elevational gradients (Sundqvist et al. 2013). We saw strong evidence for this pattern in our environmental datasets. Both long-term climate measurements from ClimateWNA and contemporary climate measurements taken onsite with temperature sensors provided similar estimates of environmental parameters. To reduce the
collinearity of this dataset, we developed synthetic environmental parameters by conducting a PCA of all the climate data along each transect. The result of this analysis highlighted the overriding influence of elevation on climate at our sample sites, limiting the interpretation of genomic correlates to specific environmental variables. For instance, both mean summer and winter temperature decrease with increasing elevation, making it difficult to determine whether selection is occurring in response to heat or cold stress. Another limitation with this approach is an inability to estimate the rate of adaptive evolution precisely. For instance, our environmental gradients covered approximately a 5.5 °C change in mean annual temperature; while we provide evidence of local adaptation over this geographic area, it is unclear over how many generations these adaptations developed. The lack of temporal scale makes it difficult to assess the speed at which species may adapt to future climate conditions, a critical question to assess the continued viability of species in a rapidly changing world (Visser 2008). Even with these caveats, GEA provides a powerful approach for detecting climate-mediated natural selection and can highlight specific genomic targets for further investigation.

4.4.1 Outlier detection

It has long been suspected that the thermal sensitivity of the American pika will threaten the species’ long-term viability in the face of climate change, which has already been implicated in local extirpations (Beever et al. 2003; Wilkening et al. 2011; Jeffress et al. 2013). In fact, we recently provided physiological evidence of climate-induced stress in pika populations at our sample sites (Waterhouse et al. 2017). What is less clear is whether these environmental stressors will result in an adaptive response. In general, outlier detection methods can suffer
from false-positives (De Villemereuil et al. 2014; François et al. 2016). We addressed this concern by considering outliers to be significant only if they were identified by multiple detection methods or across both elevational transects. This approach may have been overly conservative, potentially missing some significant outliers. However, we felt this was an acceptable tradeoff for the increased robustness to our downstream interpretations, especially as they relate to preliminary evidence for climate-mediated natural selection in the American pika.

Our combined outlier detection method highlighted 173 loci with robust evidence of divergent selection. Close to half of these outliers (45.7%) were identified in both transects and a significant portion were highlighted in both transects and by two independent analyses in one of the transects (12.1%). This congruence was especially meaningful given the strong neutral genetic divergence documented between transects, indicating that PP and TL are effectively independent replicates. This congruent genomic response in two independent transects further suggests that pikas across these elevational transects exhibit a consistent microevolutionary response to climate. The congruent patterns documented here are in contrast to other studies that looked at evolutionary responses to similar environmental change across multiple, independent locations (Muir et al. 2014; Franks et al. 2016). For example, Franks et al. (2016) found only 0.025% of outliers were identified in both of two independent populations of field mustard, Brassica rapa, concluding that these populations were largely on separate evolutionary trajectories for dealing with drought conditions.

The majority of the outliers with robust evidence for natural selection were associated with the first environmental principal component in at least one of the transects (n = 115). This ordination axis was highly correlated to a number of climate variables apparently
influenced by elevation. Two of the principal environmental conditions that change with elevation are temperature and oxygen concentration. In general, pika species are inclined to thermal and hypoxic stress (Wang et al. 2006; Yang et al. 2008; Li et al. 2009b). Our analysis indicates that these environmental stressors may be leading to an adaptive response in numerous areas of the American pika genome. These findings complement previous population genetic and transcriptomic studies of pikas along elevational gradients that found evidence for climate-induced natural selection (Lemay et al. 2013; Henry & Russello 2013).

4.4.2 Outlier annotation

A small proportion of our outlier loci returned significant annotations to known genes (6.4%). This low annotation rate is not unexpected, as the challenges related to associating DNA sequence data and gene function in non-model organisms are well-documented (Pop & Salzberg 2008; Yandell & Ence 2012). Nevertheless, several interesting patterns emerged including multiple genes associated with metabolic processes and oxygen transport. For example, scaffold_32008_8033 mapped to a gene encoding for 2-acylglycerol O-acyltransferase 3-like and scaffold_29985_7301 mapped to a gene encoding for acyl-coenzyme A thioesterase 1-like (ACOT1; Table 4.4, Table S4.3). Both of these genes are involved in metabolic processes that use Acyl-CoA, which is known to play a role in maintaining the fluidity of lipid bilayers during cold acclimatization (Nozawa 2011).

American pika are known to be thermally sensitive and inclined to cold stress (Beever et al. 2010, 2011; Ray et al. 2012; Jeffress et al. 2013; Schwalm et al. 2016), a trait shared with other pika species. In fact, the Plateau pikas (Ochotona curzoniae) show a number of seasonal regulatory responses to cold including increased activity of cytochrome c oxidase
(Wang et al. 2006). Additionally, a study of six pika species revealed evidence for positive selection acting on leptin, an important hormone involved with energy homeostasis, highlighting the role of metabolic processes in pika species to combat colder temperatures (Yang et al. 2008).

Mammalian species are often faced with hypoxic stress from diving, hibernation, and living in burrows or at high elevation (Ramirez et al. 2007). Most pika species live at high elevations and are therefore subjected to selective pressure from hypoxic stress (Hoffman & Smith 2005). Li et al. (2009b) found high expression of HIF-1α in Plateau pikas, which is a key transcription factor involved in a number of cellular and systemic adaptations to hypoxia. This metabolic pathway is mediated by dioxygenase proteins, one of which exhibited putative signature of divergent selection here in the American pika (scaffold_9501_20274). Likewise, another locus aligned with a hemeprotein with known roles in oxygen transport (scaffold_15992_4066; Zaphiropoulos 1997). Taken together, these loci may represent a genomic response in the American pika to living at high elevation brought about by cold and hypoxia stress. These genes and pathways are excellent targets for future validation studies, including elevational analysis of gene expression.

4.4.3 Population genetic analysis

There is a consistent relationship between population size and genetic diversity in wildlife populations (Frankham 1996). The American pika is a habitat specialist (Smith & Weston 1990); the higher genetic diversity along the PP transect may indicate more favorable environmental conditions supporting larger populations of pikas. This difference could reflect more favorable thermal conditions arising from the general northern aspect of the PP
For instance, both lower sites along the TL transects had higher mean annual temperatures and higher talus summer temperatures, two factors expected to induce thermal stress in American pikas (Varner & Dearing 2014; Stewart et al. 2015). We attempted to calculate effective population sizes from our genomic data to investigate this possibility. However, many of the estimates encompassed infinity, a known issue when using single sample linkage disequilibrium methods to calculate effective population sizes (Luikart et al. 2010). Future studies could test the interaction between population size, genetic diversity, and habitat quality in the American pika.

We resolved significant genetic structure between transects using the neutral dataset and extensive genetic structure among sites within transect using the outlier dataset. Adaptive genetic diversity has a higher power to resolve fine-scale genetic structure due to local adaptation (Funk et al. 2012). Moreover, pikas exhibit limited dispersal (Henry et al. 2012; Castillo et al. 2014; Robson et al. 2016). This pattern of limited dispersal was beneficial to our experimental design demonstrating that the two elevational transects represent largely independent comparisons. However, it remains possible that, despite marked contemporary structure among transects, congruent patterns could be due to ancestral polymorphism. For instance, these shared outliers could potentially represent climate adaptations that occurred lineage-wide during the American pika’s range expansion, preceding the peak of the last glacial maximum approximately 21,000 years ago (Galbreath et al. 2009). A landscape genomic study across the entire North American range of the species is currently in progress that will explicitly test this hypothesis (Russello, pers. com.).
4.4.4 Directional migration

Dispersal in American pikas is largely resource-dependent, where the primary resource is habitat (Peacock 1997). Additionally, multiple analyses have shown that dispersal capacity is lowest in warm and dry areas that are most physiologically stressful (Castillo et al. 2014, 2016; Schwalm et al. 2016). Smith (1974) reported that at higher elevation, pikas typically occupy a greater proportion of potential territories, a pattern we observed while in the field (Waterhouse, pers. obs.). Our data are consistent with the hypothesis that pika dispersal is habitat-driven; specifically, our gene flow analysis revealed one high elevation site along each transect that acted as the major source of migrants. Along the TL transect, TL03 was a source of migrants to all other sites; while TL03 was not the highest site, we believe this site did represent the highest population density along the TL transect. Along the PP transect, PP04 was the only site with significant emigration and represented the largest population we sampled in NOCA. Additionally, 5 of the 6 pikas sampled at supplemental sites were from the main sources of emigration identified from our directional migration analysis suggesting that even at lower elevations the high-elevation sites are still the major source of immigrants. Overall, these patterns suggest that the upward retreat of American pika populations with climate change is likely to occur through the extirpation of low elevational populations rather than their movement upslope (Beever et al. 2003; Grayson 2005).

Likewise, we observed relatively minimal gene flow in the outlier dataset. One of the strengths of the migration analysis employed is that it does not require loci to conform to Hardy-Weinberg expectations and can be applied to nonstationary populations (Wilson & Rannala 2003). However, since this analysis is based on detecting disequilibrium in individual multilocus genotypes, it is sensitive to the number of genetic markers used; the
lack of migration we documented in the outlier dataset could therefore have been a function of the lower number of markers (transect-specific datasets: outlier n = 123-129; neutral n = 25,211-25,297). Alternatively, the lower level of gene flow could be biologically meaningful, representing resistance to genetic introgression at locally adapted loci (Tigano & Friesen 2016).

The reduced levels of gene flow at putatively adaptive loci and the general downward direction of migration indicate potential impediments for the movement of thermal adaptations across elevational gradients of pika populations. Adaptations resulting from higher temperatures occurring at lower sites may not be able to move upward as the climate warms. This concern may be present in other alpine species as well, as lower elevation populations are expected to suffer reduced viability while higher elevation populations may persist at high densities (Parmesan & Yohe 2003). Under this scenario, we find it likely that source-sink dynamics in American pikas and other alpine species would naturally exhibit a down-slope bias, where high elevation populations act as sources of migrants to generally smaller, lower elevation populations.

4.4.5 Stress hormone analysis

Inbreeding is a common occurrence in wild populations and can have deleterious effects through inbreeding depression (Keller & Waller 2002). If severe enough, these impacts can threaten the continued viability of the species (O’Grady et al. 2006). The American pika is known to exhibit mate choice based on intermediate levels of relatedness (Peacock & Smith 1997b) and generally dispersal is dictated by competition for territory rather than inbreeding avoidance (Peacock 1997). We investigated the potential consequences of inbreeding in pika
by estimating individual levels of inbreeding and assessing if these levels had an impact on long-term chronic stress as measured by extracted corticosterone from hair samples. We documented a positive correlation between inbreeding and corticosterone levels, which indicates the potential for inbreeding depression in this species especially as it relates to climate stress. Climate change is expected to cause continued reductions in populations sizes of American pikas (Beever et al. 2013; Yandow et al. 2015) which could further exacerbate inbreeding trends (Frankham 1995). With this in mind, it may be important to continue to monitor inbreeding trends in smaller pika populations.

4.4.6 Summary

In this study, we assessed the potential for climate adaptation in a sentinel mammalian species, the American pika, by identifying genotype-environment associations in populations occurring over an environmental gradient. We resolved robust evidence of natural selection in this system and identified several gene regions potentially associated with local adaptation. Our analysis adds to the growing body of evidence for climate-induced natural selection in the American pika (Lemay et al. 2013; Henry and Russello 2013) and provides a relatively rare mammalian example of genetic adaptation to contemporary climate conditions (Boutin & Lane 2014). Future work could test the potential role of the genes identified here as outliers through broader-scale sampling or analyses of gene expression along elevational gradients. Investigation of the American pika transcriptome has already been completed (Lemay et al. 2013). Additionally, we resolved evidence for consistent directional migration, which may hold important ramifications for the movement of low elevation thermal adaptations. More broadly, the results from this study provide insights useful in applying
assisted gene flow for mitigating the negative effects of climate change (Aitken & Whitlock 2013). Continued research is needed to determine if the rate of adaptation in the American pika will be able to keep pace with a rapidly changing environment. If adaptation cannot keep pace with climate change, future conservation efforts could consider the translocation of American pika from populations highlighted as having thermal adaptations to populations suspected of undergoing reduced viability due to thermal stress. Given their thermal sensitivity and the fact that their rocky talus slope habitat is largely not impacted by direct human activities, the American pika may represent a rare mammalian model system for evaluating and implementing such conservation strategies for mitigating the deleterious consequences of contemporary climate change.
Table 4.1. Site characteristics of American pika populations sampled in the North Cascades National Park, WA. Observed heterozygosity ($H_o$), total corrected heterozygosity ($H_e$), effective number of alleles ($A_e$), and inbreeding estimates ($F_{is}$) shown for each site with a minimum sample size ($n$) of 2 along the Pyramid Peak (PP) and Thornton Lakes (TL) in North Cascades National Park, WA, USA.

<table>
<thead>
<tr>
<th>Site</th>
<th>Elevation</th>
<th>Northing</th>
<th>Easting</th>
<th>n</th>
<th>$Ho$</th>
<th>$He$</th>
<th>$Ae$</th>
<th>$F_{is}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPTrl</td>
<td>330</td>
<td>636449</td>
<td>5396874</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PPHwy</td>
<td>415</td>
<td>640203</td>
<td>5395358</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PP01</td>
<td>450</td>
<td>640367</td>
<td>5393862</td>
<td>5</td>
<td>0.27</td>
<td>0.28</td>
<td>1.426</td>
<td>0.038*</td>
</tr>
<tr>
<td>PP1.5</td>
<td>615</td>
<td>637260</td>
<td>5396080</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PP02</td>
<td>820</td>
<td>638227</td>
<td>5395281</td>
<td>6</td>
<td>0.316</td>
<td>0.292</td>
<td>1.459</td>
<td>-0.08</td>
</tr>
<tr>
<td>PP03</td>
<td>1330</td>
<td>638082</td>
<td>5393892</td>
<td>3</td>
<td>0.305</td>
<td>0.288</td>
<td>1.415</td>
<td>-0.061</td>
</tr>
<tr>
<td>PP04</td>
<td>1580</td>
<td>637027</td>
<td>5392854</td>
<td>9</td>
<td>0.302</td>
<td>0.298</td>
<td>1.475</td>
<td>-0.014</td>
</tr>
<tr>
<td>TL0.5</td>
<td>150</td>
<td>625978</td>
<td>5391339</td>
<td>2</td>
<td>0.254</td>
<td>0.264</td>
<td>1.345</td>
<td>0.039*</td>
</tr>
<tr>
<td>TL0.7</td>
<td>265</td>
<td>624461</td>
<td>5389771</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TL01</td>
<td>490</td>
<td>622400</td>
<td>5388108</td>
<td>7</td>
<td>0.286</td>
<td>0.255</td>
<td>1.406</td>
<td>-0.12</td>
</tr>
<tr>
<td>TL02</td>
<td>780</td>
<td>622818</td>
<td>5390984</td>
<td>11</td>
<td>0.26</td>
<td>0.26</td>
<td>1.415</td>
<td>-0.003</td>
</tr>
<tr>
<td>TL03</td>
<td>1390</td>
<td>623134</td>
<td>5393254</td>
<td>8</td>
<td>0.258</td>
<td>0.269</td>
<td>1.42</td>
<td>0.039*</td>
</tr>
<tr>
<td>TL04</td>
<td>1700</td>
<td>623614</td>
<td>5393933</td>
<td>4</td>
<td>0.265</td>
<td>0.255</td>
<td>1.384</td>
<td>-0.039</td>
</tr>
</tbody>
</table>

*significant at the 0.001 level
Table 4.2. Number of SNPs retained in the American pika genomic dataset after various filtering procedures. Transect specific SNP counts shown for the Pyramid Peak (PP) and Thornton Lakes (TL) transects in the North Cascades National Park, WA, USA (NOCA).

<table>
<thead>
<tr>
<th>From reads to SNPs</th>
<th>SNP count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aligned to reference (min depth 6X)</td>
<td>607,468</td>
</tr>
<tr>
<td>Max missing data of 30%</td>
<td>189,696</td>
</tr>
<tr>
<td>Minor allele frequency &gt; 0.05</td>
<td>53,162</td>
</tr>
<tr>
<td>One SNP/tag, indels and duplicates removed</td>
<td>31,298</td>
</tr>
<tr>
<td>Max of 2 alleles and 100x depth, passed HWE, minimum phred quality of 20</td>
<td>30,763</td>
</tr>
<tr>
<td>PP max missing data of 30%</td>
<td>30,128</td>
</tr>
<tr>
<td>PP Polymorphic</td>
<td>27,129</td>
</tr>
<tr>
<td>TL max missing data of 30%</td>
<td>30,411</td>
</tr>
<tr>
<td>TL Polymorphic</td>
<td>26,979</td>
</tr>
<tr>
<td>Outliers (any technique)</td>
<td>2,013</td>
</tr>
<tr>
<td>NOCA Neutral dataset</td>
<td>28,750</td>
</tr>
<tr>
<td>PP outlier dataset</td>
<td>123</td>
</tr>
<tr>
<td>PP neutral dataset</td>
<td>25,297</td>
</tr>
<tr>
<td>TL outlier dataset</td>
<td>129</td>
</tr>
<tr>
<td>TL neutral dataset</td>
<td>25,211</td>
</tr>
</tbody>
</table>
Table 4.3. Summary of outlier detection analyses in American pika. Number of putative outliers is summarized for each analysis; PC1 and PC2 refer to analyses conducted using the first and second environmental principal component, respectively. Numbers in parentheses correspond to outliers significantly correlated to both principal components.

<table>
<thead>
<tr>
<th>Method</th>
<th>Analysis</th>
<th>Number of loci</th>
<th>Percent of loci</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BAYESCAN</strong></td>
<td>PP</td>
<td>67</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>TL</td>
<td>52</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>Both</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>PP_PC1</td>
<td>493</td>
<td>1.82</td>
</tr>
<tr>
<td></td>
<td>PP_PC2</td>
<td>521 (23)</td>
<td>1.92</td>
</tr>
<tr>
<td><strong>LFMM</strong></td>
<td>TL_PC1</td>
<td>305</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td>TL_PC2</td>
<td>161 (21)</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>Both</td>
<td>54</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>PP_PC1</td>
<td>169</td>
<td>0.62</td>
</tr>
<tr>
<td></td>
<td>PP_PC2</td>
<td>124 (1)</td>
<td>0.46</td>
</tr>
<tr>
<td><strong>BAYPASS</strong></td>
<td>TL_PC1</td>
<td>180</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>TL_PC2</td>
<td>175 (1)</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>Both</td>
<td>7</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Any</strong></td>
<td>Both</td>
<td>79</td>
<td>0.34</td>
</tr>
</tbody>
</table>
Table 4.4. Summary of top BLASTN search results for outlier loci in American pika detected by BAYESCAN (BS), LFMM (LF), and BAYPASS (BP). The number after the analysis in the comparison column indicates which environmental principal components was used in the correlative analysis.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Comparison</th>
<th>SNP</th>
<th>Top blast hit (accession)</th>
<th>Abbreviated description</th>
</tr>
</thead>
<tbody>
<tr>
<td>scaffold_34750_45136</td>
<td>PP_BP_2, PP_BS, TL_LF_2</td>
<td>A/G</td>
<td>XM_012930919.1</td>
<td><em>Ochotona princeps</em> coiled-coil domain containing 77 (CCDC77)</td>
</tr>
<tr>
<td>scaffold_32008_8033</td>
<td>PP_BP_2, TL_BP_1, TL_BS</td>
<td>C/T</td>
<td>XM_004587187.1</td>
<td><em>Ochotona princeps</em> 2-acylglycerol O-acyltransferase 3-like</td>
</tr>
<tr>
<td>scaffold_15992_4066</td>
<td>PP_BP_2, PP_BS, PP_LF_2</td>
<td>T/A</td>
<td>XM_012925448.1</td>
<td><em>Ochotona princeps</em> cytochrome P450 2C18-like</td>
</tr>
<tr>
<td>scaffold_332_200703</td>
<td>PP_LF_2, TL_LF_1</td>
<td>G/T</td>
<td>LT160000.1</td>
<td><em>Macaca fascicularis</em> complete genome, chromosome chr1</td>
</tr>
<tr>
<td>scaffold_35953_8025</td>
<td>PP_LF_2, TL_LF_1</td>
<td>C/T</td>
<td>XM_004598126.1</td>
<td><em>Ochotona princeps</em> MAD1 mitotic arrest deficient-like 1</td>
</tr>
<tr>
<td>scaffold_9501_20274</td>
<td>PP_LF_1, TL_LF_1</td>
<td>G/T</td>
<td>XM_004595405.1</td>
<td><em>Ochotona princeps</em> 2-oxoglutarate and iron-dependent oxygenase domain containing 2</td>
</tr>
<tr>
<td>scaffold_34209_3344</td>
<td>PP_LF_2, TL_LF_1</td>
<td>C/A</td>
<td>XM_002711101.3</td>
<td><em>Oryctolagus cuniculus</em> tubulin alpha-1A chain</td>
</tr>
<tr>
<td>scaffold_29985_7301</td>
<td>PP_LF_2, TL_LF_1</td>
<td>G/A</td>
<td>XM_004584112</td>
<td><em>Ochotona princeps</em> acyl-coenzyme A thioesterase 1-like (ACOT1)</td>
</tr>
<tr>
<td>scaffold_3620_102829</td>
<td>TL_BP_2, TL_BS</td>
<td>T/C</td>
<td>NG_008098.1</td>
<td>TNF receptor superfamily member 11a (TNFRSF11A)</td>
</tr>
<tr>
<td>scaffold_30_208287</td>
<td>TL_BP_2, TL_BS</td>
<td>A/G</td>
<td>AL391986.12</td>
<td>Human DNA sequence from clone RP11-426E5 on chromosome 10</td>
</tr>
<tr>
<td>scaffold_590_131441</td>
<td>PP_BP_1, PP_BS</td>
<td>C/A</td>
<td>XM_004578932.1</td>
<td><em>Ochotona princeps</em> interleukin 20 (IL20)</td>
</tr>
<tr>
<td>scaffold_168552_899</td>
<td>PP_BP_1, PP_BS</td>
<td>C/T</td>
<td>XM_004597033.1</td>
<td><em>Ochotona princeps</em> DEAH (Asp-Glu-Ala-His) box polypeptide 34 (DHX34)</td>
</tr>
<tr>
<td>scaffold_4242_61990</td>
<td>TL_BP_2, TL_LF_1</td>
<td>A/T</td>
<td>XM_012755825.2</td>
<td><em>Microcebus murinus</em> adenosine monophosphate deaminase 3 (AMPD3) transcript variant X6</td>
</tr>
<tr>
<td>scaffold_7931_46314</td>
<td>PP_LF_2, TL_LF_2</td>
<td>G/A</td>
<td>CP011890.1</td>
<td><em>Ovis canadensis canadensis</em> isolate 43U chromosome 5 sequence</td>
</tr>
</tbody>
</table>

* All e-scores < 10^{-10}
Table 4.5. Pairwise comparisons of $F_{st}$ (Top diagonal; Weir & Cockerham 1984) between pika populations and associated $p$-value from a permutation analysis (1,000 permutations; bottom diagonal). Pairwise $F_{st}$ values from comparisons between transects shaded in grey. See Table 4.1 for site descriptions.

<table>
<thead>
<tr>
<th></th>
<th>PP01</th>
<th>PP02</th>
<th>PP03</th>
<th>PP04</th>
<th>TL01</th>
<th>TL02</th>
<th>TL03</th>
<th>TL04</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP01</td>
<td>--</td>
<td>0.072*</td>
<td>0.066</td>
<td>0.050*</td>
<td>0.197*</td>
<td>0.201*</td>
<td>0.173*</td>
<td>0.188*</td>
</tr>
<tr>
<td>PP02</td>
<td>0.002</td>
<td>--</td>
<td>0.049</td>
<td>0.043*</td>
<td>0.181*</td>
<td>0.186*</td>
<td>0.158*</td>
<td>0.175*</td>
</tr>
<tr>
<td>PP03</td>
<td>0.058</td>
<td>0.080</td>
<td>--</td>
<td>0.036*</td>
<td>0.189*</td>
<td>0.185*</td>
<td>0.154*</td>
<td>0.179*</td>
</tr>
<tr>
<td>PP04</td>
<td>0.004</td>
<td>0.004</td>
<td>0.044</td>
<td>--</td>
<td>0.163*</td>
<td>0.172*</td>
<td>0.143*</td>
<td>0.156*</td>
</tr>
<tr>
<td>TL01</td>
<td>0.003</td>
<td>0.001</td>
<td>0.008</td>
<td>0.001</td>
<td>--</td>
<td>0.104*</td>
<td>0.075*</td>
<td>0.107*</td>
</tr>
<tr>
<td>TL02</td>
<td>0.001</td>
<td>0.001</td>
<td>0.003</td>
<td>0.001</td>
<td>0.001</td>
<td>--</td>
<td>0.031*</td>
<td>0.061*</td>
</tr>
<tr>
<td>TL03</td>
<td>0.001</td>
<td>0.001</td>
<td>0.004</td>
<td>0.001</td>
<td>0.001</td>
<td>0.004</td>
<td>--</td>
<td>0.020*</td>
</tr>
<tr>
<td>TL04</td>
<td>0.014</td>
<td>0.006</td>
<td>0.032</td>
<td>0.004</td>
<td>0.003</td>
<td>0.004</td>
<td>0.032</td>
<td>--</td>
</tr>
</tbody>
</table>

*significant at the 0.05 level
Table 4.6. Directional migration analysis of American pika along the Pyramid Peak (PP) and Thornton Lakes (TL) transects using either the neutral or outlier datasets. Migration values are shown as proportion of the population expected to be recent migrants from the source population, value of the 95% credibility interval shown in parentheses, significant migration values shaded in grey (i.e., credibility interval does not encompass zero).

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Direction</th>
<th>Migration</th>
<th>Direction</th>
<th>Migration</th>
<th>Direction</th>
<th>Migration</th>
<th>Direction</th>
<th>Migration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PP Outlier</strong></td>
<td>PP01→PP01</td>
<td>0.854 (0.104)</td>
<td>PP02→PP01</td>
<td>0.072 (0.087)</td>
<td>PP03→PP01</td>
<td>0.037 (0.065)</td>
<td>PP04→PP01</td>
<td>0.037 (0.065)</td>
</tr>
<tr>
<td></td>
<td>PP01→PP02</td>
<td>0.034 (0.060)</td>
<td>PP02→PP02</td>
<td>0.889 (0.097)</td>
<td>PP03→PP02</td>
<td>0.033 (0.059)</td>
<td>PP04→PP02</td>
<td>0.044 (0.073)</td>
</tr>
<tr>
<td></td>
<td>PP01→PP03</td>
<td>0.048 (0.081)</td>
<td>PP02→PP03</td>
<td>0.048 (0.082)</td>
<td>PP03→PP03</td>
<td>0.857 (0.115)</td>
<td>PP04→PP03</td>
<td>0.048 (0.081)</td>
</tr>
<tr>
<td></td>
<td>PP01→PP04</td>
<td>0.026 (0.047)</td>
<td>PP02→PP04</td>
<td>0.026 (0.046)</td>
<td>PP03→PP04</td>
<td>0.026 (0.047)</td>
<td>PP04→PP04</td>
<td>0.923 (0.074)</td>
</tr>
<tr>
<td><strong>PP Neutral</strong></td>
<td>PP01→PP01</td>
<td>0.853 (0.103)</td>
<td>PP02→PP01</td>
<td>0.037 (0.065)</td>
<td>PP03→PP01</td>
<td>0.037 (0.065)</td>
<td>PP04→PP01</td>
<td>0.073 (0.086)</td>
</tr>
<tr>
<td></td>
<td>PP01→PP02</td>
<td>0.033 (0.059)</td>
<td>PP02→PP02</td>
<td>0.833 (0.098)</td>
<td>PP03→PP02</td>
<td>0.033 (0.059)</td>
<td>PP04→PP02</td>
<td>0.100 (0.090)</td>
</tr>
<tr>
<td></td>
<td>PP01→PP03</td>
<td>0.048 (0.081)</td>
<td>PP02→PP03</td>
<td>0.048 (0.081)</td>
<td>PP03→PP03</td>
<td>0.809 (0.114)</td>
<td>PP04→PP03</td>
<td>0.095 (0.104)</td>
</tr>
<tr>
<td></td>
<td>PP01→PP04</td>
<td>0.026 (0.047)</td>
<td>PP02→PP04</td>
<td>0.026 (0.046)</td>
<td>PP03→PP04</td>
<td>0.026 (0.047)</td>
<td>PP04→PP04</td>
<td>0.923 (0.074)</td>
</tr>
<tr>
<td><strong>TL Outlier</strong></td>
<td>TL01→TL01</td>
<td>0.879 (0.091)</td>
<td>TL02→TL01</td>
<td>0.030 (0.054)</td>
<td>TL03→TL01</td>
<td>0.061 (0.073)</td>
<td>TL04→TL01</td>
<td>0.030 (0.054)</td>
</tr>
<tr>
<td></td>
<td>TL01→TL02</td>
<td>0.022 (0.041)</td>
<td>TL02→TL02</td>
<td>0.889 (0.077)</td>
<td>TL03→TL02</td>
<td>0.067 (0.066)</td>
<td>TL04→TL02</td>
<td>0.022 (0.041)</td>
</tr>
<tr>
<td></td>
<td>TL01→TL03</td>
<td>0.028 (0.050)</td>
<td>TL02→TL03</td>
<td>0.056 (0.067)</td>
<td>TL03→TL03</td>
<td>0.860 (0.091)</td>
<td>TL04→TL03</td>
<td>0.057 (0.070)</td>
</tr>
<tr>
<td></td>
<td>TL01→TL04</td>
<td>0.042 (0.072)</td>
<td>TL02→TL04</td>
<td>0.042 (0.072)</td>
<td>TL03→TL04</td>
<td>0.049 (0.083)</td>
<td>TL04→TL04</td>
<td>0.868 (0.111)</td>
</tr>
<tr>
<td><strong>TL Neutral</strong></td>
<td>TL01→TL01</td>
<td>0.850 (0.095)</td>
<td>TL02→TL01</td>
<td>0.030 (0.054)</td>
<td>TL03→TL01</td>
<td>0.090 (0.085)</td>
<td>TL04→TL01</td>
<td>0.030 (0.054)</td>
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<tr>
<td></td>
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<td>0.022 (0.041)</td>
<td>TL02→TL02</td>
<td>0.867 (0.080)</td>
<td>TL03→TL02</td>
<td>0.089 (0.072)</td>
<td>TL04→TL02</td>
<td>0.022 (0.041)</td>
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<tr>
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<td>0.028 (0.050)</td>
<td>TL02→TL03</td>
<td>0.056 (0.067)</td>
<td>TL03→TL03</td>
<td>0.889 (0.085)</td>
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<td>0.028 (0.050)</td>
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<tr>
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<td>0.042 (0.072)</td>
<td>TL03→TL04</td>
<td>0.123 (0.106)</td>
<td>TL04→TL04</td>
<td>0.794 (0.107)</td>
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Table 4.7. Mixed-effects models explaining chronic long-term stress as measured by hair corticosterone in American pikas sampled in North Cascades National Park, WA, USA. Fixed effects included cranial diameter (cranial), sex, maximum ambient summer temperature (Amb_max) and individuals estimates of inbreeding ($F_{is}$). All variables loaded negatively with the exception of $F_{is}$ which loaded positively in each model.

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<th>$\Delta$AICc</th>
<th>Weight</th>
<th>Evidence Ratio</th>
<th>Marginal $R^2$</th>
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<td>6</td>
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<td>1.48</td>
<td>0.404</td>
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</table>
Figure 4.1. Map of Pyramid Peak (PP) and Thornton Lakes (TL) transects in the North Cascades National Park, Washington, USA. Topographic lines represent 100 m change in elevation. Highway 20 is indicated by the grey line.
Figure 4.2. Summary plots for genotype-environment associations in American pika. LFMM analysis for the Pyramid Peak (PP) transect (panel A) and Thornton Lakes (TL) transect (panel B); note that p-values are shown on an inverted log10 axis. Panels C and D show the regression coefficient (Beta) and Bayes Factor (BF) in decibans for the BAYPASS analysis for PP and TL transects, respectively. Analyses conducted with the first environmental principal component (circles) and second (squares) are shown for each analysis. Significant outliers identified in both transects are shown in solid blue. Locus ID is shown for all annotated outliers, blue leader lines indicate significance in both transects (See Table 4.4).
Figure 4.3. Admixture proportions for American pika along Pyramid Peak (panels A and C) and Thornton Lakes (panels B and D) transects using only neutral (panels A and B) or outlier loci (panels C and D) with inset cross-entropy (CE) estimations for each value of K. Plots show admixture proportions with the lowest CE in each case.
Table S4.1. Climate variables from iButton data obtained along the elevational transects established in the North Cascades National Park, WA, USA. See Table 4.1 and Figure 4.1 for site locations and Figure S4.1 for variable definitions. All temperatures are in degrees Celsius.

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<th>Amb_MAT</th>
<th>Amb_MST</th>
<th>Amb_MWT</th>
<th>Tal_MAT</th>
<th>Tal_MST</th>
<th>Tal_MWT</th>
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Table S4.2. ClimateWNA data for each site along the elevational transects established in the North Cascades National Park, WA. See Table 4.1 and Figure 4.1 for site locations and Figure S4.1 for variable definitions. All temperatures are in degrees Celsius, precipitation is in mm, and radiation is in MJ m\(^2\) d\(^{-1}\).

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<th>MCMT</th>
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<th>AHM</th>
<th>SHM</th>
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<th>Tmax_sp</th>
<th>Tmax_sm</th>
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Table S4.2. continued

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<th>PPT_sp</th>
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Table S4.3. Gene Ontology terms associated with significant outlier loci Blast hits.

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<td>Transferase activity</td>
</tr>
<tr>
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<td>Transferase activity, transferring acyl groups</td>
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<td>2-acylglycerol O-acyltransferase 3-like</td>
<td>Transferase activity, transferring acyl groups other than amino-acyl groups</td>
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</tbody>
</table>
Figure S4.1. Climate variables and loadings for the first (PC1) and second principal component (PC2) of the environmental PCA for the Pyramid Peak and Thornton Lakes transects. The first eight variables were directly measured using temperature sensors, and all other variables were estimated using the ClimateWNA model. Bars represent the relative magnitude (negative to the left and positive to the right of the dashed line) of loading for each variable. All temperatures are in degrees Celsius, precipitation is in mm, and radiation is in MJ m\(^{-2}\) d\(^{-1}\).
Figure S4.2. Admixture plot for all the sites sampled in NOCA (see Table 4.1 and Figure 4.1 for site locations). Inset cross-entropy (CE) estimations for each value of K show in the inset graph.
Chapter 5: Conclusion

5.1 Research findings

This thesis describes three independent approaches to assess biological responses in the American pika to rapid environmental change. The results of these chapters taken together provide consistent evidence for several biological responses occurring in pikas. Here, I will summarize how these studies add to our understanding of dispersal patterns, thermal stress, and metabolic patterns occurring in the American pika. These patterns were apparent in multiple chapters of this thesis and were delineated by using independent genetic and physiological evidence. Where appropriate, I will outline potential ramifications of each pattern for the continued viability of the species and explore management implications for the American pika and other climate-sensitive species.

5.1.1 Evidence for restricted dispersal

The limited dispersal capabilities of the American pika are well documented. Population genetic analyses have demonstrated restricted gene flow (Henry et al. 2012; Robson et al. 2015; Castillo et al. 2014), while demographic analyses have shown minimal dispersal, typically in the form of juveniles settling in the next available territory (Smith & Ivins 1983). These dispersal characteristics of American pikas have important implications to the metapopulation dynamics of the species (Peacock & Smith 1997a; Moilanen et al. 1998) and for the movement of potentially beneficial climate adaptations (Hoffmann & Willi 2008; Sgrò et al. 2011).

Human modifications to the landscape have had genetic and demographic impacts in numerous species and are a leading cause of biodiversity loss (Fischer & Lindenmayer 2007).
However, American pikas typically inhabit high alpine and relatively undisturbed habitat [although see Peacock & Smith (1997)]. In Chapter 2, I investigated the genetic connectivity of pika populations in a human-modified landscape; this approach allowed me to assess the impacts of road construction, which restricted gene flow in this species. Given the ubiquity of roads on the landscape, this finding has potential implications for range shifts associated with climate change (Opdam & Wascher 2004). Specifically, in this study, I found evidence that a major roadway severed gene flow between southern and northern pika populations. If the southern sites become uninhabitable due to climate stress, then northern migration becomes untenable if this roadway is not permeable to dispersal. On a broader scale, such restrictions to dispersal could inhibit the poleward range shift in the American pika as the climate warms (Root et al. 2003; Parmesan & Yohe 2003).

In Chapter 4, I used genome-wide markers to investigate climate-driven responses in pikas distributed along two elevational gradients. One of the strengths of using genome-wide markers is the increased power to detect demographic patterns relevant to conservation (Primmer 2009). By applying a Bayesian framework to detect directional migration in a large genomic data, I assessed the fine-scale movement of pika along elevational gradients. This analysis supported previous evidence showing resource-dependent dispersal in pika where territory availability is the primary driver of movement (Peacock 1997). Importantly, since high elevation populations typically occur at relatively higher densities (Smith 1974b), elevational dispersal patterns in pikas are likely to have a consistent downslope bias. The elevational pattern of movement in pikas has important consequences to the species’ response to climate change. It is likely that low elevation pika populations will lose viability faster than their high elevation counterparts, if dispersal in pika continues to be density
dependent then this process will likely reinforce the downward bias of gene flow. Consequently, thermal adaptations that may evolve in lower warmer sites may be impeded from upslope movement due to the downslope direction of gene flow. Since this pattern is likely present in other alpine species, this study represents an important example of elevational gene flow in a thermally-sensitive species.

5.1.2 Evidence for cold stress

American pikas typically exhibit body temperatures only a few degrees below their lethal threshold (MacArthur & Wang 1974); consequently, the thermal sensitivity of this species has received a lot of attention (Smith 1974b; Beever et al. 2011; Ray et al. 2012; Stewart et al. 2015). We resolved consistent patterns that add to the growing body of evidence for cold stress in this species (Beever et al. 2010, 2011; Ray et al. 2012; Jeffress et al. 2013; Schwalm et al. 2016). In Chapter 3, I showed a decrease in individual stress levels associated with higher ambient temperatures in early spring. Additionally, Chapter 4 highlighted several metabolic genes related to cold acclimation that appear to be under selective pressure.

Antagonistic pleiotropy is when one gene impacts two or more traits that are in opposition to one another. In the case of American pikas, it seems their thermal sensitivity may be setting up an antagonistic pleiotropic relationship. For instance, some small mammals exhibit limits to seasonal metabolic plasticity (Boratyński et al. 2016). If such limits are present in pikas, then an increase in metabolic rate to endure the cold winter could leave individuals exposed to thermal stress in the hot summer since the lower limit of their summer metabolic rate would be set by the species’ metabolic plasticity. The action of natural selection on these metabolic genes could therefore leave pika disproportionately exposed to thermal stress in
the summer. This is a concerning result for two reasons; first, climate change predictions include an increase in seasonal extremes and second, snowpack is decreasing across the United States (Karl et al. 2009). The result of climate change could therefore leave pikas without a protective snowpack in the winter, which would necessitate further cold adaptations at the cost of increased summer heat stress. Continued monitoring of winter snowpack and thermal stress in this species remain critical steps to predict the continued viability of populations.

5.1.3 Metabolic implications

The American pika has a disproportionately high metabolic rate compared to other lagomorphs and even many rodent species (Lovegrove 2003). It is likely that occupying the niche of an alpine small mammal that does not hibernate has placed unique metabolic demands on the American pika. In Chapter 3, I showed a strong relationship between body size and levels of corticosterone, a glucocorticoid stress hormone. Corticosterone is a metabolic byproduct and therefore linked to metabolic rates; among mammalian species the relationship between body size and glucocorticoids is ascribed to the species mass specific metabolic rate (Haase et al. 2016). Therefore, high levels of corticosterone in small pikas were likely due to elevated metabolic rates to compensate for heat loss due to the elevated surface area to mass ratio of smaller individuals. Interestingly, in Chapter 4, I showed evidence for several hypoxia-related genes being under natural selection from an approximate 10% reduction in available oxygen at the high-elevation sites. However, these small changes in oxygen concentrations would be exacerbated by the species’ high metabolic rate, especially in smaller individuals who exhibit elevated metabolic rates. This result adds
another dimension to the problem of living in a high alpine environment as a small mammal. Continued research is warranted to further delineate the role of hypoxia in elevational adaptation in the American pika. For example, the hypoxia inducible factor system, which was shown to be under directional selection in Chapter 4, matches metabolic demands of organisms to available oxygen (Pugh & Ratcliffe 2003; Schofield & Ratcliffe 2005). Delineating elevational patterns of the genes involved in the hypoxia inducible factor system could further highlight the role hypoxia has played in the evolution of American pika.

5.2 Limitations

5.2.1 Sampling limitations

The initial experimental design for Chapter 4 used non-invasive hair snares previously applied to genetic assessments of the American pika (Henry & Russello 2011). It was predicted that this technique could provide ample high quality genetic material suitable for genomic analysis via RAD sequencing (Baird et al. 2008). Unfortunately, I found that the non-invasive hair sampling technique delivered two orders of magnitude less genetic material than expected and suffered from cross-species contamination (Russello et al. 2015). While this technique still provided sufficient genetic material for a high resolution genomic-based population assessment, the quality and quantity of genomic information was insufficient for a genome-wide scan for climate adaptations. Overcoming this limitation necessitated live-trapping pika to obtain tissue samples, which limited the sample sizes used in Chapters 3 and 4. Additionally, the difficulty of trapping these elusive mammals and the stress induced from trapping made direct validation of the hair corticosterone measurements used in Chapter 3 infeasible.
5.2.2 Genetic limitations

In Chapter 2, I concluded the presence of a major highway restricted gene flow sufficiently for discrete genetic units of pika to develop. However, I also provided an alternative hypothesis that natural barriers to gene flow in the form of a seasonal stream and topography could be the driving factors of this genetic divergence. Coalescence theory provides a possible method to differential between these two competing hypotheses. By using approximate Bayesian computation, researchers have been able to determine the age of divergence between two populations (Beaumont et al. 2002; Cornuet et al. 2008). This method could be applied to these pika populations; if the age of divergence corresponds to the construction of the highway then the evidence would support anthropogenic barriers to gene flow. I attempted this calculation using the microsatellite dataset; however, the number of parameters needed to optimize the calculation inhibited the fine-scale assessment of divergence. Future work could incorporate genome-wide sampling to assess the fine scale genetic divergence associated with barriers to gene flow in the American pika and in other species suspected of being impacted by fragmentation.

While the American pika reference genome is an invaluable resource, it exists in a relatively incomplete form. Contigs are assembled into approximately 18,760 scaffolds with an average length of ~18,370 base pairs and are only sequenced at 1.93X coverage (http://www.ensembl.org/Ochotona_princeps/Info/Annotation). Additionally, large portions of repetitive elements and low complex regions are masked in this genome. During the reference assemble of sequencing data in Chapter 4, 76.1% (SD = 0.44%) of reads aligned to this genome potentially because of the genomes’ incomplete state. Moreover, the relatively short scaffolding lengths inhibited some powerful analytical approaches. Sliding window
analyses scan the genome for evidence of natural selection in the form of runs of decreased diversity from selective sweeps (Stephan 2016). Interestingly, I saw some evidence of this genomic pattern where sequential SNPs along several scaffolds showed outlier patterns. A more completely annotated reference genome would have further facilitated highlighting the functional role of outliers in Chapter 4.

5.2.3 Geographic limitations
The environmental pressures limiting American pika occupancy are known to vary over the broad geographic range of the species. For instance, Jeffress et al. (2013) found the relative importance of specific climate stressors changed while sampling across a bioclimatic gradient encompassing eight U.S. national parks. This study showed that heat stress was most influential on pika occupancy in the driest parks while in other parks it appeared that high elevation, cold temperatures, and high precipitation acted to limit the upper elevational distribution of pika populations. Therefore, it is likely that the selective pressures being applied to pika populations and potential resulting adaptations will also vary geographically.

Logistical considerations limited the geographic extent of our sampling for Chapters 3 and 4 to a relatively narrow segment of the American pika’s northern range. Given this northern distribution it is unsurprising that we primarily resolved evidence for cold stress. A broader geographic sampling of genomic patterns in the American pika is currently being conducted that aims to resolve the genomic response to climate stress in other regions of the pika’s distribution.
5.3 Management implications

In Chapter 2, I documented restricted gene flow associated with road development and landscape modifications that potentially caused the fragmentation of pika populations around the Highland Valley Copper mine. In this chapter, I documented extensive colonization of artificial habitat that was generated by mining activities. A study contrasting pikas on artificial sites and natural talus in the Highland Valley Copper mine found no difference in body condition or survival (Blair, *in preparation*). This indicates that artificial talus could be used to provide habitat corridors which would promote gene flow and mitigate the barrier to movement between the northern and southern sites. Such corridors have been successful in promoting movement in other small mammals (Debinski & Holt 2000), but have rarely been applied to American pika (e.g. I-90 Snoqualmie Pass East Project; Ernest, unpublished).

In Chapter 4, I documented a natural downslope bias to movement in the American pika that may act as a potential barrier to the upslope movement of individuals as the climate warms. It is likely that this biased movement will be prevalent in other alpine species where dispersal is density dependent. One potential management response is assisted gene flow from low populations suspected to contain thermal adaptations to higher populations suspected to be suffering reduced viability due to thermal stress. Aitken & Whitlock (2013) concluded that assisted gene flow between populations has the potential to address maladaptation due to climate change when it is used to introduce genotypes that are preadapted to the new local climate or increase the frequency of these genotypes in the existing population. However, one concern that needs to be carefully considered is the risk of outbreeding depression. In the case of American pika, the risk of outbreeding depression is unlikely across the restricted geographic range of elevational gradients. We resolved
significant genetic differentiation between transects in Chapter 4. However, the consistent downward movement of individuals within transects likely keeps any genomic incompatibilities to a minimum. If assisted gene flow is implemented in this system, it may be important to use individuals from the closest low elevation population to reduce the potential of outbreeding depression when they are introduced to higher elevation populations.

5.4 Significance and future directions

During this thesis, I evaluated a new sample collection method for the genomic assessment of elusive mammals, applied a novel technique of assessing long-term chronic stress in natural populations using hair samples, and provided one of the first genomic assessments of climate adaptation in a thermally-sensitive mammal. The work in this thesis will provide other researchers new and exciting genomic and physiological tools applicable to elusive mammals and other climate-sensitive species. Additionally, I have provided unique insights into several dispersal and metabolic patterns as well as providing further evidence for cold stress in the American pika. This knowledge adds to our understanding of biotic and climate stressors this species faces and will inform conservation interventions. However, more information is needed to more fully assess the continued viability of pika and other climate-sensitive species.

The geographic scope of the genotype-environment associations in Chapter 4 could be extended by incorporating lineage-wide genomic data. The Cascade lineage of pika extends from central British Columbia through central Oregon (Galbreath et al. 2009). A detailed genomic assessment of patterns along the latitudinal gradient of pika populations in the Cascade lineage presents the opportunity to scan for broad-scale genomic patterns.
associated with climate variation. Such an analysis could further highlight potential adaptations in the American pika to changing climate conditions. Furthermore, evidence of lineage-wide adaptation to climate patterns could further inform population viability analyses and niche modeling exercises aimed at forecasting future range contractions both in pika and other thermally sensitive species.
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