University of Alberta

Genetic diversity and selection in North American red squirrels (*Tamiasciurus hudsonicus*): A Hamiltonian perspective into the processes and mechanisms of evolution.

by

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ABSTRACT

The theory of natural selection has advanced our understanding in every aspect of biological sciences, yet despite this seeming ubiquity, there remain some components that are not fully resolved. Natural selection predicts the "selfish" advancement of genes that are optimally suited for their present environment. While the evolution of sexual recombination is still not fully understood, the red queen hypothesis proposes that sexual reproduction is a means to evolve resistance to parasites. In addition, the evolution of social behaviour can also contradict the basic premise of natural selection whereby helping to advance the genes of others would cost a reduction in the success of one's own genes. I investigated these issues using the North American red squirrel (*Tamiasciurus hudsonicus*) while developing and optimizing additional genetic resources for the squirrel family (Sciuridae). First, I developed a new molecular sex-typing system intended specifically for squirrels by designing new PCR primers on the Y chromosome. Secondly, I investigated the interaction between the phylogeographic history and broad scale genetic selection of the red squirrel throughout western North America. While evidence suggests the red squirrel did not have a northern refugium during the last glaciation, the strong decline in genetic diversity with latitude is likely the result of recolonization. Thirdly, I tested the genetic basis to parasite resistance and quantified the cost of parasite infection on reproductive success in male and female red squirrels. While a genetic architecture to parasite intensity was found through a negative correlation with heterozygosity in females, the influence of parasites on reproductive success

was only apparent in males, with highly successful males having higher parasite intensity. Lastly, I presented the first observation of altruism directly supported by Hamilton's rule of inclusive fitness. Surrogate females always gained a fitness advantage by adopting related juveniles when circumstances allowed for the cost of adoption to be less than the benefit multiplied by their shared relatedness ($c < rb$). These investigations into the mechanisms of genetic diversity and selection have provided much needed support for the basic assumptions of natural selection and evolutionary biology while also advancing our primary knowledge and available genetic resources.

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Chapter 1

General introduction

1.1 Introduction

Darwin's (1859) theory of natural selection proposes that individuals that are more suited for their environment are more likely to survive and reproduce, passing on their heritable traits to the next generation. While natural selection has since become the foundation of evolutionary biology, Darwin himself could not identify the means by which heritable traits were passed on. Mendel (1866) published the key to heritable variation by studying garden peas (*Pisum sativum*) in a monastery in Austria only 7 years after Darwin published his now famous book. Though Mendel's work originally went unnoticed, Fisher (1930) united Darwinism with Mendelian genetics by describing natural selection in terms of changes in allele frequencies. Alleles associated with greater individual fitness are predicted to increase in frequency, leading to an increase in mean population fitness. The mechanisms by which alleles are selected across generations, both within and between populations, are widely considered the mathematical foundation for the genetical process of evolution.

Despite widespread support and acceptance of natural selection, Darwin's theory could not explain some difficult concepts like the evolution of social behaviour, or the evolution of sexual recombination of chromosomes. The evolution of social behaviour in particular was difficult to explain via natural selection, as the latter relies on the selfish tendencies of genes to promote themselves (Dawkins 1976). Darwin himself was confused by how social insects (such as ants or honey bees) can thrive when sterile workers self-sacrifice for the colony. Altruistic behaviours should be eliminated by natural selection as helping

another individual should come with a fitness cost. It wasn't until Hamilton (1964) formulated his theory for the maintenance of altruism that kin selection was fully articulated and formulated as a mechanism for evolution (Maynard Smith 1964). *Hamilton's rule* provides the fundamental link between social interaction of related individuals and genetic fitness, and by extension of natural selection, and illustrates how altruism can be maintained and favoured over time in a population. Though Hamilton's rule is now widely accepted in evolutionary biology, there has been a shocking lack of empirical evidence supporting this theory over the last 49 years. Only a handful of studies either using computer simulation models or focused on haplodiploid eusocial insects have directly been able to show the possible evolution of altruism, but support in vertebrates is non-existent (reviewed in West *et al*. 2007, West and Gardner 2010, Abbot *et al*. 2011).

Explaining the evolution and maintenance of sexual reproduction remains one of the greatest challenges for biology, with more than 20 hypotheses having been advanced (Kondrashov 1993). Asexual organisms can thrive in optimal conditions, producing clones quickly, while sexual organisms must pay the cost of reduced fecundity in return for new chromosomal rearrangement. Therefore, asexual reproduction should become dominant over time, unless there is an advantage to sexual reproduction (Maynard Smith 1971). Today, the most accepted hypothesis to explain short-term advantages to maintenance of sexual reproduction is the *red queen hypothesis* (RQH) (Van Valen 1973). The RQH predicts that species which reproduce sexually gain an advantage from

recombining different sets of alleles in new combinations (Salathé *et al*. 2008). Specifically, this constant rearrangement of alleles provides the ability to develop resistance to parasites, which are a constant selective pressure on almost all living species (Ridley 2003). Therefore, parasites themselves could be responsible for the evolution of sexual recombination of genetic material (Hamilton *et al*. 1990). When asexually-reproducing snails are infected with a parasite they are replaced by rare variants over time due to negative frequency-dependent selection of resistant alleles, while sexually-reproducing snails remained stable over the same time period (Jokela *et al*. 2009). Hence, resistance to parasites should be genetically derived, leading to mate choice preferences and differential reproductive success between individuals. Empirical evidence of the RQH as measured by direct investigations of the cost of parasitism on reproductive success are rare in wild populations. The few studies that have attempted to quantify parasite infection and reproductive success simultaneously have not been able to reach a consensus concerning the role of parasites on fitness.

Though the use of molecular analyses to characterize genetic diversity and reproductive success has only been possible for the last 25 years (Saiki *et al*. 1988), domestic animal breeders have been studying selection on traits much longer, simply by knowing the shared ancestry between individuals. Pedigrees alone with little additional genotypic information can be enough to determine the heritability and selection on a trait without even knowing which genes are responsible. Determining the pedigree of a population is challenging and requires continuous effort across years or decades, and as such, pedigrees of wild populations are rare.

Though maternity is often determined via behavioural observations alone, paternity in wild birds and mammals is usually determined via molecular paternity testing. The application of paternity testing in wildlife has changed the field of ecology dramatically by disproving long held views such as sexual monogamy of socially pairing birds (Westneat and Stewart 2003). Genetic maternity testing has also helped clarify confusions between twinning and adoptions in the wild (Gelatt *et al*. 2001).

With advancing molecular techniques, ecologists now have the ability to quantify selection on behaviour, survival and reproduction at a molecular scale (Owens 2006). Studying selection on the molecular level increases the resolution of variance between individuals and accurately depicts which traits are under selection (Fitzpatrick *et al.* 2005). Microsatellite tandem repeats are powerful yet simple genetic markers that can be used to characterize diversity in a variety of applications including parentage analysis, assessment of relatedness, dispersal movement patterns, population structure and even determining phylogenetic lineage and speciation. Microsatellites can be found throughout the genome and provide an efficient way of characterizing genetic diversity across broad scales but also help to pinpoint the location of genomic regions under selection using association mapping. Microsatellites located near genes under selection can even be used as proxies for characterizing diversity without the need to sequence the genes themselves, as they are usually linked through coinheritance (Goldstein and Schlotterer 1999).

In contrast to broad searches across the genome, a specific genomic region of interest in ecology is the major histocompatibility complex (*MHC*) (reviewed in Bernatchez and Landry 2003, Piertney and Oliver 2006). *MHC* genes in vertebrates code specialized proteins that are essential in detecting pathogens and triggering a cascade of immunological responses, thereby constituting the genetic basis of individual immunocompetence. Each *MHC* protein has an antigen-binding site which is specific for one type of foreign antigen and is highly variable between individuals, potentially leading to differential susceptibility and infection and thereby driving selection for increased polymorphism via mate choice preference (Garrigan and Hedrick 2003). As such, *MHC*-conferred parasite resistance increases survival and reproduction leading to increased *MHC* polymorphism through both natural and sexual selection.

1.2 Thesis objectives and data chapters

During the course of this thesis, I optimized and scored a suite of microsatellites to characterize genetic diversity and perform parentage analysis in red squirrels. I successfully developed a method to multiplex 16 microsatellite primer pairs into only three separate polymerase chain reactions comprising 8, 4 and 4 pairs per reaction. Using this multiplex system to generate multilocus genotypes, I have assigned paternity to sires and constructed a full pedigree which has been fruitful in helping to address a number of evolutionary questions, e.g. reproductive success. Since assuming responsibility of the Kluane red squirrel pedigree, I have genotyped 2700 individuals at 16 loci (43,200 individual

genotypes). I have also seen the pedigree grow from 4400 individuals (between 1985-2005) to over 8000 (up to 2012) with 6200 maternal links. I have performed parentage analysis and assigned paternity to siring males, adding 1500 paternal links which brings the total to 1800. In addition to the data chapters that follow in this thesis of which I did most of the work, I was also a collaborator on a number of other publications as a result of my work maintaining the Kluane red squirrel pedigree (see McFarlane *et al*. 2011, Taylor *et al*. 2012, Williams *et al*. 2013).

Apart from microsatellite genotyping and pedigree reconstruction, I also attempted to sequence novel portions of the red squirrel genome to better characterize genetic diversity and investigate selection within and between species of squirrels. I successfully sequenced the minor histocompatibility complex (*Smcy*) and confirmed that the *Smcy* is only present on the Y chromosome in squirrels. I also attempted to sequence the *MHC* in order to study the relationship between genetic diversity and balancing selection for parasite resistance, survival and mate choice. Despite my best efforts to sequence *MHC de novo* by using conserved mammalian primers, developing new primers, cloning, and attempting next-generation sequencing, I was not able to confidently and reliably isolate *MHC* haplotypes for red squirrel at either the *DRB* or *DQA* loci. The primary impediment to my success was the duplication of these genes which resulted in more than two alleles per individual, which also included a non-functional pseudogene which contained a 20-base deletion midway through the sequence, resulting in a stop codon. Thus, although the above constituted a considerable portion of the work associated with my thesis, it figures little in the thesis per se.

Instead I have compiled a series of chapters which present the development and optimization of genetic resources for characterizing genetic diversity followed by implementing these resources to study selection acting across the genome as a whole in a wild population of red squirrels.

Squirrels (Rodentia, Sciuridae) are great model organisms to study a wide suite of ecological, behavioural and evolutionary questions as they usually occur in abundant numbers and are easy to capture, mark and observe. But more importantly, they also show a remarkable range of different levels of social interaction and variation across their mating systems throughout the family. The North American red squirrel (*Tamiasciurus hudsonicus* Erxleben, 1777) (Fig. 1-1) is particularly well suited for use in studying fundamental questions in population genetics and evolutionary ecology as they have an abundance of parasites to examine in the interests of the red queen hypothesis. Red squirrels are also typically asocial compared to other squirrels, which makes rare observations of social interaction ideal for testing the assumptions of Hamilton's rule and examining the evolution of social behaviour in an asocial environment. Researchers involved with the Kluane red squirrel project (Yukon Territory, Canada) have been studying red squirrels since 1985 and have both the experience and the data to test the assumptions of Hamilton's rule and the red queen hypothesis. For my doctoral thesis, I tested for the presence and underlying mechanisms behind selection for the evolution of social behaviour and parasite-resistance. In addition, I developed and optimized genetic resources along the way

to aid both my own and future research on squirrels as well as to deepen our understanding of their natural and phylogeographic history.

The thesis is divided in four data chapters, one of which develops genetic resources for many species of squirrels across North America (2) while three are focused solely on the red squirrel (3, 4 and 5). Each successive chapter investigates mechanisms of genetic diversity and selection at finer scales, starting at the species level, then metapopulations across their distribution, then a localized population, and finally at the individual level.

In **chapter 2**, I sequence for the first time the *Smcy* in seven species of North American squirrels from four genera. By comparing the aligned sequences, I was able to design new primer sets that when paired with internal microsatellite control markers present the very first molecular sex-typing system developed for squirrels. Sequences were conserved within but were divergent between genera, allowing for genera-specific but not family-specific primer pairs.

In **chapter 3**, I investigate broad scale patterns of genetic diversity and how they relate to the historical biogeography of the red squirrel throughout western North America. I planned to also test for the presence of historical balancing selection on the *MHC* by comparing the patterns of genetic diversity at the *MHC* to neutral genetic variation across the red squirrel's distribution. Though *MHC* was not available for comparison, broad genetic diversity still allowed me to test for the presence of a northern refugium during the last glaciation. I also test the assumptions of the central-marginal hypothesis which predicts levels of

genetic diversity and gene flow between populations that are dependent on their location throughout the species' range distribution.

In **chapter 4**, I quantify the endoparasite load in one population of Kluane red squirrels while examining both physical and genetic determinants of parasite infection intensities. I also quantify reproductive output of all males and females and test for correlations between parasite intensity and reproductive success as predicted by the red queen hypothesis. I originally hypothesized that increasing genetic diversity at the *MHC* would confer parasite resistance, however I was only able to investigate broad scale patterns of neutral genetic diversity on parasite resistance.

In **chapter 5**, I present the first empirical test of Hamilton's rule in a wild population of vertebrates using the Kluane red squirrel. I estimate the reproductive cost incurred by surrogate mothers in adopting orphaned juveniles and test if this cost is overcome by the genetic benefits of inclusive fitness as predicted by Hamilton's rule.

Figure 1-1. Female red squirrel prepares to move a juvenile between nests.

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Chapter 2

Sexing the Sciuridae: A simple and accurate set of molecular methods to determine sex in tree squirrels, ground squirrels and marmots

A version of this chapter has been published.

Gorrell JC, Boutin S, Raveh S, Neuhaus P, Côté SD, Coltman DW. 2012.

Molecular Ecology Resources. 12: 806-809.

2.1 Introduction

Knowledge of individual sex is often critical in studies of behaviour, ecology, life history and conservation. Male and female squirrels (Sciuridae, Rodentia) are usually similar in size and colouration and may be difficult to sex visually. With 285 extant species endemic to all continents except Antarctica, Australia and Madagascar (Thorington *et al*. 2012), squirrels are the second most diverse family of rodents and are useful models in behavioural and evolutionary ecology (e.g. Gorrell *et al*. 2010, Raveh *et al*. 2011) as well as physiology and medicine (e.g. Fredes *et al*. 2012, Williams *et al*. 2011). However, until now there has not been a specialized and reliable molecular method to sex individuals from the family Sciuridae.

Three Y chromosome genes are commonly targeted for sex determination in mammals: zinc-finger-Y (*Zfy*), testis-determining factor (*Sry*) and minor histocompatibility complex antigen (*Smcy*). Squirrel karyotypes are highly conserved with many species having small Y chromosomes, almost dot-like, and presumably many Y-specific genes have relocated to other areas of the genome (Li *et al*. 2004, Li *et al.* 2006). The *Zfy* gene does not reliably indicate sex in ground squirrels (*Spermophilus sp.*), as *Zfy* appears to have migrated to the X chromosome (Ermakov *et al.* 2006). *Sry* was believed to be widely male-specific, however studies have shown that *Sry* can be either copied to the X chromosome or can be lost entirely across different rodent lineages (Fernandez *et al*. 2002, Graves 2002). Kusahara *et al*. (2006) used conserved *Sry* primers to sex the Pallas tree squirrel (*Callosciurus erythraeus*), however we found this method unreliable

in North American red squirrels (*Tamiasciurus hudsonicus*). For example, we observed several bands with fluctuating intensities across both sexes and could not accurately sex individuals (Gorrell unpubl. data).

Smcy is the minor histocompatibility complex antigen and is male-specific in four species of *Spermophilus* ground squirrels (Ermakov *et al*. 2006). Here, we designed new PCR primers complimentary to conserved regions inside the *Smcy* sequence for sex determination in seven squirrel species. These seven species provide a cross-section of the diverse squirrel family with representatives from Tamiasciurini, Sciurini and Marmotini. We describe a simple and robust protocol for sex determination with co-amplification of nuclear loci as internal controls. Our goal was to provide a fast and reliable method for sex determination by simple PCR using short fragments that are easily separated using conventional gel electrophoresis.

2.2 Methods

Tissue samples were collected throughout Alberta and Yukon, Canada and stored at -20ºC until DNA was extracted using DNeasy Tissue extraction kits (Qiagen) and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific). We sequenced the entire 8^{th} intron and partial flanking exons (~600 bp) of the *Smc* gene on the Y chromosome for two to three males of each species using primers Smc8D and Smc9R (Ermakov *et al* 2006). PCR was carried out in a final volume of 25 µL, containing 1X PCR buffer (50 mM Tris-HCl, pH 9.2, 10 mM ammonium sulfate, 100 µg/ml bovine serum albumin, 2.5 x 10^{-3} v/v beta-

mercaptoethanol), 1.8 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, 0.02 U of Taq polymerase and 40 ng of genomic DNA. We used a touchdown PCR approach by dropping the annealing temperature by 1 ºC every other cycle starting at 62 ºC until reaching 58 ºC. The conditions for PCR amplification were as follows: a denaturing step at 95 ºC for 4 min, followed by 8 cycles of 95 ºC for 15 s, 62-59 ºC (2 cycles at 62 ºC, 61 ºC, 60 ºC, 59 ºC) for 15 s, 72 ºC for 60 s, and then another 30 cycles of 95 ºC for 15 s, 58 ºC for 15 s, 72 ºC for 60 s and a final extension at 72 ºC for 5 min. All PCRs were carried out using an Ep Gradient Mastercycler (Eppendorf). We included known males, known females and negative controls in the PCR to confirm amplification was male specific and ensure reactions were contaminant free.

10 μL of PCR product was electrophoresed on a 1% agarose gel stained with ethidium bromide. 5 μL of PCR product was purified with 2 μL of ExoSap-IT (USB) following the manufacturer's protocol. 1.5 μL of purified product was used in a 10 μL sequencing reaction with BigDye Terminator 3.1 cycle sequencing kit (Applied Biosystems) following the recommended cycling parameters and then sequenced on a 3730 DNA Analyzer (Applied Biosystems). Products were sequenced in both directions (~600 bp in total length) and aligned using BioEdit 7.0 (Hall 1999). Sequences were submitted to Genbank for North American red squirrel (*Tamiasciurus hudsonicus*, acc. no. JQ361901), Eastern gray squirrel (*Sciurus carolinensis,* JQ361902), Columbian ground squirrel (*Urocitellus columbianus,* JQ361904), Arctic ground squirrel (*Urocitellus parryii,* JQ361905), Hoary marmot (*Marmota caligata,* JQ361906), Yellow-bellied

marmot (*Marmota flaviventris,* JQ361907) and Woodchuck (*Marmota monax,* JQ361908).

From these sequences, we used Primer3 (v.0.4.0; Rozen and Skaletsky 2000) to design new genera-specific primers to amplify a short male-specific product (126-145 bp) that could be co-amplified with a non-Y-linked microsatellite locus as an internal control (Table 2-1). We intentionally designed the male-specific product to be shorter than the microsatellite control to mitigate the effects of amplification bias in product length on our ability to detect the male-specific product (Waits and Paetkau 2005). We included the previously established microsatellite primers *Thu32* (~282 bp, Gunn *et al*. 2005) as internal control for *Tamiasciurus* and *Sciurus*, and microsatellite primers *GS14* (~243 bp, Stevens *et al*. 1997) for *Urocitellus* and *Marmota*.

Sex-determining PCR was carried out in a final volume of 10 μL, containing 1x PCR buffer (50 mM Tris-HCl, pH 9.2, 10 mM ammonium sulfate, 100 μg/ml bovine serum albumin, 2.5 x 10^{-3} % beta-mercaptoethanol), 1.8 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μM of each male-specific primer, 0.5 μM of each microsatellite primer, 0.03 U of Taq polymerase and 20 ng of genomic DNA. The cycling parameters for PCR amplification were as follows: a denaturing step at 95 ºC for 4 min, followed by 35 cycles of 95 ºC for 15 s, 54ºC 15 s, 72 ºC for 60 s, and a final extension at 72 ºC for 5 min. A negative control was run with each primer set to detect contamination and ensure amplification was not due to primer interaction. Products were electrophoresed at 110 V (7.3 V/cm) for 45 min on a 1% agarose gel stained with ethidium bromide and sex was

determined based on the banding pattern. Males show both the short male-specific product and the longer microsatellite product while females only show the microsatellite product. We considered the reaction a failure if neither product was observed.

2.3 Results and discussion

Primers *ThuSmc* successfully co-amplified with *Thu32* in *T. hudsonicus* and *S. carolinensis*. *UroSmcF* and *UroSmcR* successfully co-amplified with *GS14* in both species of *Urocitellus* while *UroSmcF* and *MarSmcR* successfully co-amplified with *GS14* in all three species of *Marmota*. Figure 2-1 provides a representative banding pattern of each genera-specific primer set which appeared identical within all species tested for each primer set including both the male-specific product and the microsatellite product.

We tested the accuracy of our method using samples collected from long-term study populations. Sex is known with certainty as these studies employ frequent mark-recapture of individuals throughout their lifetime and monitor annual reproductive success. We tested 83 tree squirrels (*T. hudsonicus*, Gorrell *et al*. 2010) and 83 ground squirrels (*U. columbianus*, Raveh *et al*. 2011). A blind observer correctly identified the sex of all 166 squirrels from a single round of PCR. We also tested 114 marmots (*M. caligata*) captured from Caw Ridge, AB, Canada, although these animals are captured less frequently and field identification of sex is less certain. Our PCR-based sex determination matched the sex determined in the field in 106 individuals (93%) and the results were

consistent with a second round of PCR. Therefore, we remain confident in our method to accurately and reliably determine sex across a range of species.

The multiplex method developed here is the simplest and most efficient approach for sexing squirrels to date. These PCR products are easy to visualize and interpret, as the male-specific product and the control product are easily separated using agarose gel electrophoresis (>100 bp difference, Fig. 1). With a single round of PCR, we were able to reliably determine the sex of *T. hudsonicus* and *U. columbianus* in 100% of our samples.

Band intensities and sharpness seen in Fig. 2-1 were consistent across known males and females of *S. carolinensis*, *U. parryii, M. flaviventris* and *M. monax* demonstrating that primers designed across conserved regions consistently co-amplified with internal controls across several taxa and would likely amplify across other species from the same genera.

We have developed a variety of primer sets that could potentially amplify across, and help sex, several genera encompassing a diverse range of squirrel species from several major phylogenetic lineages. We believe this method is a useful molecular resource that could extend across a wide array of studies throughout the world. Since our method produces short products by simple PCR, this approach could be applied to circumstances with low quality DNA from non-invasive sampling (Waits and Paetkau 2005).

Table 2-1. Primer sequences for male-specific and control loci according to genera.

Genera	Forward	Sequence	Reverse	Sequence	Size
Tamiasciurus, Sciurus	*ThuSmcF*	CCTTCCTGACATTCCTAGAG	*ThuSmcR*	TTTAGTTTCCCTTGGTTCAG	145
Tamiasciurus, Sciurus	*Thu32F*	GCACTTGGCTGGTACGTATG	*Thu32R*	CCCCTTTTAAAACAATGACTGTTAC	~282
Urocitellus	*UroSmcF*	TCTGTCATTTGTCTTCCTGA	*UroSmcR*	GTCCTTTTTCTCTGGTTGTC	140
Marmota	*UroSmcF*	TCTGTCATTTGTCTTCCTGA	*MarSmcR*	GTTGTTCCCATCTTATGGAT	126
Urocitellus, Marmota	*GS14F*	CAGGTGGGTCCATAGTGTTAC	*GS14R*	TTGTGCCTCAGCATCTCTTTC	~243

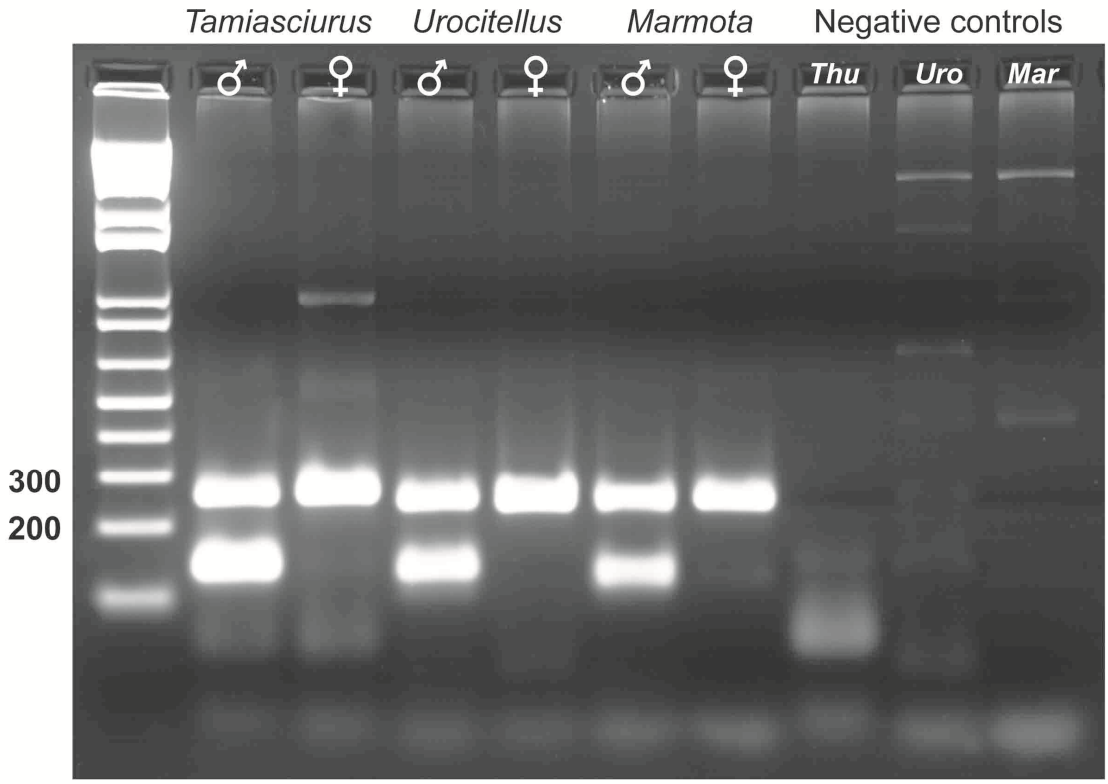

Figure 2-1. Co-amplification of male-specific PCR product and microsatellite control across representatives of each genera-specific primer sets. Negative controls Thu (Tamiasciurus hudsonicus), Uro (Urocitellus columbianus), Mar (Marmota caligata) show genera-specific primers in the absence of genomic DNA.

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Chapter 3

Clinal reduction of genetic diversity in North American red squirrels

3.1 Introduction

The ubiquitous pattern of clinal reduction in species richness and biodiversity from the equator to the poles is better known as the latitudinal diversity gradient (reviewed in Willig *et al*. 2003). At present, there are over 30 hypotheses to explain this phenomenon, which is common across almost all forms of life. Studies involving both vertebrates and plants have found greater intraspecific genetic divergence among populations at lower latitudes supporting the idea of increased molecular evolution near the equator as one of the leading hypotheses (Martin and McKay 2004, Eo *et al*. 2008). For example, insects have shown evidence of thermal selection in candidate genes with clinal variation in metabolic, immunity and circadian rhythm genes, but not in pigmentation or developmental genes (Fabian *et al*. 2012, De Jong *et al*. 2013). Gillman *et al*. (2009) found that the rate of evolution in mammals is increased in warmer latitudes leading to greater evolutionary independence and ultimately greater speciation. Hence, greater within species genetic diversity in southern latitudes can partly be explained by the same reasons responsible for the latitude diversity gradient (Mittelbach *et al*. 2007). However, there is debate whether this latitudinal cline in genetic diversity is the product of increased evolution with temperature or simply a historical artifact from the (re)colonization pattern of species from different refugia.

Howes and Lougheed (2008) demonstrated that not only can genetic diversity decline with latitude but can also vary with other ecological variables that in turn contribute to genetic diversity. For example, Dionne *et al*. (2007)

showed that major histocompatibility complex diversity in Atlantic salmon (*Salmo salar*) declines with latitude but increases with temperature (temperature was negatively correlated with latitude) with temperature being the better predictor of diversity. Johansson *et al*. (2006) showed that while population differentiation in the common frog (*Rana temporaria*) increased with latitude, both population size and genetic diversity decreased with latitude. However, diversity does not always decrease with latitude, sometimes increasing instead. Both genetic diversity and effective population size in fisher (*Martes pennanti*) increased northwards from their southern periphery into the core of their range (Wisely *et al*. 2004) suggesting the commonly observed latitudinal cline could be confounded by core-periphery dynamics.

Indeed, Hardie and Hutchings (2010) showed that most plants and animals exhibit a reduction in genetic diversity from the core to the periphery of species' range regardless of latitude. Range expansion reduces genetic variation in populations at the margins of species' geographic distributions as a result of colonization bottlenecks (Eckert *et al*. 2008). Wolverines (*Gulo gulo*) have shown increased population structure at their southern periphery while Canada lynx (*Lynx canadensis*) and mountain goats (*Oreamnos americanus*) had reduced genetic diversity in peripheral areas compared to core populations (Kyle and Strobeck 2001, Schwartz *et al*. 2003, Shafer *et al*. 2010a).

Gene flow dynamics between the core and periphery of a species' range is formalized as the central-marginal hypothesis which has two major predictions (Eckert *et al*. 2008). First, that neutral genetic diversity should be reduced in

peripheral compared to central populations, and second, that genetic differentiation should be greater among peripheral compared to central populations. However, Guo (2012) highlights how in some studies, latitude and core-periphery dynamics can explain the same patterns and that sometimes these effects are confounded and cannot be easily separated. Indeed, some studies use latitude as a proxy for recolonization history after the last glaciation where latitude is invariably tied to the expanding periphery of a species' range (e.g. Johansson *et al*. 2006).

An in-depth review of the phylogeographic history of plants and animals in northwestern North America has revealed that while most species have recolonized from southern refugia after the Pleistocene, many species held northern refugia and descended southwards after the glacial retreat (Shafer *et al*. 2010b). Recolonization would result in a step-wise reduction in genetic diversity among advancing peripheral populations as a result of founder effects, however refugial mixing would provide new genetic variation and result in increased genetic diversity (Petit *et al*. 2003, Hewitt 2004). Most phylogeographic studies use mitochondrial DNA to investigate historical movement patterns and expose multiple refugia, however the inclusion of nuclear DNA can reveal more detail than mitochondrial DNA alone. Shafer *et al*. (2010a) used both nuclear and mitochondrial DNA from mountain goats to discover novel evidence for a northern refugium in Alaska despite fossil evidence suggesting only a southern refugium existed.

Ideally, studies focused on species with a wide distribution, abundant population size and limited potential for long-distance dispersal have the best opportunity to detect historical gene flow patterns and cryptic refugia. Members of the family Sciuridae have these characteristics and are ideal species for inferring the phylogeographic history of northwestern North America. However, compared to some species, squirrels have received much less attention into their phylogeographic history, with only a few studies focusing on mitochondrial DNA. These studies suggest that Alaska marmots (*Marmota broweri*) and Arctic ground squirrels (*Urocitellus parryii*) had northern refugia in Beringia but that red squirrels (*Tamiasciurus hudsonicus*) and northern flying squirrels (*Glaucomys sabrinus*) recolonized the north from southern refugia (Arbogast 1999, 2001, Steppan *et al*. 1999, Eddingsaas *et al*. 2004). However, red squirrels could have potentially survived in northern refugia of white spruce (*Picea glauca*) and lodgepole pine (*Pinus contorta*) (Anderson *et al*. 2006, Godbout *et al*. 2008).

The North American red squirrel is an ideal species for studying recolonization history as they have a vast range within the boreal forest spanning most of Canada, are extremely abundant in numbers and have short dispersal distances of <100 metres on average (Larsen and Boutin 1994, Steele 1998). Furthermore, red squirrels from Alaska had the same cytochrome-*b* mitochondrial haplotype as individuals in Michigan despite being listed as separate subspecies (Steele 1998, Arbogast *et al*. 2001). This is in contrast to more recent studies of red squirrels where between 35 and 62 unique mitochondrial control region (D-loop) haplotypes were found across Colorado, Idaho, Illinois, Indiana, Minnesota,

Montana, Utah and Wyoming (Wilson *et al*. 2005, Beatty *et al*. 2011). Therefore, further studies utilizing nuclear genetic markers are warranted to help better understand the phylogeographic history of these squirrels.

In this study, we examined the genetic diversity of eight populations of red squirrels (*Tamiasciurus hudsonicus* Erxleben, 1777) spanning a latitudinal gradient. We sampled across a 2200 km stretch, from the core to the periphery of their range, and used nuclear genetic markers to determine genetic diversity within populations and differentiation among populations. Based on the central-marginal hypothesis, we predicted that core populations would have increased diversity compared to peripheral populations and that differentiation would be higher among peripheral populations. At the same time, we also predicted that red squirrels would show a clinal reduction in diversity indicative of a lack of northern refugia.

3.2 Methods

3.2.1 Field methods

We collected ear-tissue samples from 114 adult red squirrels across eight populations ranging from southern Alberta to northern Yukon Territory, Canada between October 2007 and March 2008 (Fig. 3-1). Time of sampling at the sites was staggered by latitude in order to compensate for seasonal effects as part of another study (Ben-Ezra 2009), and global positioning system (GPS) coordinates were recorded at each of the eight sites. Squirrels were trapped in Tomahawk live

traps baited with peanut butter and collected tissue samples were stored in 70% EtOH. See Ben-Ezra (2009) for full details of field procedures.

3.2.2. Genetic analyses

DNA was extracted using DNeasy extraction kits (Qiagen) following the manufacturer's instructions. We generated genetic profiles for all individuals using 16 polymorphic microsatellite loci (Gunn *et al*. 2005). Multiplex polymerase chain reactions (PCR) were carried out in a final volume of 10 μL, containing 1X PCR buffer (50 mM Tris-HCl, pH 9.2, 10 mM ammonium sulphate, 100 μg/mL bovine serum albumin, $2.5x10^{-3}$ v/v beta-mercaptoethanol), 1.8 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, 0.02 U of Taq polymerase and 30 ng of genomic DNA. PCR cycling parameters were as follows: a denaturing step at 95 ºC for 4 min, followed by 30 cycles of 95 ºC for 15 s, 60ºC for 15 s, 72 ºC for 60 s and a final extension at 72 ºC for 5 min for all primer sets. Amplified products were visualized on a 3730 DNA Analyzer (Applied Biosystems) and genotyped using GENEMAPPER 4.0. All profiles were manually inspected for scoring consistency.

We tested for linkage disequilibrium and deviation from Hardy-Weinberg equilibrium using GENEPOP 4.2 (Rousset 2008). Allelic richness (A) and private allelic richness (PA) were rarefied using HP-RARE 1.0 (Kalinowski 2005) to account for unequal sample sizes at each location. We used MSA 4.05 (Dieringer and Schlötterer 2003) to calculate unbiased expected heterozygosity (H_E) and GENEPOP to calculate Wright's inbreeding coefficient (F_{IS}) within each

population. We also used MSA to calculate two metrics of pairwise genetic distance among populations including Nei's genetic distance D_S (1978) corrected for small sample sizes, and F_{ST} (Weir and Cockerham 1984). Significance of unbiased pairwise genetic distance F_{ST} was tested with 10 000 permutations. We bootstrapped Nei's D_S 100 times in MSA in order to construct neighbor-joining (NJ) trees with PHYLIP 3.69 (Felsenstein 1989) and the final consensus tree was visualized with TreeView 1.6 (Page 1996).

Partial Mantel tests with F_{ST} and D_S were performed using IBDWS 3.23 (Jensen *et al*. 2005) with 5000 permutations to test for isolation by distance (IBD). Euclidian distance between populations was calculated from GPS coordinates using ArcGIS 10. We also included mean latitude for each pair of populations to test the prediction of increased differentiation at the range periphery while controlling for isolation by distance. Lastly, we used simple linear regressions to determine the relationship between H_E, A and PA with latitude.

3.3 Results

We found no significant deviation from Hardy-Weinberg equilibrium or linkage disequilibrium after Bonferroni correction (Rice 1989). The total number of alleles per locus ranged from six (Thu25 & Thu37) to 18 (Thu40) and population estimates of A ranged from 4.9 to 7.0 (Table 3-1). Population H_E was lowest for Rock River (0.70) and highest for Kananaskis (0.82) while F_{IS} varied from -0.066 to 0.033. Population differentiation overall was moderate (F_{ST}=0.061) and statistically significant ($p<0.001$), and all pairwise F_{ST}

comparisons were significant after Bonferroni correction (0.022-0.111, $p<0.05$). Nei's D_S showed a similar pattern, albeit with a wider range of estimates (0.08-0.38) (Table 3-2).

Partial Mantel tests showed significant isolation by distance using both F_{ST} ($r^2=0.55$, $p<0.001$) and D_S ($r^2=0.51$, $p<0.001$) (Fig. 3-2). Mean latitude was not correlated to F_{ST} ($r^2=0.11$, $p>0.14$) or D_S ($r^2=0.01$, $p>0.59$) (Fig. 3-3) after correcting for distance indicating no change in differentiation from core to periphery. Our NJ tree provided further support showing a clinal relationship with similar branch lengths between populations (Fig. 3-4). Latitude was a significant predictor of H_E ($F_{1,6} = 44.3$, $r^2=0.86$, $p<0.001$), A ($F_{1,6} = 38.3$, $r^2=0.84$, $p<0.001$) and PA ($F_{1,6} = 31.3$, $r^2=0.81$, $p<0.002$), demonstrating a reduction in genetic diversity with increasing latitude (Fig. 3-5).

3.4 Discussion

We quantified genetic diversity across eight populations of red squirrels along a latitudinal gradient and tested the two primary predictions of the central-marginal hypothesis. We found a strong reduction in genetic diversity moving from southern Alberta to northern Yukon Territory, which simultaneously spans from the core to the periphery of the red squirrel's range. Though latitude and distance to northern edge of range (Eckert *et al*. 2008) are collinear in this circumstance, they are equal predictors of genetic diversity (results for distance to edge not shown). Hence, our data supports the first prediction of reduced genetic

diversity at the periphery compared to the core of a species' range while also showing a latitudinal cline in genetic diversity.

The other prediction is that genetic differentiation will be greater among peripheral populations than core populations. However, we found no effect of the location along the distribution range (represented by mean latitude) on differentiation among core or peripheral populations. Though both assumptions have been supported in previous studies, there is no statistical correlation between support for one assumption and simultaneous support of the other (Eckert *et al*. 2008). Hence these two assumptions are not seemingly mutually inclusive.

Guo (2012) proposed to use among-population differences in genetic diversity to separate core-periphery effects from latitude effects. However, red squirrels show no change in among-population genetic differentiation from their core to their periphery which makes separating these confounding effects difficult. Core-periphery effects can be difficult to separate from latitude effects when the latitudinal range overlaps both the core and periphery of a species range, such as in this study, as both predict reduced genetic diversity (Guo 2012).

Reduced population size can lead to stronger effects of genetic drift and loss of diversity (Johansson *et al*. 2006). Extreme northern populations of red squirrels are smaller due to habitat fragmentation with pockets of forests separated by large expanses of tundra. Ben-Ezra (2009) showed that red squirrel population density was lowest in the two most northern Yukon populations as would be expected at the periphery of a species' range and hence could partly explain the loss of genetic diversity northwards (Guo 2012).

Our results suggest that red squirrels likely did not have a northern refugium as reduced genetic diversity in the Yukon is likely the result of range expansion from the South. Had red squirrels survived in Beringia we would expect increased genetic diversity in the Yukon where the two lineages would mix upon recolonization (Petit *et al*. 2003). Similarly, northern populations had lower private allelic richness than southern populations. Smaller northern populations could mask the effects of refugial mixing; however, the three most northern populations had negative F_{IS} values indicating an excess of heterozygosity and likely little inbreeding. Additionally, global F_{ST} differentiation was moderate while global F_{IS} variation was negligible suggesting random mating within populations but marginal differentiation among them. This is likely the result of contiguous species range, high abundance and no major physical barriers to dispersal (e.g. mountains), leading to high levels of gene flow and only recent establishment of geographic patterns of genetic variation (Arbogast *et al*. 2001).

The absence of a northern refugium is interesting given previous evidence that white spruce had a refugium in Alaska (Anderson *et al*. 2006) and lodgepole pine had a refugium in the Yukon (Godbout *et al*. 2008). These occurrences could seemingly provide the opportunity for squirrels to persist in these pockets during glaciation provided these conifers did not enter a prolonged state of dormancy. An alternative method to investigate the historical presence of a northern refugium relies on determining the mitochondrial lineage of red squirrels in the North. Indeed Arbogast *et al*. (2001) found that red squirrels in Alaska had the same

cytochrome-*b* mitochondrial haplotype as red squirrels from Michigan, though they did not include any samples from Canada.

In conclusion, we found partial support for the central-marginal hypothesis as well as a latitudinal cline in genetic diversity indicating that peripheral red squirrel populations had lower diversity than core populations which is also suggestive of a lack of northern refugia. However, despite significant isolation by distance, peripheral populations were not more differentiated than core populations and therefore we did not find support for the second major prediction of the central-marginal hypothesis. As this is the first study to use nuclear genetic markers to investigate latitudinal effects in a squirrel, we recommend future phylogenetic studies employ a combination of both nuclear and mitochondrial data to best resolve historical and current gene flow patterns.

Table 3-1. Site descriptions from eight populations of red squirrels including sample size (*N*), allelic richness (*A*), private allelic richness (*PA*), expected heterozygosity (H_E), Wright's inbreeding coefficient (F_{IS}).

Population	Latitude	Longitude	*N*	*A* ± SE	*PA* ± SE	H_E ± SE	F_{IS}
Rock River, YT	66.9	-136.4	10	4.94±0.39	0.04±0.03	0.70±0.04	-0.031
Stewart Crossing, YT	63.4	-136.7	10	5.00±0.22	0.04±0.03	0.73±0.02	-0.065
Montague, YT	61.8	-136.1	18	5.69±0.52	0.07±0.05	0.72±0.05	-0.025
Kluane Natl Park, YT	61.0	-138.0	20	6.06±0.36	0.23±0.07	0.73±0.03	0.005
Prophet River, BC	58.3	-122.7	12	5.69±0.36	0.17±0.09	0.76±0.03	-0.066
Hinton, AB	53.5	-117.6	20	6.31±0.37	0.31±0.10	0.78±0.02	0.033
Nordegg, AB	52.5	-116.1	10	6.50±0.43	0.41±0.12	0.77±0.04	0.022
Kananaskis, AB	51.0	-115.0	14	6.97±0.33	0.60±0.16	0.82±0.01	-0.025

Table 3-2. Pairwise genetic distances among eight populations of red squirrels. All of the pairwise F_{ST} values in the top matrix were statistically significant ($p<0.05$). Bottom matrix shows D_S values.

	KN	ND	HN	PR	KNP	MT	SC	RR
KN	-	0.051	0.051	0.064	0.092	0.092	0.087	0.111
ND	0.21	-	0.028	0.037	0.055	0.050	0.065	0.080
HN	0.20	0.10	-	0.022	0.064	0.055	0.078	0.082
PR	0.26	0.13	0.08	-	0.066	0.059	0.067	0.073
KNP	0.33	0.17	0.20	0.20	-	0.037	0.038	0.073
MT	0.31	0.14	0.17	0.17	0.10	-	0.035	0.082
SC	0.32	0.21	0.26	0.21	0.11	0.10	-	0.050
RR	0.38	0.23	0.25	0.21	0.20	0.22	0.13	-

Figure 3-1. Map of eight red squirrel populations sampled across a 2200 km stretch from southern Alberta, British Columbia and Yukon Territory, Canada.

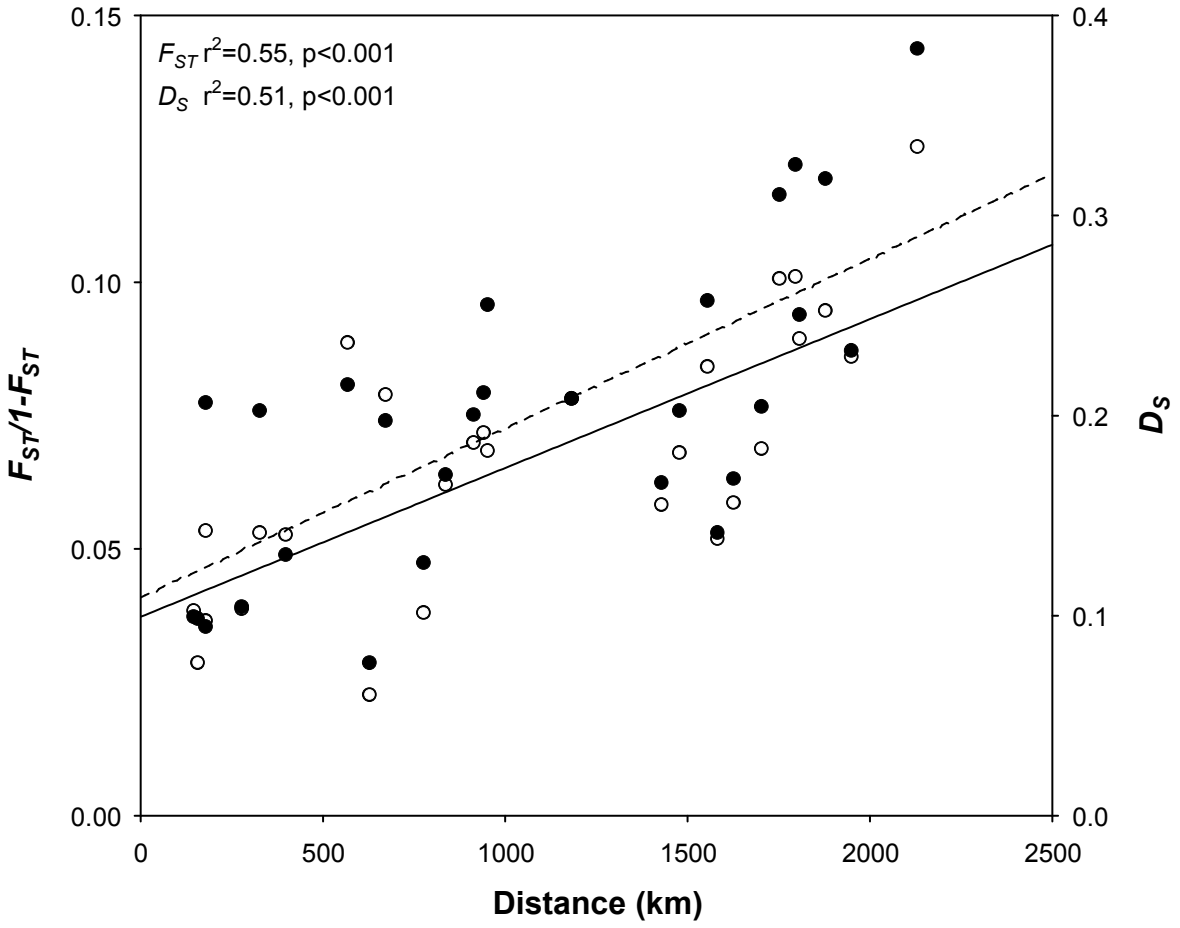

Figure 3-2. Isolation by distance across eight populations of red squirrels. Open circles and solid line indicate F_{ST} while D_S is noted by closed circles and a dotted line. Genetic distance was characterized using 16 neutral microsatellite loci.

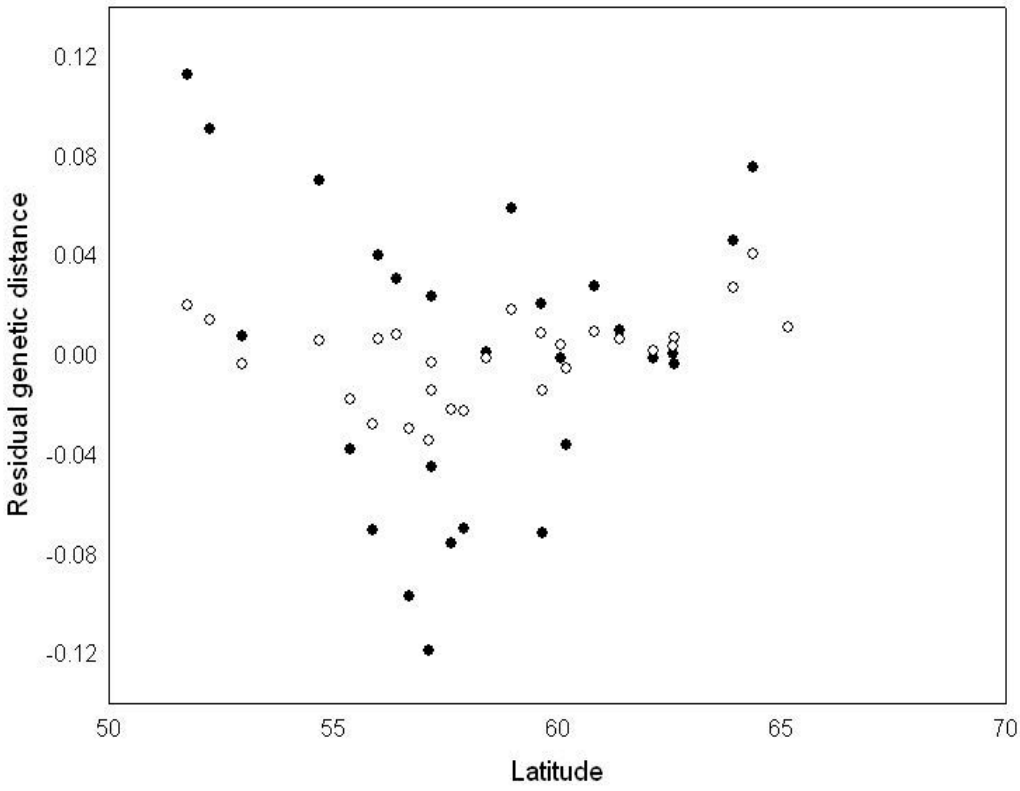

Figure 3-3. Partial Mantel across eight populations of red squirrels showing genetic distance corrected for geographic distance plotted against mean latitude between sites. Open circles indicate F_{ST} while D_S is noted by closed circles.

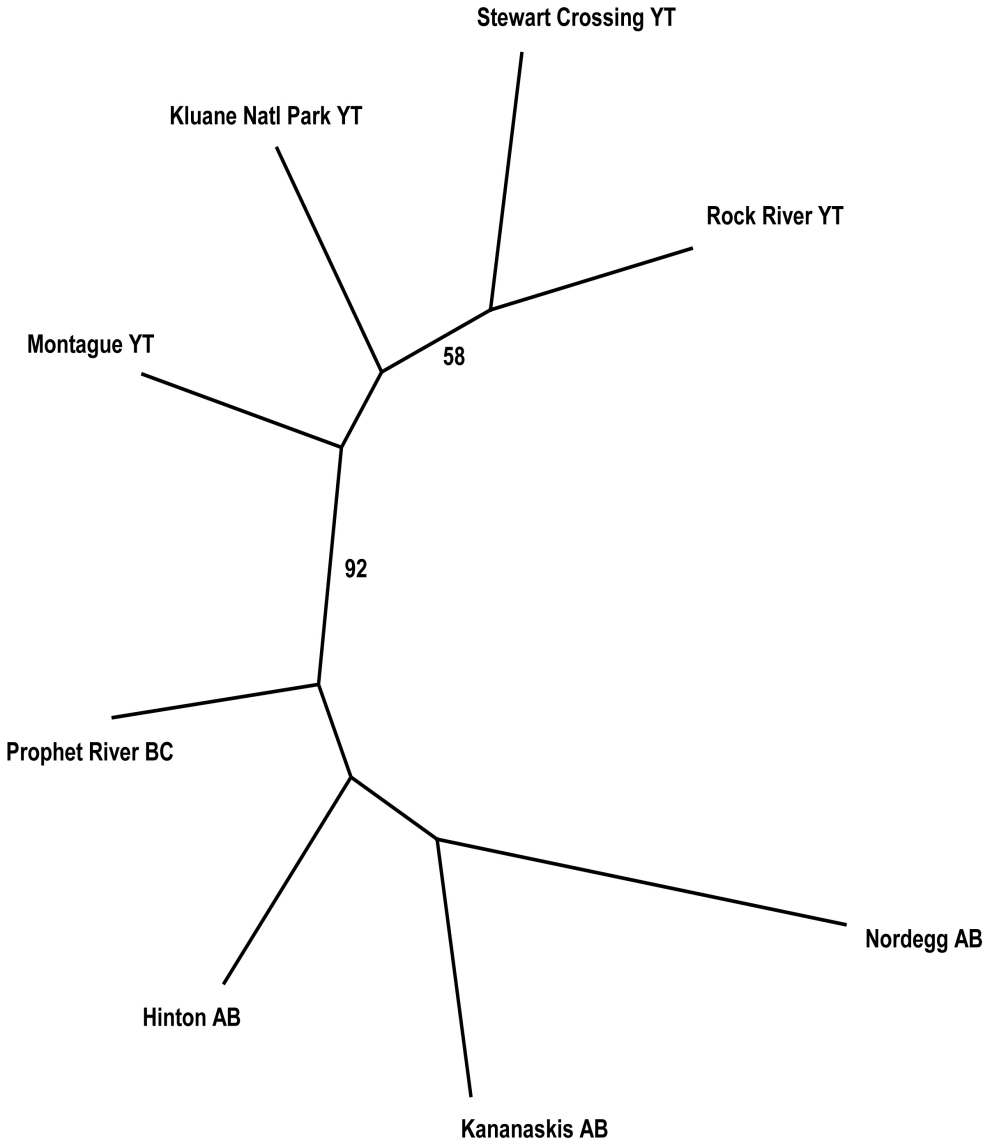

Figure 3-4. Neighbor-joining dendogram of the genetic relationships among eight red squirrel populations inferred from a matrix of Nei's D_S using 16 neutral microsatellite loci. Bootstrap values greater than 50% are shown.

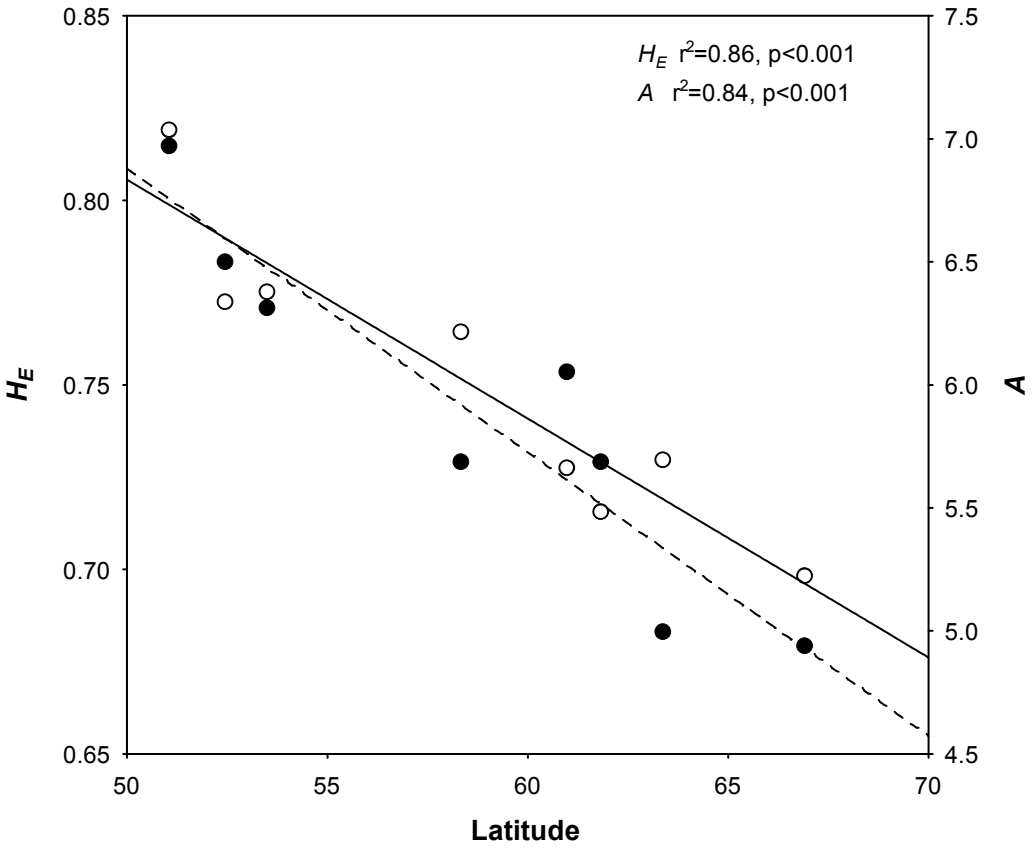

Figure 3-5. Genetic diversity of red squirrels decreased with latitude. Open circles and solid line indicate H_E while A is noted by closed circles and a dotted line. Genetic diversity was characterized using 16 neutral microsatellite loci.

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Chapter 4

Reproductive success, heterozygosity and endoparasitism in red squirrels

4.1 Introduction

Parasites are expected to be costly to the hosts they infect in a variety of ways including loss of nutrients, increased disease transmission, reduced longevity and decreased fitness. Parasites can be such a powerful selective force, that the evolution of sexual recombination of chromosomes was likely facilitated as a response to parasitism (Hamilton *et al*. 1990). As stated by the red queen hypothesis, all species are under pressure to constantly evolve just to prevent their current rate of infection from becoming more severe (Salathé *et al*. 2008). Parasites should impose a selection pressure favouring genetic diversity in their hosts whenever parasites reduce host fitness and resistance to parasites is acquired through genetic diversity. Examples of reduced parasite loads in animals with greater heterozygosity include both song birds and large mammals (Gulland *et al*. 1993, Coltman *et al*. 1999, Acevedo-Whitehouse *et al*. 2003, MacDougall-Schackleton *et al*. 2005, Luikart *et al*. 2008, Rijks *et al*. 2008, Voegeli *et al*. 2012) and additional studies have shown that inbred individuals have reduced cell-mediated immune response to pathogens (Reid *et al*. 2003, Hawley *et al*. 2005, Fossøy *et al*. 2009, Gompper *et al*. 2010). However other studies have failed to find any correlation between heterozygosity and parasite load (Côté *et al*. 2005, Acevedo-Whitehouse *et al*. 2009, Pujolar *et al*. 2009, Smith *et al*. 2009), suggesting that host heterozygosity does not invariably provide resistance. Furthermore, reviews on heterozygosity-fitness correlations by Chapman *et al*. (2009) and Hedrick (2012) call for additional studies to shed more light on the subject.

Females of many species use honest indicators of male quality, including parasite resistance, in mate choice decisions (Hamilton and Zuk 1982). The immunocompetence handicap hypothesis predicts that these indicators are costly and therefore remain honest. Only males of superior quality will be able to overcome the immunosuppressive effects of testosterone and parasite burden to successfully attract and mate with females (Folstad and Karter 1992). Alternatively, the energy allocation hypothesis predicts that investing energy into reproduction is done at the detriment of less energy being allocated towards immune function (Sheldon and Verhulst 1996, Bachman 2003). Furthermore, as males of most vertebrates have higher concentrations of testosterone and also invest less in immunity than females, parasite burden is traditionally male-biased (Moore and Wilson 2002, Nunn *et al.* 2009, Bordes *et al*. 2012). However, sex-biased parasitism can fluctuate between the sexes, depending on the host or parasite species and can also be the result of size dimorphism, body condition, home range or seasonality (Krasnov *et al*. 2012, Kiffner *et al*. 2013).

The costs of parasitism on reproduction are not confined to males, as females can also suffer from decreased energy reserves leading to reduced offspring growth rates or female-biased sex ratios (Trivers and Willard 1973, Charmantier *et al*. 2004, Reed *et al*. 2008). Soay sheep survival was lower among parasitized sheep than unparasitized individuals. There are examples of parasites reducing host fitness by decreasing survival in Soay sheep (*Ovies aries*) and harbour seals (*Phoca vitulina*) (Gulland 1992, Gulland *et al*. 1993, Rijks *et al*. 2008, Hayward *et al*. 2011). However, direct evidence of parasites reducing

reproductive success is limited and misleading at times. Male siring success in red flour beetles (*Tribolium castaneum*) declined when infected with rat tapeworm (Pai and Yan 2003). However, most studies focus on female birds and are confined to measuring clutch size, fledging mass and survival, culminating with mixed results (Korpimaki *et al*. 1993, 1995, Sundberg 1995, Dufva 1996, Pacejka *et al*. 1998, Bouslama *et al*. 2001, Marzal *et al*. 2005, Heylen *et al*. 2009). Proxies of male success in birds (e.g. pairing or mate choice) are inconsistently correlated to parasite load and do not measure true male success when extra-pair paternity is extremely variable among species of birds (Westneat and Stewart 2003). Fish and mice have also shown mate choice preference for non-parasitized partners (Ehman *et al*. 2002, Kavaliers *et al*. 2003, Deaton 2009) while non-parasitized female squirrels had higher reproductive success (Neuhaus 2003, Hillegass *et al*. 2010, Patterson *et al*. 2013).

However, there are far fewer studies focusing on the costs of parasitism on male siring success using molecular paternity testing. One study found that male siring success was both positively correlated with ectoparasite intensity and negatively correlated with endoparasite richness while an experimental study showed that parasite-free males did not gain any reproductive advantage (Gooderham and Schulte-Hostedde 2011, Raveh *et al*. 2011). Despite these efforts, there is a still a lack of studies on wild populations that simultaneously quantify parasite intensity and reproductive success.

Here, we investigate the physical and genetic correlates of parasitism in a wild population of North American red squirrels (*Tamiasciurus hudsonicus*

Erxleben, 1777) and using molecular parentage analysis, determine the association between reproductive success and parasite load in both males and females. Red squirrels are solitary and territorial rodents that remain active throughout the winter months. They are not sexually dimorphic in body size or mass and fluctuate little in body condition apart from female gestation. Most females reach sexual maturity by two years of age and then breed annually until death (McAdam *et al*. 2007). Females exhibit asynchronous estrous for one day each spring with earlier estrous dates being favoured (Boutin *et al*. 2006). Males remain scrotal for several months throughout the length of the breeding season which usually lasts March to June (Lane *et al*. 2010). Red squirrels have a scramble competition mating system, resulting in polygynandry and multiple paternity, with male search effort having the greatest contribution to male reproductive success (Lane *et al*. 2008, 2009). Females produce a single litter of 3 offspring on average after a 35-day gestation, which emerge and wean at 42 and 70 days respectively (McAdam *et al*. 2007).

A common endoparasites of the Sciuridae are the coccidia *Eimeria sp*. (Protozoa). *Eimeria* have a direct life-cycle making self-infection a likely contributor to high infection rates. After being excreted in the host feces, oocysts quickly develop into sporocysts which are capable of infecting a susceptible host upon ingestion. Once sporulated, sporozoites are known to remain infective after even 6 months of winter conditions, and can survive at 4 ºC for 3-4 years (Thomas *et al*. 1995, Duszynski and Wilber 1997). Sporozoites invade the epithelial cells of the intestine where they reproduce asexually releasing merozoites which penetrate

new cells and subsequently reproduce further. Severe infections (known as coccidiosis) can result in villous atrophy, crypt hyperplasia, lymphocytopenia and capillary hyperaemia which lead to malabsorption of nutrients, diarrhea, loss of body condition and immunosuppression (Pellerdy 1954, Rose and Hesketh 1982). Red squirrels are also host to a variety of helminths including nematodes and cestodes as well as ectoparasites from Siphonaptera and Acarina (Steele 1998).

We hypothesized parasitism to vary between the sexes and that resistance is influenced by host genetic diversity. Therefore, we predicted parasitism to be male-biased but also to be lower in individuals with greater genetic diversity, measured here as heterozygosity. We also hypothesized that reproductive success comes as a trade-off with immunity and therefore predicted that more parasitized squirrels will have higher reproductive success than less parasitized individuals.

4.2 Methods

4.2.1 Field methods

Red squirrels near Kluane National Park, YT, Canada have been intensively trapped since 1989 with every individual being marked with unique ear tags. Kluane red squirrels are non-hibernating and defend year-round food-based territories with a central underground cache (midden) where they preferentially store white spruce (*Picea glauca*) cones in the fall as their main food source (Fletcher *et al*. 2010). Annual fluctuations in cone production have direct impacts on the reproductive output of red squirrels in the current and following year, with a high cone production leading to an increase in reproduction

(Boutin *et al*. 2006). The fall of 2011 (before our data collection) had the fewest amount of cones recorded since 1989 (1.1 cones per tree on average) while the fall of 2012 had the median number of cones over the last 23 years (8.0 cones per tree on average) (unpublished data, see Fletcher *et al*. 2010 for annual variation).

A complete description of field procedures is available in McAdam *et al*. (2007) but briefly, squirrels were repeatedly trapped using Tomahawk live traps baited with peanut butter from March 1 to August 31, 2012. Adults were weighed to the nearest gram using a Pesola ® spring balance and we used increasing weight and abdominal palpation followed by sudden weight decrease and lactation to determine female pregnancy and parturition. We subsequently used a combination of radio-telemetry and behavioural observations to locate natal nests soon after birth, where offspring were counted, sexed, weighed using a digital balance to the nearest 0.1 gram and uniquely marked before tissue samples were collected for paternity analyses. Offspring were ear-tagged in the natal nest 25 days post-partum when we weighed them a second time to determine growth rates (Boutin *et al*. 2006). By tagging squirrels still in their natal nest, we are confident of their age as recruiting adults in the population. We also collected feces from adults after release from the traps and stored them in 2.5% potassium chromate (K_2CrO_4) until further processing. Adult body mass on the day of fecal collection was used in subsequent statistical analyses. All fecal samples were collected between the 3rd and 25th of June, 2012 as we wanted to capture the seasonal peak of endoparasite egg shedding while simultaneously minimizing seasonal variation (Gorrell and Schulte-Hostedde 2008).

4.2.2 Paternity analyses

We extracted genomic DNA from tissue samples using DNeasy tissue extraction kits (Qiagen) and amplified 16 polymorphic microsatellite loci (Gunn *et al*. 2005). Polymerase chain reaction (PCR) was carried out in a final volume of 10 μL, containing 1X PCR buffer (50 mM Tris-HCl, pH 9.2, 10 mM ammonium sulphate, 100 μg/mL bovine serum albumin, $2.5x10^{-3}$ v/v beta-mercaptoethanol), 1.8 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, 0.02 U of Taq polymerase and 30 ng of genomic DNA. PCR cycling parameters were as follows: a denaturing step at 95 ºC for 4 min, followed by 30 cycles of 95 ºC for 15 s, 60ºC for 15 s, 72 ºC for 60 s and a final extension at 72 ºC for 5 min for all primer sets. Amplified products were visualized on a 3730 DNA Analyzer (Applied Biosystems) and genotyped using GENEMAPPER 4.0. Microsatellite genotypes were used for paternity analyses and to calculate individual heterozygosity averaged over loci, which we used as an overall index of genome-wide genetic diversity. Paternity was determined using CERVUS 3.0 (Kalinowski *et al*. 2007) by matching a suitable male candidate with the offspring's genotype while considering the mother's genotype. We determined assignment confidence by first running a simulation with 10,000 offspring, assuming that 90% of the males in the population had been sampled and we accounted for a genotyping error of 1%. All sire-offspring matches were determined with 99% confidence with not more than one locus trio-mismatch allowed.

4.2.3 Parasite analyses

We crushed 0.5 g of feces in 4 mL of K_2CrO_4 using a mortar and pestle before straining the mixture through 4 layers of cheese cloth into a petri dish. Samples were covered and left to sporulate at room temperature (22 °C) for 6 days before being centrifuged at 2500 rpm for 2 min (Duszynski and Wilber 1997). We decanted the supernatant and resuspended the pellet in 1.5 mL of H_2O and centrifuged again before decanting. We resuspended the pellet in 1.2 mL of zinc sulfate ($ZnSO_4$, specific gravity 1.18) and centrifuged for 5 min before loading the supernatant into both chambers of a standard McMaster counting slide. After 5 minutes, we examined slides at 100X magnification and counted the number of parasite eggs inside both grids and multiplied the total by 100 to give an estimate of eggs per gram (e.p.g.) of feces which we used as our measure of parasite intensity. Parasite eggs were identified and taxonomic groups were counted and analyzed separately.

4.2.4 Statistical analyses

Fecal egg counts were ln-transformed to improve the normality of the data. We used a general linear model (LM) to determine if age, sex, mass, heterozygosity, or the date when the fecal sample was collected influenced excretion intensity for the most common parasite. Because of confounding between squirrel sex and the date of collection (male samples were collected two days later on average), we removed males sampled after the last female in the dataset and resampled parasite intensity with 1000 bootstraps to verify that a sex

effect would be genuine. We included female breeding status in a second model that contained the same parameters mentioned above. Lastly, we used a generalized linear model (GLM) with a binomial error structure to test if parasite prevalence could be predicted by the same parameters mentioned above.

To determine the influence of our index of parasite burden on male reproductive success, we used a GLM with Poisson error structure to model the number of offspring sired which also included age, age^2, mass and heterozygosity as predictors. The quadratic term for age was included to model any potential curvilinear relationship in male performance with age (i.e. senescence)(Lane *et al*. 2009). To determine the influence of parasite intensity on female reproductive success, we used a suite of GLMs with Poisson error structure (where appropriate) to model several proxies of female performance including litter size, parturition date, sex ratio, average litter growth rate, and proportion of litter survival to 25 days of age. The dispersion parameter was estimated for each model using the quasipoisson family and in cases where the dispersion parameter exceeded 1.2, we tested the significance of the variables in the quasipoisson model using an F-test. We present the coefficient ± standard error for each parameter in the LMs and the χ^2 for each term in the GLMs. All analyses were implemented in R (ver. 2.15.3; R Core Development Team, The R Foundation for Statistical Computing, Vienna, Austria).

4.3 Results

4.3.1 Correlates of parasitism

We collected fecal samples from 88 adult males and 75 adult females. We identified three taxa through egg shedding from intestinal parasites. The most common were the oocysts from the coccidian *Eimeria tamiasciuri* (Levine *et al*. 1957). Oocysts were present in 97.5% of squirrels in our study with a mean of 77,004±9,127 (SE) oocysts per gram of feces. We also discovered both cestode and nematode eggs with a prevalence of 6.1% and 3.7% respectively. The cestodes and nematodes were not identified to genus due to the difficulty in identification from egg morphology alone. Because of the low prevalence of cestodes and nematodes, we restricted most of our analyses to only include the intensity counts of *Eimeria*. Therefore, we excluded three males and one female that had zero *E. tamisciuri* oocysts as we considered them outliers and not fitting with the standard definition of parasite intensity (>0). These four outliers did not differ from the remaining dataset in age (t_{161}=2.0, P>0.14), mass (t_{161}=0.3, P>0.79) or heterozygosity (t_{161}=-0.3, P>0.82). Means and variances of data used in all subsequent analyses are summarized in Table 4-1.

Eimeria fecal oocyst count (FOC) was correlated to several of the traits we examined (LM: $F_{5,153}$ =7.46, R^2 =0.17, P<0.0001, Table 4-2). Females had ~42% higher FOC than males (93,382±11,677 vs. 65,848±14,173 oocysts per gram, Fig. 4-1). We found that FOC varied with date of sampling with samples collected earlier in June having higher values. In addition, we found that our female samples tended to be collected two days earlier on average than male samples

(t_{157}=2.4, P<0.02). However, date of sampling remained significant only in males when sexes were analyzed separately (Males: $F_{4,80}$=4.03, R^2=0.13, P<0.01). To disentangle the confounding effects between date and sex, we bootstrapped confidence intervals from the restricted dataset (excluding eight males sampled later than 166 Julian days) and found the difference between males and females remained significant. We also found that heterozygous squirrels had lower FOC than homozygous individuals when sexes were analyzed together (Fig. 4-2) but this correlation held only for females when the sexes were analyzed separately (Females: $F_{5,68}$=3.27, R^2=0.13, P<0.01). Neither age, nor mass were correlated with FOC. Female breeding status (bred or not bred) was not correlated to FOC. In order to investigate the factors that might predict presence/absence of less common parasites, we grouped cestodes and nematodes into one group representing 'helminth' prevalence to increase our statistical power. Helminth prevalence was not correlated to any of our host measurements (full GLM: χ^2=5.37, N=159 individuals, P>0.50).

4.3.2 Costs of parasitism

We genotyped 85 adult males, 89 adult females and 202 offspring. However, we did not collect parasite samples from 14 breeding females though we still determined paternity of their offspring. We matched a sire to 180 of the 202 offspring born in our study area, while the remaining 22 offspring had more than one trio-mismatch with the closest male candidate. Offspring maternity was known with certainty as offspring were tagged in the natal nest. Sixty-nine

females produced a litter (average litter size was 3.0±0.1 offspring for all 69 females and remained consistent when only including the 55 females from which we had collected a parasite sample). This lead to 164 offspring with a mother that had a parasite sample, while 20 females did not breed (13 of these were yearlings, six were two-year olds and one was seven years old). Table 4-1 summarizes the results for the 74 females which we had parasite samples for, of which 54 had a litter after removing the one female with a parasite count of zero. We were able to calculate growth rate for 43 litters. Our data also included 49 males that sired at least one offspring and 36 males with zero siring success. We defined male reproductive success as the number of offspring sired across the population, which ranged from 0-15 offspring with a mean of 2.1±0.3 per male. Average heterozygosity of adults was 0.748±0.009 SE.

Male reproductive success varied by age (χ^2=15.09, P<0.001) with middle-aged males having the most success indicated by the age^2 term (χ^2=60.54, P<0.001). Male FOC was positively correlated to the number of offspring sired (β=0.14±0.05, χ^2=7.41, P<0.01, N=85 males, Fig. 4-3a) and this relationship remained significant when we excluded males with zero siring success (β=0.15±0.05, χ^2=10.32, P<0.01, N=49 males). Neither mass (χ^2=0.58, P>0.44) nor heterozygosity (χ^2=0.09, P>0.76) correlated with FOC. Female FOC was not significantly associated with female litter size (Fig 4-3b) or any of our other four measurements of female reproductive success (Table 4-3).

4.4 Discussion

We quantified the fecal oocyst count in a wild population of red squirrels in the Yukon Territory, Canada while exploring physical and genetic correlates of FOC and reproductive cost of parasitism. Our study demonstrates how FOC can vary depending on host sex and host genetic diversity. Additionally, we show that FOC can be either directly or indirectly linked to reproductive effort and that the trade-offs between FOC and reproduction can affect the sexes differently.

The most prevalent and abundant endoparasite was the coccidian *Eimeria tamiasciuri* which was found in 97.5% of squirrels in our study. Our findings mirror those of previous studies which found similar prevalences in red squirrels from Wisconsin (98%, Dorney 1966), Ontario (92%, Soon & Dorney 1969), Alberta (95-98%, Mahrt and Chai 1970, 1972) and Alaska (91%, Seville et al. 2005). The overwhelming and consistent prevalence amongst red squirrels suggests that *E. tamiasciuri* is a widespread parasite and likely has a long co-evolutionary history with the red squirrel.

Heterozygous females had lower FOC than homozygous females suggesting that genetic heterozygosity might confer greater resistance in red squirrels. Luikart *et al* (2008) also found a negative correlation between microsatellite heterozygosity and fecal lungworm counts in bighorn sheep (*Ovis canadensis*). Homozygous individuals could be selected against if the cost of parasitism is severe enough to cause a differential fitness outcome among highly parasitized and less parasitized individuals (Coltman *et al*. 1999, Rijks *et al*. 2008, Hayward *et al*. 2011). Parasite-mediated selection against inbred individuals has

been documented in populations of Soay sheep (*Ovis aries*, Gulland *et al*. 1993, Coltman *et al.* 1999, Hayward *et al*. 2011) and harbour seals (*Phoca vitulina*, Rijks *et al*. 2008). Similar to our study, heterozygous sheep were less parasitized than homozygous sheep; however, Coltman *et al*. (1999) also found that parasitized and homozygous sheep had lower overwinter survival. While mean heterozygosity remains relatively high in our population, parasite-mediated selection could maintain these levels of polymorphism.

Contrary to previous findings (e.g. Raveh *et al.* 2011), males with higher FOC sired more offspring. Gooderham and Schulte-Hostedde (2011) found that male red squirrels with higher ectoparasite (but not endoparasite) intensity sired more offspring.

Our positive correlation supports the idea of a trade-off between immune function and reproduction (Manjerovic and Waterman 2012). This trade-off could be the result of the immunocompetence handicap hypothesis (Folstad and Karter 1992) which proposes that the increased expression of testosterone to boost reproductive effort leads to a suppression of the immune system. Higher quality males would be able to remain scrotal longer during the breeding season despite the parasite burden and still achieve higher reproductive success. However, male red squirrels do not have higher concentrations of testosterone than females (Gorrell 2006) suggesting that the trade-off seen here is perhaps due to finite energy allocation rather than immune suppression (Sheldon and Verhulst 1996, Bachman 2003). Male red squirrels participate in a scramble competition, raising their metabolism 2.5X their resting metabolic rate and increasing their home range 10-fold from

March to June (Lane *et al.* 2010). This increased energy investment towards searching for females, copulation and spermatogenesis could be at the detriment of energy allocated to immunity. Hence, males that invested more energy into reproductive effort (and subsequently gained more siring success) could then become burdened with higher parasite loads late in the breeding season. The positive relationship could also be driven by increased transmission risk. Males that increase their home range in search of females, and subsequently encounter more females, could be increasing their exposure risk through increased contact rates with conspecifics which suggests that parasitism is an indirect cost of reproductive effort (reviewed in Altizer *et al*. 2003).

Females however, did not show a relationship between FOC and any of our estimates of reproductive success. Our results are contrary to parasite removal experiments where females treated for parasites had higher litter survival (Neuhaus 2003, Hillegass *et al*. 2010, Patterson *et al*. 2013) but similar to that of montane voles (*Microtus montanus*) that showed no association between *Eimeria* infection and reproductive status (Winternitz *et al*. 2012). Antiparasite medication is expected to relieve the host of all species of parasites. However Pedersen *et al*. (2013) found that while an anthelminthic did reduce nematode infection, cestode and coccidian infection actually increased after treatment suggesting that parasite removal experiments may not be as straightforward as they claim. Since all animals in our study were infected with parasites and we did not administer an antiparasite medication to remove parasites, we did not have parasite-free females to compare against parasitized females. Instead, we measured the relative

difference in natural levels of FOC between females. These findings indicate that any differential reproductive success as a result of parasites is only detectable when all parasites are removed completely. Hence, if parasites do reduce reproductive success in females, there does not seem to be a difference between low or high parasitism.

Female-biased endoparasite load contradicts the immunocompetence handicap hypothesis but highlights the misunderstanding between parasite resistance and tolerance and how sexual dimorphism in immunity can explain differences in longevity and fitness (Råberg *et al*. 2009, Nunn *et al*. 2009). For example, coccidian infections in grey squirrels (*Sciurus carolinensis*) reduced spleen mass in males but not in females indicating differential energy investment in reproduction over immune function (Scantlebury *et al*. 2010). Studies of female Cape ground squirrels (*Xerus inauris*) have shown higher intensity of endoparasites and higher percentage of neutrophils compared to males (Hillegass *et al*. 2008, Manjerovic and Waterman 2012). Finally, Bordes *et al*. (2012) found no difference in helminth infection between male and female wood mice (*Apodemus sylvaticus*) but did notice that females invested more in immune defence and also appeared to be more tolerant of parasitic diversity.

One possibility for female-biased parasitism is the increased transmission risk imposed on breeding females when sharing the same territory and nest with emerged offspring. Though the age of first infection is unknown in red squirrels, we opportunistically examined the feces of one emerged offspring that was positive for coccidia by 61 days of age (unpublished data). Additionally, we have

on occasion observed underweight offspring at 25 days of age with anal blockages which may be the result of coccidiosis-induced diarrhea.

Our study highlights the differential effects parasites can exert across the sexes. We found that fecal oocyst counts correlated with male but not female reproductive success. As we found some support for the immunocompetence handicap hypothesis, our prediction that reproductive success is a trade-off with immunity was supported. Male red squirrels seem to allocate energy towards reproduction at the expense of immune function. As well, our results agree with a genetic basis to parasite resistance as heterozygosity was negatively correlated with parasite intensity. Knowing the effects of parasites on adults is compelling but we lack understanding of the parasite dynamics in offspring. Future studies should investigate the effects of offspring parasite load on development and survival.

Table 4-1. Means (± one standard error) and range of untransformed parasite intensity, fecal collection date (Julian date), age, mass and heterozygosity across both male and female red squirrels. Siring success reports the number of offspring sired in the population while female reproductive success was estimated by litter size, parturition date (Julian date) litter sex ratio, litter growth rate to 25 days of age and litter survival to 25 days of age. Sample sizes (*N*) are reported at the right of each row.

	Males			Females		
Variable	Mean (SE)	Range	*N*	Mean (SE)	Range	*N*
Parasite intensity (e.p.g.)	65848.2 (14173.2)	800-833,700	85	93382.4 (11677.4)	1200-503,100	74
Fecal collection date (Julian)	160.2 (0.5)	154-176	85	158.7 (0.4)	154-166	74
Age (years)	1.96 (0.09)	1-6	85	2.03 (0.14)	1-7	74
Mass (g)	263.3 (1.8)	190-300	85	264.6 (2.7)	215-328	74
Heterozygosity	0.738 (0.012)	0.313-0.938	85	0.759 (0.012)	0.5-0.938	74
Siring success	2.12 (0.31)	0-15	85	-	-	
Litter size	-	-		3.04 (0.10)	1-4	54
Parturition date (Julian)	-	-		121.7 (2.3)	95-172	54
Sex ratio	-	-		0.48 (0.4)	0-1	54
Growth rate (g/day)	-	-		1.60 (0.05)	0.72-2.78	43
Litter survival (%)	-	-		0.93 (0.03)	0-1	54

Table 4-2. Coefficients, standard errors and statistical significance for terms included in general linear models on fecal *Eimeria* parasite intensity across both male and female red squirrels (sexes analyzed together and separately). Heterozygosity of adult squirrels was calculated from 16 neutral microsatellites, fecal date is the Julian date of when the feces were collected from each squirrel and female breeding status was binary (1 bred, 0 did not breed).

	Coefficient	SE	t	*P*	*N*
Age	0.05	0.11	0.44	0.66	159
Sex	-0.64	0.21	-3.03	<0.01	159
Mass	0.01	0.01	1.76	0.08	159
Heterozygosity (sexes pooled)	-2.12	0.97	-2.20	<0.03	159
Males	-1.69	1.32	-1.28	0.20	85
Females	-2.80	1.39	-2.01	<0.05	74
Fecal collection date (sexes pooled)	-0.09	0.03	-3.64	<0.001	159
Males	-0.12	0.03	-3.81	<0.001	85
Females	-0.04	0.05	-0.86	0.39	74
Female breeding status	0.22	0.40	0.57	0.57	74

Table 4-3: Likelihood ratio χ^2 test results from generalized linear models for reproductive success in female red squirrels showing the effect of mass, age, heterozygosity and parasite intensity on female litter size (*N*=54), litter growth rate (*N*=43), litter survival to 25 days of age (*N*=54), litter sex ratio (*N*=54) and female parturition date (*N*=54).

	Litter size	Growth rate	Litter survival	Sex ratio	Parturition date[1]
Mass	0.12	0.01	0.08	0.01	2.50
Age	0.13	0.17	0.03	0.05	9.99*
Heterozygosity	0.01	0.06	0.01	0.01	0.23
Parasite intensity	0.12	0.25	0.01	0.03	5.89

* indicates $P<0.05$ with 1 d.f., [1]Parturition date was assessed using an F test instead of χ^2 due to overdispersion in the model.

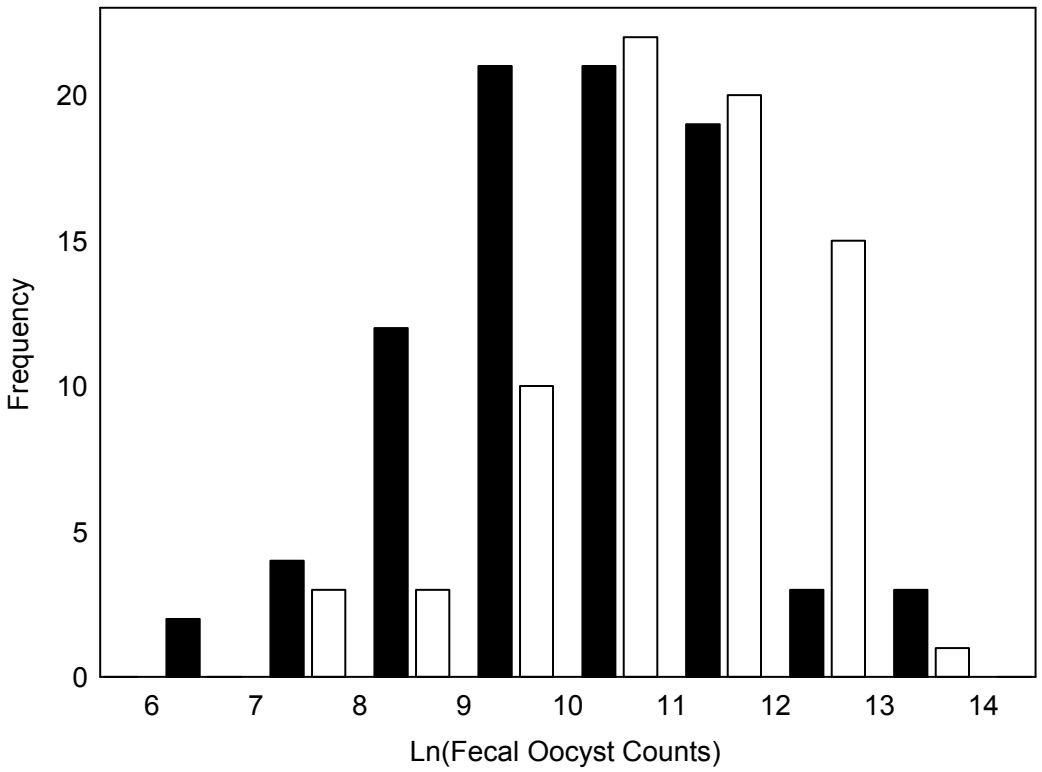

Figure 4-1. Histogram showing female bias of fecal *Eimeria* fecal oocyst counts in red squirrels. Males are black bars, females denoted by white bars. Oocyst counts are ln-transformed.

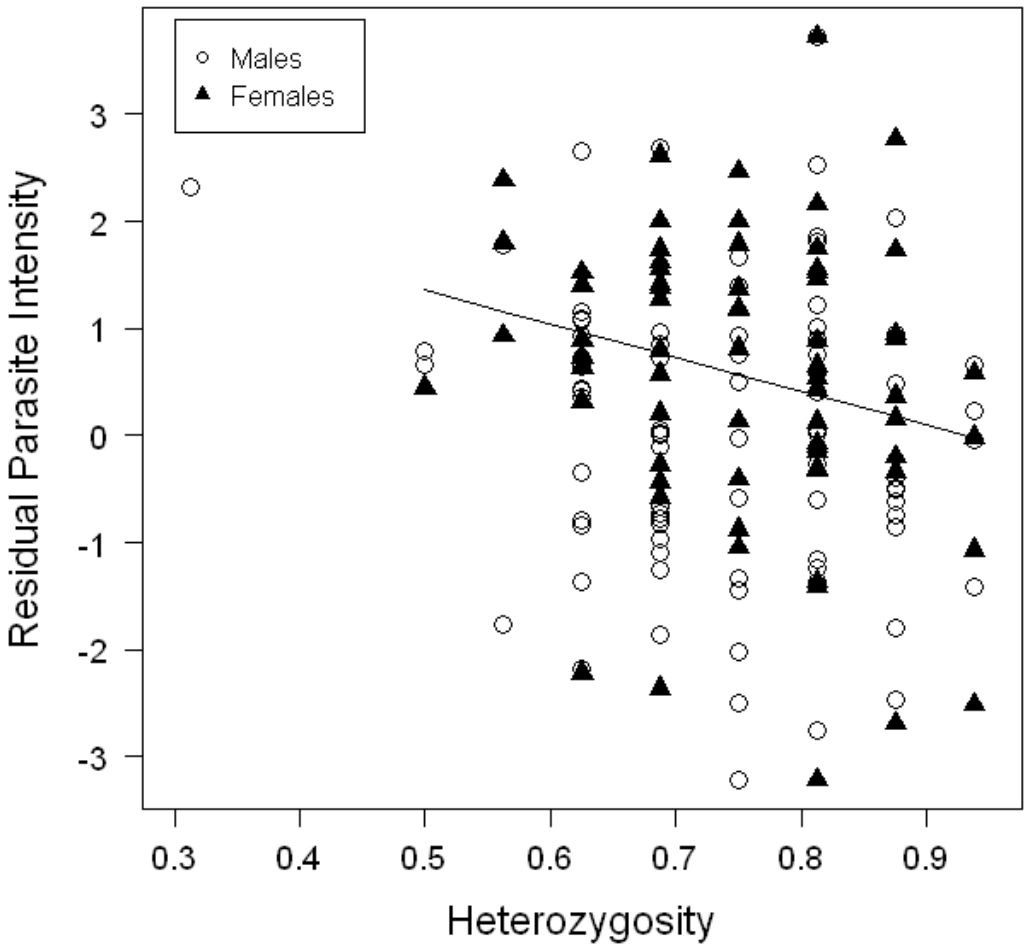

Figure 4-2. Heterozygosity was negatively correlated with fecal *Eimeria* parasite intensity in 74 female (▲, solid line) but not 85 male (○) red squirrels after accounting for age, mass and Julian date of fecal collection. Heterozygosity was calculated from 16 neutral microsatellites.

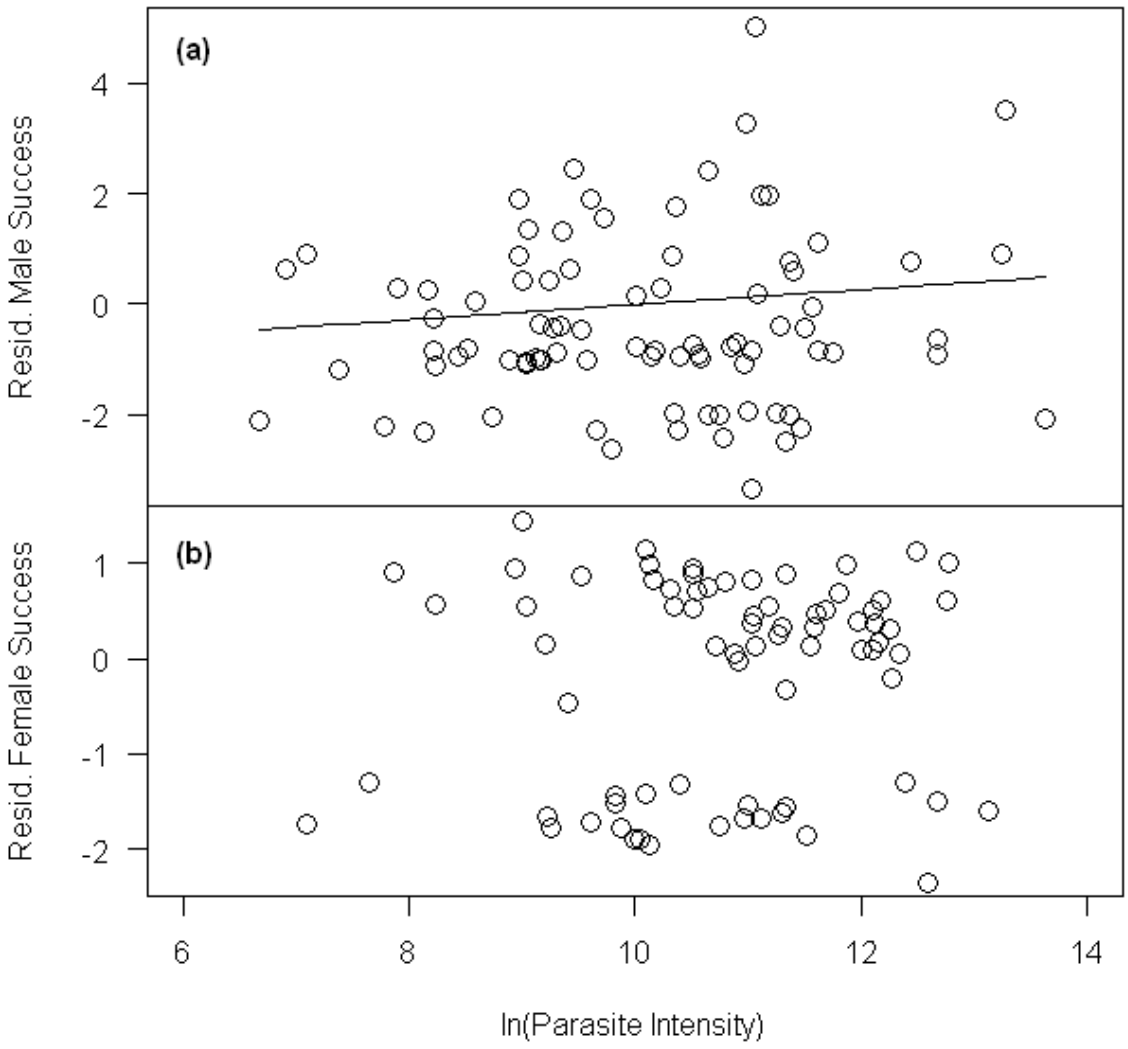

Figure 4-3. Residual reproductive success of male and female red squirrels as a function of their fecal *Eimeria* parasite intensity. (a) Parasite load positively correlated with the number of offspring sired amongst 85 males while (b) parasite load did not correlate with litter size amongst 54 female red squirrels. Residual reproductive success was determined through separate GLMs including age, mass and heterozygosity.

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Chapter 5

Adopting kin enhances inclusive fitness in asocial red squirrels

A version of this chapter has been published.

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5.1 Introduction

When J.B.S. Haldane was asked if he would lay down his life to save his brother, he famously replied "No, but I would to save two brothers or eight cousins" (Lewis 1974). More formally, Hamilton's rule states that altruism should evolve when the fitness cost to the actor (*c*) is less than the fitness benefit to the recipient (*b*) multiplied by their coefficient of relatedness (*r*) (Hamilton 1964). As a result, providing costly assistance to another individual is likely only when the two individuals are closely related.

Adoption (the act of solely and completely caring for another female's offspring as your own until weaning) is often cited as an example of altruism and has been reported in at least 62 mammal species. Most of these cases are among cooperatively breeding and/or highly social species where groups consist of kin (Riedman 1982). Although many of these studies put their findings in the context of Hamilton's rule, none have actually been able to calculate the fitness cost to the actor (*c*) in these circumstances (Heinsohn and Legge 1999, Eberle and Kappeler 2006). Fitness costs to the adopting parent are often confounded with potential benefits such as increased survival, rank or reproductive success through effects on group size and social relationships brought about by adopting any individual, even unrelated orphans (Silk *et al*. 2009*,* Boesch *et al*. 2010). In addition, given that most social groups normally consist of closely related individuals, there is often no opportunity for non-kin to be adopted (Eberle and Kappeler 2006). These confounding factors would not be present in asocial species but the three reported

cases of adoption by asocial species have all been attributed to misdirected care rather than kin selection (Lunn *et al*. 2000).

Here, we report instances of adoption in an asocial mammal for which we can directly quantify all components of Hamilton's rule. We found that orphans were always adopted by closely related kin despite the odds favouring adoption by unrelated females. Adopting females increased their inclusive fitness because the benefits gained by adopting one closely related orphan outweighed the survival costs to the litter incurred by adding an additional individual. We also observed instances where orphaned young were not adopted but the potential surrogate mothers in these cases were not closely related enough to the orphans for the indirect fitness benefits to outweigh the costs of adoption. Together, these findings provide strong empirical support for Hamilton's rule.

5.2 Methods

5.2.1 Field methods

Since 1989, we have studied red squirrels (*Tamiasciurus hudsonicus*) in Yukon, Canada using complete enumeration to track the population size, survival and reproductive output of all females (Boutin *et al*. 2006, McAdam *et al.* 2007). We repeatedly live-trapped squirrels using Tomahawk traps and followed the pregnancy progression of all females through palpation and weight gain. Lactation or a sudden weight decrease indicated parturition and we used behavioural observations and/or radio-telemetry to locate nests. We temporarily removed juveniles from their nests immediately after parturition to count, ear-notch, weigh,

and sex each individual in the litter. We re-entered each nest at 25 days post-partum when juveniles were weighed again and given ear-tags. Juveniles were then followed through observation and live-trapping for as long as they were alive and remained in our study area (Boutin *et al*. 2006, McAdam *et al.* 2007). Using these natal relationships, we constructed a complete maternal pedigree spanning multiple generations. Across 19 years (1989-2008), we monitored the survival and reproductive success of 6,793 juveniles in 2,230 litters produced by 1,101 free-ranging females.

5.2.2 Cost/benefit analyses

We constructed a general linear mixed-effects model for female annual reproductive success (ARS) with litter size, litter size^2, parturition date and juvenile growth rate as fixed effects. The quadratic term for litter size was added to investigate whether reproductive success was maximized at some intermediate litter size as a result of offspring size-number trade-offs (see below for results). ARS was measured as the number of juveniles born in the current year that survived to the following spring (individuals are capable of breeding in the spring following their year of birth) using 753 observations on 502 females in 19 years. We only included control females that we knew had a single litter that year and for which we measured their date of parturition, litter size and juvenile growth rate. Previous analyses found that these variables significantly influenced female annual reproductive success (e.g., McAdam and Boutin 2003, Réale *et al.* 2003). Parturition date and juvenile growth rate were standardized within grid-year

combinations and the intercept of the model was forced through zero to correspond to the biologically relevant model in which females who did not produce any juveniles had zero ARS. Grid-year combinations were also fitted as a random effect to account for non-independence of ARS within grid-years.

Since our goal was to estimate parameters for the influence of litter size on ARS, and not to make inferences regarding their significance, we fit a general linear mixed-effects model (Gaussian error distribution) using the lmer function (Bates and Maechler 2009) in R (R Development 2009), rather than a generalized linear mixed-effects model (Poisson error distribution), because of the complications of back-transforming predicted values based on parameters estimated on a transformed (i.e. link) scale. In this model litter size had a positive effect on ARS (parameter ± one s.e.m.; b = 0.39 ± 0.06), but the quadratic term indicated that the rate of increase in ARS with litter size declined as litter size increased (b = - 0.04 ± 0.01). This relationship can also be visualized as a decline in the probability of each individual offspring surviving until spring as litter size increased (Fig. 5-1). These analyses of long-term observational data are supported by previous experimental litter augmentations, which reduced juvenile survival but did not decrease maternal survival or future reproductive success (Humphries and Boutin 2000).

We calculated the predicted survival probability (P_x) for individual offspring within a litter of size x as the predicted ARS for a litter of size x divided by litter size. We defined the fitness cost to the adopting female to be the difference between the probability of each juvenile surviving to spring based on

her natal litter size (P_x) and the probability of survival based on her new litter size (P_{x+1}) multiplied by the number of juveniles in her natal litter (x) and her degree of relatedness to her offspring (0.5). The fitness benefit to the juvenile of being adopted was the probability that the adopted juvenile would survive to the following spring (P_{x+1}), where x represents the natal litter size of the surrogate female. We then calculated the minimum relatedness between the adopting female and the adopted juvenile that would be required for the benefits (discounted by r) to exceed the costs of adopting according to Hamilton's rule (Fig. 5-1) (Hamilton 1964). Here we have calculated the costs and benefits of adoption based on ARS and have not considered costs of adoption reflected in the survival of the adopting female or her future reproductive success. Previous analyses have indicated that future breeding success is unaffected by previous breeding history and that survival costs of reproducing compared to not-reproducing are restricted only to yearling females and females six years of age or older (Descamps *et al*. 2009). Furthermore, these survival costs of reproducing are independent of how many juveniles are reared at the time of weaning (Descamps *et al*. 2009). Surrogate females only adopted a single juvenile even though multiple juveniles were likely available to be adopted at the time they were orphaned (with the exception of case A4). The costs of adopting two juveniles were large enough to decrease inclusive fitness in five of the seven potential adoption cases (Table 5-1).

5.3 Results

5.3.1 The asocial nature of red squirrels

Individual red squirrels (*Tamiasciurus hudsonicus*) defend food-based territories year round using territorial vocalizations (Price *et al*. 1990, Humphries and Boutin 2000). Removal experiments of territory owners show that squirrels quickly recognize the absence of a neighbour (Boutin and Schweiger 1988), yet behavioural observations clearly demonstrate the lack of physical interactions between adults. From 1989 to 2008, we have recorded 54,785 red squirrel behaviours and only 307 (0.6%) were classified as physical interactions between adults (excluding male-female matings, dam-offspring and sibling interactions prior to dispersal from the natal territory). All of these interactions were aggressive territorial chases against intruders.

5.3.2 Cases of adoption

During detailed study of red squirrels at a single site over the past 19 years (2,230 litters), we have detected five instances of adoption (a lactating dam nursing another dam's juvenile until weaning) for which we knew the ancestry of the dam and the adopted juvenile. These adoptions occurred in different years with different females but always involved the adoption of kin (Table 5-1). Four adoptions occurred when the juveniles were between 43 and 63 days old. The individuals (identified by unique ear-tags) were seen nursing from and/or nesting with the surrogate dam and her offspring. Nest emergence occurs between 42-65 days of age while weaning occurs at 70 days of age (Stuart-Smith and Boutin

1995, McAdam *et al*. 2007). The fifth adoption was detected when we entered a nest to find one juvenile that was unusually large relative to its littermates (20.6g vs. 13.7g ± 0.7 s.e.m.). The mass, sex and presence of an ear-notch (juveniles are marked with ear-notches just after birth) suggested that the large juvenile originated from a neighbouring litter that had been born and sampled six days earlier. This was confirmed using tissue samples collected from both litters which included identical genotypes across 16 microsatellite loci (Gunn *et al*. 2005) between this larger juvenile and one of the offspring from the previously sampled litter. This juvenile also mismatched the surrogate dam at three loci but at none with the original dam. To confirm identical genotypes were not from different individuals, we used the software package Cervus 3.0 (Kalinowski 2007) to determine the probability of identical genotypes using allele frequencies estimated from 100 adults from the same grid and year. The probability of another individual matching this genotype was small (1.17×10^{-19}), whereas the probability that a full sibling would have this identical genotype remained unlikely (4.90×10^{-7}). Both of these probabilities were smaller than the average probabilities of identity across all genotypes in the population (1.93×10^{-17}; 5.20×10^{-7} respectively). Since a six day-old juvenile would have to be carried to a new nest, we parsimoniously assumed that the surrogate female initiated the adoption rather than the orphaned juvenile.

5.3.3 Relatedness of adopted juveniles and surrogates

Since we knew the maternal ancestry of all squirrels born within our study area, we were able to estimate the minimum relatedness between the surrogate dam and the adopted juvenile using the maternal pedigree in all cases (Table 5-1). We consider this a minimum degree of relatedness because our maternal pedigree could not identify individuals related through paternal lineages. We define non-kin to be individuals that were related less than 0.125 through our maternal pedigree. Four cases of adoption occurred in years before we began collecting tissue samples for paternity analyses, while the fifth case occurred as we began sampling both adults and juveniles. In this latter case, the maternal pedigree confirmed the adopted juvenile and surrogate dam were non-uterine siblings ($r \geq 0.25$). Still, we used the software package SPAGeDi 1.3 (Hardy and Vekemans 2002) to calculate their coefficient of relatedness based on genetic similarity at 16 microsatellite loci (Gunn *et al*. 2005, Lane *et al*. 2007). Here we present the Queller and Goodnight (Queller and Goodnight 1989) estimator, which was calculated using allele frequencies estimated from 100 adults from the same grid and year, although other relatedness estimators produced comparable results. The original dam and adopted juvenile were highly related as expected ($r = 0.560 \pm 0.099$; standard error derived from jackknife resampling over loci) while the surrogate dam and adopted juvenile were also highly related ($r = 0.368 \pm 0.158$). One sample t-tests revealed that both of these estimates were significantly greater than zero ($t = 5.65$, $df = 15$, $P < 0.00003$; $t = 2.33$, $df = 15$, $P < 0.02$ respectively).

5.3.4 Who adopts?

In all adoption cases the original and surrogate dams occupied territories ≤ 75 m apart (measured from centre of territories) and gave birth within 19 days of each other. We used these two search criteria to identify potential surrogates and cases where adoption could occur through the death of one of the lactating females. In the five cases of adoption, the number of potential surrogates surrounding each adopted litter ranged from two to four females, with only one female being kin ($r \geq 0.125$) in each case (Table 5-1). We calculated the probability of the litter being adopted by kin based on the proportion of potential surrogates made up by kin. The probability of all five adoptions being by kin was only 1.0%. We identified 29 additional litters where a dam died and a surrogate was available, but no adoption occurred. In only two of these 29 cases were kin females available to adopt orphaned litters, indicating a strong relationship between adoption and kinship (Exact logistic regression odds ratio = 56.3, 95% CI 6.2 to unknown, $P < 0.0002$).

5.3.5 Testing Hamilton's rule (rb > c)

Adopting additional juveniles is expected to decrease the surrogate dam's direct fitness as juvenile survival declines with increasing litter size (Fig. 5-1) (Humphries and Boutin 2000). We determined the relationship between litter size and annual reproductive success using 19 years of data for our study population. The fitness cost to the surrogate dam of adoption (c) was represented by the reduction in survival of her natal litter as a result of litter size being increased by

one juvenile. The benefit to the adopted juvenile (*b*) was its probability of survival given the size of its new litter. We then determined the minimum relatedness (*r*) necessary between the surrogate dam and adopted juvenile for the benefits of adoption to outweigh the costs according to Hamilton's rule (Fig. 5-1). In all five cases of adoption, surrogate dams were sufficiently related to their adopted juveniles to enhance inclusive fitness, whereas in the two cases when a kin neighbour was present and lactating but did not adopt, adoption would have reduced the female's inclusive fitness (Table 5-1). Although we were unable to confirm the availability of multiple offspring at the time of adoption, there were additional littermates present on the last occasion that we entered the nest. Females never adopted more than one orphan where by doing so would have made the costs of adoption out-weigh the indirect benefits in most cases (Table 5-1).

5.4 Discussion

Contrary to other documented cases of adoption by asocial species, adoptions by red squirrels in our study did not represent misdirected parental care but rather a form of altruism that follows Hamilton's rule of kin selection. Studying an asocial species gave us a unique opportunity to calculate the fitness components of Hamilton's rule. We found that females did suffer fitness costs of adoption through reduced survival of their own juveniles, but this cost was offset by the inclusive fitness gained when the adopted juvenile was closely related. Further, although females had the opportunity to contravene Hamilton's rule by

adopting unrelated juveniles or more than one related juvenile, they never did so. These results provide a particularly clear test of Hamilton's rule.

While the opportunity to increase inclusive fitness through adoption arises rarely (only 5 cases of adoption out of 2,230 litters) the clear bias toward adopting kin suggests that kin selection has been strong enough to favour the persistence of this unique behaviour. The mechanism by which female red squirrels assess their relatedness to juveniles or their dams is unknown and is a current focus of research at our study site. While not specifically designed to study kin discrimination, previous cross-fostering experiments found that genetic and foster offspring grew at the same rate (Humphries and Boutin 2000, McAdam and Boutin 2003), suggesting that female red squirrels do not differentially invest in genetic offspring and unrelated foster offspring present in their nest. This leads us to hypothesize that females assess relatedness of orphaned juveniles indirectly through recognition of their dam prior to her death, but this awaits experimental testing.

Table 5-1. Probability and inclusive fitness of adopting kin. Cases A1-A5 were adopted while cases NA1 and NA2 were not adopted even though there were lactating kin ($r \geq 0.125$) nearby. The number of lactating females nearby was estimated relative to the orphaned juvenile and not the surrogate dam. The probability of adoption by kin is the proportion of total females made up by kin. Relatedness was assessed between the surrogate dam and the adopted juvenile by assuming Mendelian inheritance through a maternal pedigree (twice the coefficient of coancestry) and by pairwise estimates using microsatellite allele frequencies ("-" indicates no data). Surrogate females A1 and A3 had recently lost their own litters but were still lactating at the time of adoption so their litter size was scored as zero. Inclusive fitness was calculated based on the minimum relatedness estimated from the maternal pedigree or from genetic similarity when available.

Case	Surrogate's original litter size	No. of lactating kin nearby	Total no. of lactating females nearby	Probability of adoption by kin	Relatedness from maternal pedigree	Relatedness from genetic similarity	Inclusive fitness of adopting one juvenile (*rb-c*)	Inclusive fitness of adopting two juveniles (*rb-c*)
A1	0	1	2	0.50	≥0.125	-	0.0441	0.1120
A2	2	1	4	0.25	≥0.125	-	0.0002	- 0.0003
A3	0	1	2	0.50	≥0.250	-	0.0882	0.1120
A4	2	1	3	0.33	≥0.125	-	0.0002	- 0.0003
A5	4	1	2	0.50	≥0.250	0.368	0.0079	- 0.1028
NA1	3	1	2	0.50	≥0.125	-	- 0.0217	- 0.0528
NA2	3	1	2	0.50	≥0.125	-	- 0.0217	- 0.0528

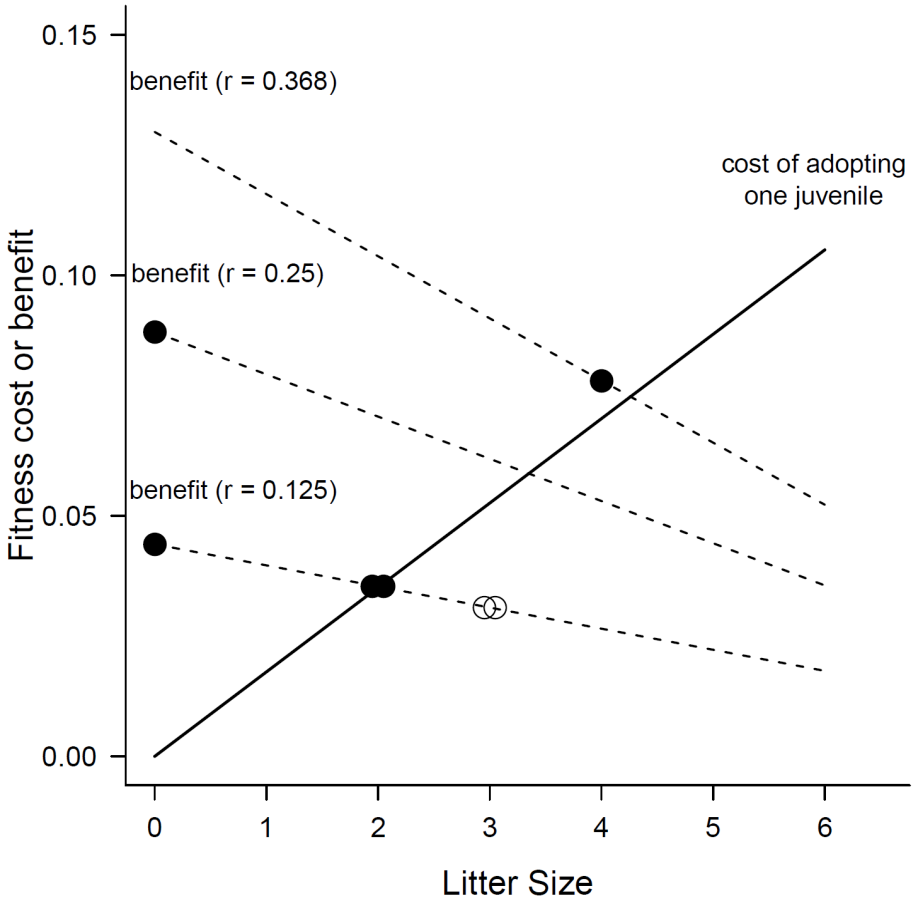

Figure 5-1. Adoptions increase inclusive fitness consistent with Hamilton's rule. Predicted fitness cost (solid line) of adoption to the surrogate dam increases while fitness benefit (dash line) to the adopted juvenile decreases with litter size. Dashed lines represent three different degrees of relatedness calculated from a maternal pedigree or microsatellite allele frequencies and multiplied by the benefit to the juvenile (*rb*). Adoptions (closed circles) increased inclusive fitness while unadopted litters (open circles) would have reduced inclusive fitness if adopted.

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Chapter 6

General conclusion

6.1 Conclusion

My doctoral thesis was centered on the importance of genetic diversity and its role in selection. I developed novel genetic resources and optimized others to improve the suitability of the red squirrel to become a model system. I expanded the current knowledge on the natural history of my study species while also using them as a model system to test evolutionary theory. Throughout my time, I also managed the Kluane red squirrel pedigree which resulted in several successful publications and even more studies not yet published by the time this thesis reached completion.

In **chapter 2**, I developed the first molecular sex-typing system for squirrels by determining novel genetic sequence data from the *Smcy* gene on the Y chromosome. I designed new genera-specific primers that when multiplexed with microsatellite primers and separated using gel electrophoresis provided a distinct banding pattern with two bands for males and one band for females. In spite of the simple application of this sequence data towards laboratory diagnostics, characterizing nuclear diversity right across a 30 million year-old family group is rare (Mercer and Roth 2003). Future studies should use this sequence data to investigate how the evolution of the Y chromosome might differ from that of the mitochondrial genome given such a long time period. The importance of this work has already been validated through peer-reviewed citation as well as personal communication with two different research groups in the United Kingdom that are developing genetic resources to help stop the invading

Eastern grey squirrel (*Sciurus carolinensis*) and save the Eurasian red squirrel (*Sciurus vulgaris*).

In **chapter 3**, I described the broad scale patterns of genetic diversity across the North-South distribution of red squirrels. The decline in genetic diversity with increasing latitude coupled with strong isolation by distance and lack of private alleles in northern populations suggest that red squirrels did not survive in the Canadian North during the Pleistocene and likely recolonized from the southern United States. The intention of this study was to compare the decline in neutral genetic diversity to the pattern of *MHC* diversity with latitude. Had I been able to show that *MHC* was not correlated with latitude, despite genome-wide decline in diversity, then I would have concluded that *MHC* is under balancing selection. However, without *MHC* data I was still able to test the assumptions of the central-marginal hypothesis, though I found mixed support. This study would be improved by adding sequence data from a hypervariable mitochondrial region, such as D-loop, to strengthen the investigations into the phylogeographic history.

In **chapter 4**, I investigated the mechanisms that relate to the contemporary maintenance or function of genetic diversity. This study is one of only a few that directly tests the Red Queen hypothesis by quantifying parasite intensity, genetic diversity and reproductive success simultaneously. I found support for the red queen hypothesis with parasite intensity decreasing as host genetic diversity increased in females and therefore conclude that parasites could be a significant selective pressure on the evolution and maintenance of sexual

reproduction. Still, the direct influences of parasites on reproductive success were different from my original expectations in that males with more parasites also sired more offspring while females had no correlation between parasites and reproduction. These findings suggest that either the priorities of the immunocompetence handicap hypothesis can be reversed (such that reproduction takes priority over immune function rather than vice versa) or that coccidian parasites do not impinge on their hosts' ability to successfully reproduce. Since I only examined the correlation between parasites and genetic diversity in adults, future studies should explore these findings further by investigating if and when parasites begin to portray a cost to juveniles during their growth and development. Having *MHC* diversity to compare with parasite load would have been ideal, as this region is often associated with differential parasite intensities (e.g. Westerdahl *et al*. 2012).

In **chapter 5**, I examined the consequences of genetic diversity and relatedness, demonstrating how kin selection in the past could have lead to the evolution of altruistic behaviour. Surrogate females always gained a fitness advantage from adopting related orphans by satisfying the $c<rb$ conditions for altruism, while simultaneously avoiding fitness traps where adopting would have reduced their fitness. This study was likely the first to show empirical support of Hamilton's rule of inclusive fitness in an asocial wild vertebrate due to the traditional challenges of quantifying the benefits and costs associated with behaviour.

The field of population genetics has changed remarkably in the time since I began this thesis. Though microsatellites are still relevant today, many new projects are now using next-generation sequencing to discover single nucleotide polymorphisms within functional genes (Stapley *et al*. 2010). Undoubtedly the red squirrel *MHC* will be sequenced with more success at some point with new advancing technologies. However, it will remain to be seen if these questions still remain to be answered and what role the red squirrel will be able to play in the future. Despite these set backs, my thesis has still been able to advance the frontiers of knowledge with substantiating evolutionary theory as well as developing novel genetic resources. The Kluane red squirrel project has become one of the most unique and valuable long term projects in the world and will hopefully continue to serve as an arena to train young scientists and scintillate older ones. Where the project goes now and what questions will be answered next will depend on the curious minds of those who find passion in studying little furry creatures with big attitudes.

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APPENDIX 1

Allele frequencies across loci and populations from chapter 3.

Locus	Populations.....							
03a	HN	KN	MO	ND	PR	RR	SC	KL
219	2.50	10.71		20.00	8.33			
221		21.43		5.00				
223	15.00	14.29	11.11	5.00	16.67	15.00	20.00	2.50
225	2.50	7.14						5.00
227	32.50	25.00	19.44	45.00	33.33	15.00	10.00	15.00
229		14.29		15.00			10.00	12.50
231	27.50	7.14	30.56	5.00	20.83	55.00	30.00	40.00
233	12.50		38.89		16.67	15.00	30.00	15.00
235	7.50			5.00	4.17			10.00
08a	HN	KN	MO	ND	PR	RR	SC	KL
180	5.00			5.00	12.50			
188				5.00				
190			5.56					
194	15.00	10.71	38.89	20.00	16.67		30.00	10.00
196	12.50		2.78		8.33		15.00	5.00
198	2.50	10.71	2.78					15.00
200	27.50	14.29	13.89	30.00	25.00	45.00	30.00	25.00
202	17.50	14.29	19.44		12.50			5.00
204	2.50	25.00				5.00		2.50
206	17.50	17.86	8.33	35.00	25.00	50.00	20.00	30.00
208		3.57	8.33	5.00			5.00	7.50
210		3.57						
14a	HN	KN	MO	ND	PR	RR	SC	KL
266		7.14		5.00			5.00	
268	2.50		5.56			15.00		7.50
270	7.50	7.14	11.11	5.00	8.33			
272	12.50	3.57						7.50
274	2.50	32.14		20.00				7.50
276			11.11	10.00		10.00		15.00
278	12.50	7.14	13.89		41.67	45.00	40.00	7.50
280	27.50	3.57	16.67	5.00	29.17	15.00	15.00	30.00
282	27.50	25.00	30.56	45.00	12.50	10.00	20.00	17.50
284	7.50	10.71	5.56	10.00	8.33		10.00	7.50
286			5.56			5.00	10.00	
288		3.57						

23a		HN	KN	MO	ND	PR	RR	SC	KL
	180	20.00		11.11	15.00				2.50
	182		32.14	5.56	25.00	16.67	5.00	10.00	
	184	12.50	14.29	13.89	5.00			10.00	2.50
	186	7.50	14.29	2.78		4.17		5.00	5.00
	188	10.00	3.57	11.11	5.00	20.83			7.50
	190		3.57	5.56		4.17		5.00	
	192	2.50			5.00				
	193	2.50	3.57						2.50
	195	7.50	10.71	5.56	15.00	20.83	10.00	20.00	5.00
	196	2.50	3.57						
	197	12.50	3.57	13.89	10.00	12.50	10.00	5.00	12.50
	199	17.50	7.14	30.56	15.00	16.67	45.00	45.00	57.50
	201	5.00	3.57		5.00	4.17	30.00		2.50
	203								2.50

25a		HN	KN	MO	ND	PR	RR	SC	KL
	176	2.50	32.14		5.00				
	184	62.50	17.86	19.44	30.00	29.17	25.00	5.00	10.00
	186	7.50	10.71	25.00	15.00	25.00	30.00	25.00	10.00
	188	22.50	32.14	19.44	20.00	37.50		10.00	30.00
	190	5.00	7.14	36.11	30.00	8.33	35.00	40.00	37.50
	192						10.00	20.00	12.50

31a		HN	KN	MO	ND	PR	RR	SC	KL
	127				5.00				
	129	2.50							
	131	5.00	17.86		5.00			20.00	2.50
	133	2.50	7.14		15.00				
	135	35.00	28.57	11.11	25.00	41.67	45.00		5.00
	137	15.00	21.43	33.33	30.00	45.83	15.00	45.00	50.00
	139	15.00	14.29	13.89	10.00	4.17	15.00	20.00	15.00
	141	22.50	3.57	36.11	5.00	8.33	25.00		20.00
	143	2.50	7.14	2.78				15.00	2.50
	145			2.78	5.00				5.00

32a		HN	KN	MO	ND	PR	RR	SC	KL
	271	5.00	10.71	2.78		12.50			2.50
	273				10.00				
	275	2.50		2.78					
	277				5.00	8.33	20.00		2.50
	279	5.00	10.71	11.11	15.00		5.00		20.00
	281	32.50	32.14	13.89	35.00	33.33	30.00	50.00	15.00
	283	12.50	10.71	25.00	20.00	33.33	25.00	15.00	25.00
	285	20.00	28.57	19.44	10.00	12.50	15.00	15.00	20.00
	287	7.50	3.57	16.67			5.00	20.00	15.00
	289	10.00	3.57	8.33	5.00				
	291	2.50							
	293	2.50							

33a		HN	KN	MO	ND	PR	RR	SC	KL
	143				5.00				2.50
	145		3.57		10.00			5.00	
	147	20.00	3.57	16.67		4.17			
	149	35.00	35.71	38.89	20.00	25.00		30.00	32.50
	151	12.50	21.43	5.56	5.00	8.33	35.00	5.00	17.50
	153	2.50	14.29	5.56	15.00	4.17	10.00	15.00	7.50
	155	20.00	3.57	25.00	20.00	20.83	15.00	30.00	32.50
	157	5.00	10.71	8.33	15.00	25.00	25.00	15.00	5.00
	159	5.00	3.57			12.50	10.00		2.50
	161				10.00		5.00		
	163		3.57						

37a		HN	KN	MO	ND	PR	RR	SC	KL
	159	52.50	39.29	91.67	85.00	70.83	80.00	55.00	72.50
	168	7.50	17.86			4.17			
	170		3.57						
	172	2.50	28.57	8.33			10.00	20.00	17.50
	174	10.00	7.14			4.17		10.00	
	182	27.50	3.57		15.00	20.83	10.00	15.00	10.00

38a		HN	KN	MO	ND	PR	RR	SC	KL
	294	10.00	39.29					5.00	
	296	7.50	3.57		15.00	12.50	50.00	30.00	7.50
	298		3.57		10.00				5.00
	300	50.00	25.00	75.00	50.00	50.00	30.00	55.00	65.00
	302	7.50	7.14	25.00	10.00	16.67	10.00	10.00	17.50
	304	22.50	21.43		15.00	20.83	10.00		
	306								5.00
	308	2.50							

40a		HN	KN	MO	ND	PR	RR	SC	KL
	374		3.57						
	376		3.57		10.00				
	378		3.57						
	380	12.50		11.11	10.00	12.50	10.00	5.00	10.00
	382	17.50	14.29	41.67	25.00	25.00	25.00	45.00	17.50
	384	2.50	17.86	8.33	10.00	8.33	5.00		25.00
	386	20.00	14.29	5.56		33.33	5.00		7.50
	388			2.78	5.00		10.00		7.50
	390	2.50		11.11		16.67	15.00	10.00	7.50
	392	2.50	7.14	2.78	5.00		15.00	30.00	2.50
	394	10.00		2.78	20.00		10.00	10.00	10.00
	396	12.50	10.71	8.33	15.00				
	398	7.50	21.43	5.56		4.17			7.50
	400	5.00					5.00		2.50
	402	2.50							2.50
	404	2.50							
	406		3.57						
	408	2.50							

41a		HN	KN	MO	ND	PR	RR	SC	KL
	238	2.50	3.57	2.78	25.00			20.00	7.50
	255	45.00	35.71	5.56	25.00	41.67	30.00	10.00	15.00
	257	25.00	14.29	19.44	20.00	25.00	10.00	5.00	12.50
	259	5.00		2.78	5.00		15.00		
	261	10.00	7.14	30.56	20.00	4.17	15.00		10.00
	263	5.00	3.57	27.78		16.67	30.00	45.00	40.00
	265	2.50	3.57	11.11	5.00	8.33		20.00	15.00
	267	2.50							
	269	2.50	3.57						
	271		17.86						
	273		7.14			4.17			
	275		3.57						

42a		HN	KN	MO	ND	PR	RR	SC	KL
	232	12.50	17.86	13.89	10.00	4.17	25.00	10.00	21.05
	234		3.57						
	236								5.26
	244					4.17			
	246	20.00	28.57	16.67	5.00	16.67		5.00	2.63
	248	7.50		5.56		16.67	15.00		10.53
	250	52.50	28.57	38.89	65.00	20.83	5.00	30.00	47.37
	252	5.00	7.14	19.44		16.67		30.00	5.26
	254	2.50	14.29	2.78	10.00		25.00	25.00	5.26
	256			2.78	10.00	20.83	25.00		
	258						5.00		2.63

49a		HN	KN	MO	ND	PR	RR	SC	KL
	188	10.00	7.14	5.56	35.00	8.33	5.00		5.00
	190		3.57		15.00	12.50	5.00		2.50
	192	15.00	17.86		5.00	4.17	5.00	10.00	5.00
	194	32.50	32.14	69.44	30.00	20.83	70.00	70.00	22.50
	196		3.57	2.78	5.00		5.00		5.00
	198	2.50		2.78				10.00	10.00
	200	22.50	10.71	13.89	10.00	37.50		10.00	45.00
	202	12.50	21.43	5.56		4.17			2.50
	204	5.00				8.33	10.00		2.50
	206		3.57						
	207					4.17			

50a		HN	KN	MO	ND	PR	RR	SC	KL
	270	2.50	3.57						
	273	10.00	53.57	25.00	5.00	12.50	15.00	15.00	20.00
	274		3.57		15.00				
	275	2.50							2.50
	279	10.00	10.71	8.33	20.00	16.67		10.00	5.00
	281			5.56		4.17			2.50
	283	17.50	10.71	38.89	25.00	4.17	65.00	50.00	60.00
	285	30.00		16.67	20.00	54.17	20.00	15.00	2.50
	287	2.50	14.29		10.00	8.33		10.00	2.50
	289	25.00	3.57	5.56					2.50
	293				5.00				2.50

55a		HN	KN	MO	ND	PR	RR	SC	KL
	260							5.00	
	261					4.17			
	262	30.00	14.29	19.44	20.00	25.00	20.00	45.00	47.50
	264	22.50	3.57	16.67	30.00	16.67	10.00		7.50
	266	2.50		5.56	5.00				2.50
	267			11.11		4.17		10.00	
	268			8.33	5.00	12.50			
	269	2.50	10.71	5.56					5.00
	270	7.50	10.71	2.78	5.00	12.50	10.00		5.00
	271		17.86		5.00				
	272	32.50	17.86	8.33	20.00	12.50	50.00	20.00	22.50
	274			11.11	10.00			5.00	5.00
	276		17.86	8.33		4.17	10.00	15.00	2.50
	278		3.57	2.78		8.33			
	280	2.50	3.57						2.50

APPENDIX 2

PCR conditions for microsatellite multiplexing.

- Preparation of PCR buffer

250 mL of 1M KCl
25 mL of 2M TRIS pH 8.8
5 mL TRITON X-100
8 mL 100mg/mL BSA
Add water to 500mL

$MgCl_2$ stock solution is 25mM
dNTP stock solution is 2mM
Taq stock solution is 3U/uL
Primer stock solutions are 100uM

- Making primer mixes

Add the following volumes of primer stocks (forward & reverse) in one tube so that final concentration of each primer is 2uM.

Primer mix 1
20uL of Thu03, Thu25, Thu33, Thu42, Thu49, Thu 50, Thu55 = 280 uL
10uL of Thu31 = 20 uL
Add 700uL of water to fill to 1000uL final volume.

Primer mix 2
20uL of Thu08, Thu 23, Thu41 = 120uL
10uL of Thu37 = 20uL
Add 860uL of water to fill to 1000.

Primer mix 3
20uL of Thu14, Thu32, Thu38, Thu40 = 160uL
Add 840uL of water to fill to 1000.

- PCR reaction volumes per tube or plate. Repeat each plate for primer mix 1, 2 and 3.

	1x reaction	115x for one entire plate
Buffer	1	115
$MgCl_2$	0.6	69
dNTP	1	115
Primer mix	1	115
Water	4.3	495
Taq	0.1	12
Template DNA	2	-
Total volume	10uL	

- Multiplex Cycling conditions:

95ºC hot start
95ºC 2 min
94ºC 30 sec
60ºC 90 sec — repeat 33 times
72ºC 60 sec
72ºC 10 min

- Single locus cleanup conditions:
(primer pairs at 10uM, 3uL DNA)

94ºC hot start
94ºC 60 sec
94ºC 30 sec
60ºC 20 sec — repeat 3 times
72ºC 5 sec
94ºC 15 sec
60ºC 20 sec — repeat 30 times
72ºC 1 sec
72ºC 10 min

- Dilution and fragment analysis

1. Add 20uL of water to all wells from primer mix plates 1, 2 and 3
2. Transfer 2uL of primer mix 1 to a dilution plate 1
3. Add 38uL water to dilution plate 1
4. Transfer 2uL of dilute to final plate 1
5. Transfer 2uL of primer mix 2 to dilution plate 2
6. Transfer/co-load 2uL of primer mix 3 to dilution plate 2
7. Add 56uL water to dilution plate 2
8. Transfer 2uL of dilute 2 to final plate 2
9. Add 8uL of HiDI and LIZ600 to both final plates
   1 plate = 850uL HiDi + 27uL LIZ600
10. Program 3730 with instrument protocol "DS33_RCT_50cm_Pop7"

- Dilution of single locus cleanup

Add 20uL to PCR product, transfer 2uL, fill to 60uL with water, transfer 2uL to final plate

Genotypes are used in parentage analysis and pedigree reconstruction using CERVUS 3.0 and COLONY 2.0.

Use CERVUS to determine paternity of known sires after accounting for maternal genotypes.

Running CERVUS

```
1. Run Allele Frequency Analysis
2. Run Simulation of paternity
      Offspring = 10000
      Candidate fathers = Number of males in analysis
      Prop. sampled = 0.9 (90% of males sampled)
      Prop. loci typed = (precalculated in Allele Freq Analysis
      Prop. loci mistyped = 0.01
      Calculate confidence = delta
      Relaxed = 95
      Strict = 99
3. Run Paternity Analysis
      For each offspring include = Two most likely parents

Criteria for accepting paternity: 99% confidence and no more than
1 trio mismatch
```

Use COLONY to determine if pups/litters from unknown sires are singly or multiply sired and to connect paternal sibs from different litters.

Running COLONY

```
Polygamy for both species
Diploid and dioecious
Short run
Full likelihood
Medium precision
Do not update freq.
Without prior
Prob of sire/dam = 0.9
Excluded paternity/maternity/sibships = 0

Start by looking under Best (ML) Cluster to see potential sires
for unknown offspring. Double check this assignment under the
Halfsib dyad and only accept assignments greater than 90%.
```

APPENDIX 3

- Major Histocompatibility Complex in red squirrel

This section is meant to provide a record of lab protocols and efforts towards novel sequencing. I tried sequencing both DRB exon 2 as well as DQA exon 2, both containing the active site of the protein for antigen-binding. My primers for DRB always picked up a pseudogene along with the target gene. The pseudogene had a 20 base deletion right in the middle of the sequence which resulted in a stop codon. Isolating colonies and Sanger sequencing revealed that DRB had approximately 20 SNPs within a 200 base region, while DQA was less variable with only ~7SNPs within the <200 base sequence. Attempts to differentiate the variability using IonTorrent NGS resulted in massive variation (likely error) with some individuals having 200+ unique alleles.

Novel sequencing of DRB exon 2

DRB primers to produce ~200 bp product.
RSL3 designed by Gorrell. SusR2 from Biedrzycka *et al*. 2008.

RSL3
ACCAGATCTCGCTTCTTGGA
SusR2
TCACCTCTCCKCTCCACAGTGAA

	1X
Water	16
Buffer	2.5
dNTP	2.5
$MgCl_2$	1.8
Primer stock at 10uM	1
Taq	0.2
DNA	1
Total Volume	25uL

PCR cycling parameters used a touchdown technique for the annealing temperature from 62ºC to 58ºC. See Gorrell *et al*. 2012 for full details.

Products where then gel-extracted after running in a 2.5% gel for 4 hours to separate the pseudogene from the target product which was then cloned and sequenced.

E.g. sequence
GCTTCAGCTGAGTGCCATTTCTACAACGGGACGCAGCAGGTGCGGTTC
CTGGAGAGACACTTCTACAACAGGGAGGAGTTCCTGCGCTTCGACAGC
GACGTGGGCGTGTACCGCGCGGTGACCGAGCTGGGGCGGCCCGAGGC
CAAGTCCTGGAACAGCCAGGAGGACTTCATGGAGCGGAAGCGGGGCC
AGGTGGACAATTACTGCAGACACAACTACGGGGTT

E.g. pseudogene
TCTACAACGGGACGCAGCGGGTGCGGTACTTGCAGAGATACTTCTACA
ACCGGGAGGAGTTCGTGCGGTTCGACAGCGACGTGGGAGAGTTCCGC
GCGG----------GACGCCAAGTACTTTAACAGCCAGGAGGACTTCA
TGGAGCAGAGACGGGCGGCGGTGGACAACTACT

N.B.
Both primer pairs JS1/JS2 and GH46/GH50 gave clean bands in an agarose gel but were messy when sequenced. Should try isolating colonies amplified with these pairs and then sequencing again.

	1X
Water	15.25
Buffer	2.5
dNTP	2.5
$MgCl_2$	1.8
Primer stock at 10uM	1
Formamide (HiDi)	0.7
Taq	0.2
DNA	1
Total Volume	25uL

PCR cycling parameters used a touchdown technique for the annealing temperature from 62ºC to 58ºC. See Gorrell *et al*. 2012 for full details.

Novel sequencing of DQA exon 2

Original DQA primers produce 700 bp product
DQA1 CCGGATCCCAGTACACCCATGAATTTGATGG
DQA2 CCGGATCCCCAGTGCTCCACCTTGCAGTC

E.g. sequence
GGACCAGTTGTTCTCTGTGGACCTGAAGAACGGGGAGGTTGAGTGGCG
ACTGCCTGAATTTGGTGATTTCGCGCACTTTGACCCACAGAATGGACT
GGCCAGCATCGCCTCGATCAGAGCCCATCTGGATGTCCTGGTGGAACG
CTCCAACCACACCAGAGCCACCAGCGGTACCTGCCCTGCCCCCATCCA
GCGAAGCAGGAGATAGAGACGCTTCTCCCCCTCCCTGGCCTCCCTCAC
TCTGGCACAAGCTCTCACCGGCCCCCTTCTTTCCCTGGAAGCTCTAGGT
CTTCCCAGACCCCTCAGCATCCTCCCCCTCYTCAGCCTCCCTTGTCCCA
GCTTTCCTCCCCCATGGTCCAGGGTCTATCATCCCAGGGCACCCCCTGC
CTGCAGATGTCTAAGTGGGAAGAAGTGCCCCTTAATCCTGGTTCTAAT
AAGGCCCCGCTRCACGGGATGAGCCTTTGGGATTCAGAGCTTCGTTCC
TCTTAGTGTCTCCAAGGGCGACTGTGCTCCCCAAGTCTCGAGTGGAGC
TGGGCGAGCCCAACATCCTCATCTGCATCGTGGACAACATCTTCCCCC
CCGTGATCAACGTCACCTGGCTGCGCAATGGCCGAACCGTCACTGAGG
GAGCAGCTCAGACCAGCTTCTACTCCCAGCCTGACCACTTRTTCCKCA
AGTTCTCTTACCTGACCTTCGTGCCTTCGGCAGATGATGTCTAC

Redesigned reverse primer to give short (193bp) product
DQAr3 GCTCTGGTGTGGTTGGAG

Glenn & trP1 sequences were added to the original primers to allow for IonTorrent sequencing.
RS_GLENN_DQA1
CAGTCGGGCGTCATCACCGGATCCCAGTACACCCATGAATTTGATGG
RS_trP1_DQAr3
CCTCTCTATGGGCAGTCGGTGATGCTCTGGTGTGGTTGGAG

2 step PCR procedure with dilution in between, gel extract using QiaExII, quantify with Qubit, pool individuals at equal concentrations, run mixed sample on e-gel, sequence on IonTorrent.

PCR step 1, annealing temperature of 59ºC

	1X
water	8.75
5x HF buffer	4
dNTP	2
Primer RS_Glenn_DQA1	1
Primer RS_trP1_DQAr3	1
Phusion high fidelity Taq	0.2
DNA	3
Total volume	20uL

3 rounds of 10x serial dilution of product = 1/1000 concentration

PCR step 2, annealing temperature of 59ºC

	1X
water	8.75
5x HF buffer	4
dNTP	2
Primer Barcode	1
Primer trP1	1
Phusion high fidelity Taq	0.2
DNA	3
Total volume	20uL

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