MECHANISMS OF SELECTION AND MHC DIVERSITY IN THE MONTANE VOLE AND OTHER WILD MAMMALS

by

JAMIE CAROLINE WINTERNITZ

(Under the Direction of SONIA ALTIZER)

ABSTRACT

The loss of genetic diversity due to reduced gene flow, inbreeding and genetic drift can result in reduced fitness for individuals and extinction risks for populations and species. One locus for which genetic diversity is vitally important is the Major Histocompatibility Complex (MHC). The MHC is renowned for its unparalleled allelic diversity which facilities recognition of diverse parasites by vertebrate immune systems, and this genetic diversity has been maintained even in the face of repeated population bottlenecks in some species. Two selective processes could maintain this diversity: mate choice and parasitism. This research investigates the relative roles of sexual selection and parasite-mediated selection in maintaining MHC diversity both within populations and across species. At the population level, I employed both field work and molecular genetics using Second Generation (454) sequencing technology to investigate host-parasite interactions and neutral and adaptive genetic diversity in a cyclic montane vole population. For interspecies analysis, I employed comparative phylogenetic tests to ask whether parasites drive MHC diversity across species, and for the first time, determine if sexual selection is broadly important in explaining MHC diversity across a range of vertebrate species,. Knowledge obtained here will inform efforts to conserve genetic diversity in small

wildlife populations and can suggest processes that can promote natural MHC diversity (e.g. mate choice coordinated through captive breeding).

INDEX WORDS: Major Histocompatibility Complex, parasite-mediated selection, sexual selection, host-parasite relationship, population dynamics, balancing selection, population bottlenecks, conservation genetics

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DEDICATION

To all the people who have helped me along the way. To my parents and my sisters for providing limitless moral support.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

"The Nothing is spreading," groaned the first. "It's growing and growing, there's more of it every day, if it's possible to speak of more nothing. All the others fled from Howling Forest in time, but we didn't want to leave our home. The Nothing caught us in our sleep and this is what it did to us…You don't feel a thing. There's just something missing. And once it gets hold of you, something more is missing every day. Soon there won't be anything left of us."

Michael Ende, *The Neverending Story*

The loss of genetic diversity due to reduced gene flow, inbreeding, and genetic drift is a potential problem for every natural population, and can result in fitness consequences for individuals and extinction risks for populations and species (Saccheri *et al.* 1998; Crnokrak & Roff 1999; Frankham *et al.* 2002; Reed & Frankham 2003). Random genetic drift in particular can fix deleterious mutations and remove adaptive rare alleles in small populations, resulting in reduced reproductive potential of populations and increased threat of extinctions (Lande 1994; Whitlock 2000). While genetic drift is a given for natural populations due to finite size, the rate of genetic loss, from a snail's pace to cheetah speed, is determined by the size of the population, and smaller populations are particularly vulnerable to faster loss of genetic diversity. Natural selection can be a force opposing genetic drift and the loss of genetic diversity. Conservation genetics and evolutionary biology is concerned with understanding patterns of variation in genetic diversity and identifying modes of selection opposing drift to maintain variation

(Frankham *et al.* 2002). If selection is strong enough, it can maintain adaptive variation in populations even when neutral genetic diversity is depleted (e.g. Wenink *et al.* 1998; Hedrick *et al.* 2000a; Aguilar *et al.* 2004). For this reason, variation at functional genes may have greater relevance for adaptive potential (Hoffmann & Willi 2008; Gebremedhin *et al.* 2009), and can help buffer populations against biotic and abiotic threats (Altizer *et al.* 2003a). The overarching goal of this thesis is to understand the mode and strength of selection that maintains functional genetic variation in natural populations, focusing primarily on immunogenetic variation and the role of parasites as selective agents.

The major histocompatibility complex (MHC) is the most variable region in the vertebrate genome and is vital for effective immune response (Klein 1986). Class I and II MHC molecules are responsible for presenting peptides to T cells of intracellular and extracellular parasites, respectively (Knapp 2005). Different MHC alleles recognize and bind to specific pathogen proteins, so multiple alleles are required to confer resistance to different pathogen genotypes and species (Klein 1986). Therefore, animals that show higher levels of diversity across MHC loci should recognize and present a greater diversity of pathogen peptides than homozygous individuals. High heterozygosity and hundreds of alleles have been observed in human and primate populations, and there is great allelic variability across species (Hedrick *et al.* 1991; Edwards & Hedrick 1998; Mikko *et al.* 2006). In fact, the MHC is arguably the best studied gene of the vertebrate immune system, and it is associated with both parasite resistance and reproduction across many species, including humans (reviewed by Potts & Wakeland 1990; Hedrick 1994b; Apanius *et al.* 1997; Wedekind & Furi 1997; Ober *et al.* 1998; Bernatchez & Landry 2003). This complex serves as an outstanding model for deepening understanding of the natural processes promoting adaptive variation in wild populations.

Because of its role in immune response, MHC polymorphisms are attributed to antagonistic coevolution with parasites (Potts & Wakeland 1990; Bernatchez & Landry 2003; Spurgin & Richardson 2010). Parasites can have large impacts on individuals, populations, and even ecosystems (reviewed in Hudson 2002b; Ostfeld *et al.* 2008), and can serve as powerful agents of natural selection. Parasite-mediated selection for balanced polymorphisms at the MHC (balancing selection) can be subdivided into three non-exclusive mechanisms: heterozygote advantage (Doherty & Zinkernagel 1975), rare-allele advantage (Takahata & Nei 1990), and temporal and spatial fluctuation in selection pressures driven by variation in parasites (Hill 1991). The relative importance of each mechanism for parasite-mediated selection is still unknown (Spurgin & Richardson 2010), and other forces of selection may also be important for conserving MHC diversity.

Theoretical studies have shown that disassortative mating could also preserve allelic diversity across MHC loci (Hedrick 1992), and mate choice for MHC compatibility and maternal-fetal associations are two proposed mechanisms with empirical support (Wedekind & Furi 1997; Tregenza & Wedell 2000; Radwan *et al.* 2008; Cutrera *et al.* 2012). Both of these processes generate offspring with high MHC diversity, which could result in greater offspring fitness due to genome-wide diversity (heterosis) or to greater parasite resistance. Animals can discriminate between potential mates based on MHC-specific individual odors (Yamazaki *et al.* 1978; Boyse *et al.* 1987; Potts *et al.* 1994; Carroll *et al.* 2002; Penn 2002; Milinski *et al.* 2005), or by selecting mates with other phenotypic signals of MHC diversity (Schantz *et al.* 1996; Ditchkoff *et al.* 2001). However, the evidence supporting sexual selection as a general phenomenon is mixed, and may depend on ecological context and mating system constraints (Paterson & Pemberton 1997; Roberts 2009). For example, mating system is often determined

by the availability of resources (Clutton-Brock & McAuliffe 2009), and direct benefits (e.g. territory, protection) may be more compelling to a female's mate choice than indirect benefits (e.g. good genes).

To comprehensively investigate the opposing forces of genetic drift and selection on adaptive immune genes of the MHC, my dissertation work spans the scale from individuals and populations, to species. The majority of studies described in this thesis (Chapters 1, 2, and 3) focus on a wild rodent species characterized by dramatic fluctuations in abundance that court random genetic drift. The montane vole (*Microtus montanus*) inhabits alpine grassy meadows of North America ranging from Colorado to Utah (Sera & Early 2003). Montane voles undergo high-amplitude and frequent population cycles, peaking in abundance every three to four years (Pinter 1986; R. Smith unpublished data). They also have a diversity of parasites (Winternitz *et al.* 2012) and a promiscuous mating system, thus potentially enabling selection to maintain high MHC diversity through mate choice and parasite interactions.

In Chapter 1, colleagues and I examine the individual and population level predictors of gastro-intestinal parasitism on wild cyclic montane voles to determine if evidence was consistent with theory implicating parasites as selective agents driving population cycles. I sampled three sites in central Colorado for the duration of a multi-annual cycle and recorded the prevalence and intensity of *Eimeria* and cestodes. Results showed significant associations between host infection status, individual traits (sex, age, and reproductive status) and population-level variables (site, trapping period, and population density), including a positive association between host density and cestode prevalence, and a negative association between host density and *Eimeria* prevalence. Both cestode and *Eimeria* intensity correlated positively with host age, reproductive status and population density, but neither parasite was associated with poorer host

condition. Overall this indicated that parasites likely had little effect on the fitness of individual montane voles or their overall population dynamics, although cumulative, sublethal effects could influence lifetime reproduction.

Chapter 2 focuses on characterizing variation at the MHC locus in montane voles. Colleagues and I tested for evidence of historic balancing selection, recombination, and gene duplication to identify mechanisms maintaining allelic diversity. Counter to expectations, we found strong evidence of purifying selection acting on the DRB locus in montane voles. To further explore this idea, I conducted a phylogenetically controlled comparative analysis across 16 rodent species with varying demographic histories and MHC duplication events. I found evidence that the number of duplicated loci was positively related to allelic diversity and strength of purifying selection at the DRB locus. Analyses also revealed that the tendency for cyclic population dynamics was positively correlated with high values of Tajima's D, an indicator of balancing selection. This study highlights the necessity of considering demographic history and genetic structure alongside patterns of natural selection to understand resulting patterns of genetic variation at the MHC.

Though I found some evidence of historical balancing selection at the MHC in montane voles, an outstanding question involves the degree to which balancing selection on the MHC can oppose genetic drift and maintain genetic variation in the face of severe population bottlenecks. Chapter 3 addresses this question by examining genetic diversity, population structure at neutral and MHC genes, and measures of parasitism in a fluctuating population of montane voles. Colleagues and I found high measures of neutral and MHC allelic variability, indicating genetic drift has had little impact on this vole population, despite regular population bottlenecks. MHC diversity did not predict overall parasite richness or the presence/absence of two common

endoparasites. Two specific MHC alleles predicted *Eimeria* intensity and cestode load, and measures of neutral genetic diversity based on microsatellites were positively associated with host body condition, total parasite richness and cestode load. One explanation for greater cestode loads among hosts with greater genome-wide and MHC diversity is that these individuals might better tolerate heavy infections than more inbred hosts. Overall, these results suggest that the parasite examined here do not have a strong role maintaining the high observed MHC variation in cyclic wild voles, and other potential mechanisms of selection such as mate choice should be considered.

Finally, Chapter 4 looks for selective mechanisms maintaining variability in MHC diversity across species in a comparative analysis. Parasites are a major evolutionary force driving MHC polymorphisms across species, but sexual selection is another likely mechanism. In reality, both parasite-mediated selection and sexual selection may act in concert in wild populations, but the majority of empirical studies have considered each separately. Colleagues and I used comparative methods to assess the relative contribution of both mechanisms on MHC diversity across carnivores, chiroptera, primates, rodents, and ungulates. Specifically, we tested whether parasite species richness and relative testes size (as an index of sexual selection) were correlated with two measures of MHC class II DRB diversity: allelic richness and nucleotide diversity. Controlling for phylogeny and confounding ecological variables (i.e. population size, body mass, and sampling effort), we found that parasite species richness was only positively correlated with nucleotide diversity for rodents and ungulates, and negatively correlated for carnivores. In contrast, relative testes size was positively correlated with nucleotide diversity for all taxa. Allelic richness was only significantly associated with taxon, with ungulates having

lower diversity in general. This study provides broad support for both parasite-mediated selection and sexual selection in shaping functional MHC polymorphism across mammals.

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CHAPTER 2

PARASITE INFECTION AND HOST DYNAMICS IN A NATURALLY FLUCTUATING RODENT POPULATION[1]

[1] J.C. Winternitz, M.J. Yabsley and S.M. Altizer (2012). *Canadian Journal of Zoology*, 90:1149-1160. Reprinted here with permission of publisher.

ABSTRACT

Parasites can both influence and be affected by host population dynamics, and a growing number of case studies support a role for parasites in causing or amplifying host population cycles. In this study we examined individual and population level predictors of gastro-intestinal parasitism on wild cyclic montane voles (*Microtus montanus* (Peale, 1848)) to determine if evidence was consistent with theory implicating parasites in population cycles. We sampled three sites in central Colorado for the duration of a multi-annual cycle and recorded the prevalence and intensity of directly-transmitted *Eimeria* and indirectly-transmitted cestodes from a total of 267 voles. We found significant associations between host infection status, individual traits (sex, age, and reproductive status) and population-level variables (site, trapping period, and population density), including a positive association between host density and cestode prevalence, and a negative association between host density and *Eimeria* prevalence. Both cestode and *Eimeria* intensity correlated positively with host age, reproductive status and population density, but neither parasite was associated with poorer host condition. Our findings suggest that parasites are common in this natural host, but determining their potential to influence montane vole cycles requires future experimental studies and long-term monitoring to determine the fitness consequences of infection and the impact of parasite removal on host dynamics.

Key words: *Microtus montanus*, Montane vole, *Eimeria*, helminths, population cycles, parasite regulation

INTRODUCTION

Understanding parasite impacts on host populations can inform a basic understanding of population regulation (Elton 1924) and can aid efforts in wildlife conservation and management (Obrien & Evermann 1988; Scott 1988; Smith *et al.* 2009). For example, population declines can be caused or exacerbated by infectious diseases, as evidenced by endangered Tasmanian devils and transmissible facial cancer (McCallum 2008), amphibian population declines due to chytridiomycosis (Skerratt *et al.* 2007), and for great ape populations suffering from Ebola and other emerging diseases (reviewed in Leendertz *et al.* 2006). More generally, parasites can have both regulatory and destabilizing effects on the dynamics of vertebrate host populations (reviewed in Hudson 2002a). However, only a handful of studies have directly implicated parasites as causing or amplifying host population cycles. These include the classic examples of red grouse and their ceacal nematodes on the Scottish moors (Dobson & Hudson 1992; Hudson *et al.* 1998), feral Soay sheep and gastrointestinal nematodes on the island chain of St. Kilda (Gulland 1992), and deer mice and their gastrointestinal helminths in eastern North America (Pedersen & Greives 2008).

The relationship between host abundance and parasite transmission is a core concept in epidemiology (Anderson & May 1978; Lloyd-Smith *et al.* 2005). For parasites to regulate their hosts, their transmission must increase with host density (Anderson & May 1978). However, in natural systems, parasite life history, transmission mode, host recruitment, behavior, and climate can obscure the relationship between host density and parasite prevalence or even cause a negative relationship to appear (Montgomery & Montgomery 1988; Haukisalmi & Henttonen 1990; Lloyd-Smith *et al.* 2005). For example, increased range size (Ostfeld *et al.* 1985) or territorial exclusion (Boonstra *et al.* 1998) at low densities could lead to individuals foraging

further distances, increasing their exposure to parasites in the environment. Beyond reductions in abundance, mathematical models suggest three conditions under which parasites can induce host population cycles: 1) delayed density-dependence in parasite recruitment, 2) moderate to low degree of parasite aggregation among hosts, and 3) negative effects of parasitism on host fecundity or the recruitment of juveniles (Anderson & May 1978; Dobson & Hudson 1992; Hudson *et al.* 1998).

Microtine rodent population cycles have fascinated population ecologists since Elton (1924) first observed them almost 100 years ago, and yet the mechanisms for these cycles are still unknown (Boonstra *et al.* 1998). Some likely drivers are variation in climate, resource abundance, predators, parasites, and the interactive effects of multiple drivers (Turchin 1993). Montane voles (*Microtus montanus*) exhibit frequent population cycles and can fluctuate from 300 to less than 25 animals per hectare (Smith & Merrick 2001). Montane voles have short lifespans (6-12 months) and can produce up to four litters per year (Jannett 1977), giving them potential for explosive growth. Montane voles are also infected with a range of parasites that can reach high prevalence in the populations, including cestodes, nematodes, and protozoans (Kinsella 1967; Timm 1985). Although the fitness consequences of parasite infection have never been assessed in montane voles, these parasite taxa are known to reduce host fitness in numerous vertebrate species through reducing body condition, survival, and reproductive success (Scott & Lewis 1987; Scott 1988; Hakkarainen *et al.* 2006; Turner *et al.* 2011), thus creating the potential for effects on host population cycles.

The goals of this study were to examine the potential for individual and population level impacts of parasitism on montane voles. We first characterized the community of gastrointestinal parasites at three sites inhabited by voles near the Rocky Mountain Biological

Laboratory in Colorado, USA. We next asked whether individual host traits, including age, sex, body condition, and reproductive status were associated with measures of parasitism. We further explored population-level changes in parasitism, including within- and between-year changes in prevalence and host abundance, and tested for host density effects on the spatial distribution of infection. We predicted that for parasites to influence host dynamics, either parasite prevalence or infection intensity should increase with vole population density. We also examined whether parasites were weakly aggregated among hosts, as has been shown for other regulating parasites. Finally, we tested for negative effects of parasites on host body condition or reproductive status, as would be expected if parasites negatively affect host fitness.

MATERIALS AND METHODS

Sites and field sampling

Voles were trapped for three consecutive years (2008 – 2010) at three replicate sites within 5 km of the Rocky Mountain Biological Laboratory, located in the Upper East River Valley, Colorado, U.S.A. (39°N, 107°W). Trapping sites (A = Kettle Ponds 1, B = Kettle Ponds 2, C = Research Meadow) were separated by a minimum of 0.5km at approximately 2900m elevation. Vegetation at each site was dominated by the grasses *Festuca thurberi*, *Agropyron trachycaulum*, *Bromus polyanthus*, *Bromopsis inermisi*, and the forb *Lupinus argenteus*. Voles were captured using Longworth live traps (23x8x8cm) spaced 10m apart, with the size of each sampling grid based on available open space. Site A encompassed a 60 x 80m trapping grid (7x9 trap stations: 63 total traps), Site B encompassed a 60 x 40m trapping grid (7x5 trap stations: 35 total traps), and Site C contained 60 x 60m trapping grids (7x7 trap stations: 49 total traps). Trapping grids were baited with a mix of peanut butter, rolled oats and millet and a piece of polyfill bedding

material was added to each trap for warmth. Traps were opened between 18:00-20:00 and checked the following day between 6:00-9:00. Animals were captured 4-5 consecutive days per site every two weeks throughout the breeding season (Jun 15 – Aug 15).

Animals were uniquely identified with numbered eartags (National Brand Tag Company). We recorded individual sex, reproductive status, weight (to the nearest 0.5g) using a spring balance and body length (to the nearest mm) using digital calipers. Males were considered to be reproductive if a scrotal sac was visible and females if they had a perforate vagina, visible nipples or were pregnant by palpation. Body weight combined with evidence of sexual maturity was used as an index of age class (juvenile = < 15g and not reproductively active; sub-adult = 16-30g; adult = > 30g) following Negus, Pinter (1966) and Sullivan *et al.* (2003). A 2mm tail tip was collected from non-juveniles and stored in 95% ethanol after anesthetizing the animals with isoflurane gas for 1 min. Fecal samples were collected from the used traps and traps were scrubbed with 20% bleach solution to reduce parasite transmission. Animals were processed with permission by the Colorado Department of Natural Resources Division of Wildlife (Scientific Collections Licenses #08-10TR2006) and handled in accordance with the animal care guidelines of the American Society of Mammalogists (Sikes & Gannon 2011), the Rocky Mountain Biological Laboratory Animal Care and Use Committee, and the University of Georgia Institutional Animal Care and Use Committee (AUP#A2010 5-092).

Parasite identification and quantification

Fecal samples were stored in 10% formalin with half the sample stored in 2.5% potassium dichromate for coccidia sporulation and identification (Duszynski & Wilber 1997). Salt flotation using sodium nitrate solution (specific gravity 1.2-1.5) was used to isolate intestinal parasites

eggs (Dryden *et al.* 2005). Coverslips were scanned in five replicate zig-zag transects at both 100X and 400X; parasite oocytes and eggs were quantified per gram of feces. Non-invasive fecal egg counts are commonly used in longitudinal studies as an approximation of worm burden, and have been shown to correlate with the ability of the host's immune system to regulate worm burden and fecundity (Stear *et al.* 1995; Nielsen *et al.* 2010). While the relationship between fecal egg counts and the actual number of adult worms is system specific, this measure has been shown to be useful in other wild rodent systems and can provide a valuable non-invasive approximation of worm burden (Brenner 1970; Keymer & Hiorns 1986; Scott 1988; Ferrari *et al.* 2004; Froeschke & Sommer 2005; Harf & Sommer 2005).

Morphological measurements were used to identify three major taxonomic groups of intestinal parasites (Table 1.1): coccidia, cestodes and nematodes. *Eimeria* is the only intestinal coccidian genus reported from voles (Levine & Ivens 1965). Oocysts of this protozoan parasite are shed in the host feces and transmitted via a fecal-oral route. *Eimeria* can reduce over-winter survival of rodents (Fuller & Blaustein 1996), increase susceptibility to predation (Vorisek 1998), and reduce breeding success (Hakkarainen *et al.* 2006). We identified five *Eimeria* morphospecies in montane voles following sporulation and these were designated as *Eimeria* A through E. The most common *Eimeria* sp. found in our study (*Eimeria* A) was not similar to any of the previously described *Eimeria* spp. of *Microtus* based on measurements and general morphology of 325 oocycts (Levine & Ivens 1965). All *Eimeria* spp. have direct life cycles. We identified two species of cestodes in this study that were identified as *Andrya* and *Paranocephala* spp. based on the size and morphology of eggs. In particular, comparison of morphometric evidence with reports in the primary literature indicated that these species were *Andrya macrocephala* or *A. primordialis* (Rausch & Schiller 1949; Rausch & Tiner 1949) and

Paranoplocephala infrequens (Rausch & Kuns 1950). Because both of these species have similar transmission (ingestion of infected intermediate hosts (orabatid mites) from the environment), data on all cestodes were combined for most analyses. We also observed eggs of one nematode species (2% prevalence) morphologically similar to *Syphacia obvelata* (Kinsella 1967).

Multiple measures of infection status were examined for individual voles: parasite species richness, and for each parasite, the presence/absence and intensity of infection. Because multiple parasite species could infect any single host, we summed the number of intestinal parasite species (counting separately each morphospecies of *Eimeria*, cestodes and nematodes, for a maximum of 8 species) as one measure of parasitism. Analyses of parasite prevalence and intensity were conducted for each parasite type (combining data for all morphospecies) separately. Intensity was recorded for the two most prevalent parasite groups: cestode intensity was estimated as the number of oocytes/eggs per gram of feces, and *Eimeria* intensity was scored categorically on a scale of 0-3 per scan of the entire coverslip (1 = 1-10 oocysts, 2 = 11-100 oocysts, and 3 = > 100 oocysts).

STATISTICAL ANALYSIS

Estimation of host density using Capture-Mark-Recapture

Estimates of population size were calculated using the Schnabel method, based on recapture data and assuming closed populations (Sutherland 1996). Individual-level methods to estimate population size were not employed due to the low recapture rate per individual (mean captures per animal = 2.92 ± 0.1; Range: 2-9). We divided the breeding season into five, two-week long

trapping intervals and estimated population size separately for each trapping period and site. Population size was divided by site grid size, giving vole density per hectare.

Correlates of parasitism: effects of individual host traits, density and time

To test the level of aggregation among hosts for each type of parasite, we examined the *k* parameter of the negative binomial distribution (Wilson 2002), and the index of discrepancy (*D*) of (Poulin 1993) using the program *Quantitative Parasitology* by (Rózsa *et al.* 2000). Measures of aggregation were examined for the most common parasite morphospecies (*Eimeria A* and cestode A) owing to sample size limitations for the less common parasites. To test for potential association between the presence of cestodes and *Eimeria*, we used Chi-squared tests, and we also used Pearson's correlations to test for associations between cestode and *Eimeria* intensity focusing on the subset of animals positive for both parasites.

To examine how individual vole traits and population-level variables influenced the risk of infection, we ran generalized linear models (GLMs) with the presence of *Eimeria* and the presence of cestodes as separate dependent variables (assuming a binary logistic link function). The independent predictor variables were age, sex, site, trapping period, and year with vole population density as a continuous covariate, and we included all biologically relevant two-way interactions. To examine factors associated with parasite intensity, we ran a series of GLMs with the same independent predictors (listed above) and the following dependent variables, in separate analyses: endo-parasite species richness (assuming a Poisson distribution and loglinear link function), cestode intensity (assuming a negative binomial distribution and log link function) and *Eimeria* intensity (using a linear link function). We ran a separate analysis using similar predictor variables with a reduced sample set of only adults with reproductive scores ($N = 70$) to

consider the relationship between adult reproductive status and parasite prevalence and intensity. Host breeding status was scored as follows: 0 (non-reproductive; $N = 7$), 1 (perforate vaginal opening or visible nipples; $N = 25$), 2 (pregnant or lactating; $N = 20$), 3 (scrotal; $N = 16$). We log-transformed host density prior to all analyses and we simplified the model using AICC (Akaike information criteria corrected for smaller sample sizes) following (Crawley 2002). Unless otherwise stated, all analyses were performed using the statistical software SPSS v. 19.0, and results are reported as likelihood ratio χ^2 for analyses of continuous dependent variables (e.g., body condition, parasite intensity), and as Wald χ^2 for analyses of binomial dependent variables (e.g., presence of infection).

Association between parasitism and host condition

To test whether infection measures were associated with poorer host body condition, we estimated body condition in four ways: as a relative index (Ri) using the residuals of an ordinary least squares (OLS) regression of Ln(mass) against Ln(length) (Schulte-Hostedde *et al.* 2005), as Fulton's Index (untransformed mass/length^3) (Fulton 1904), as the body-mass index (BMI = untransformed mass/length^2) (Garrow & Webster 1985) and as a scaled index (SMI) using the Scaled Mass Index (Peig & Green 2009). The SMI uses the population mean, a scaling exponent estimated from an OLS regression, and the length and body mass off each individual to produce a predicted body mass (Peig & Green 2009). We ran multiple GLMs using linear link functions on each condition index, with the following infection measures as independent variables: Model 1: endoparasite species richness; Model 2: *Eimeria* presence + cestode presence, Model 3: *Eimeria* intensity (using data from positive animals only) and Model 4: log cestode intensity (again, using data for positive animals only). Other independent variables included in all GLMs

were age, sex, site, trapping period, log-density, and year. We excluded pregnant females (N = 16) from these analyses as their weight was grossly affected by the size of their gestational mass, and their condition would thus not provide a biologically meaningful comparison to other groups. Pregnant females were included in a separate analysis to explore the effects of reproductive status on the association between infection and body condition in adults only (N = 70). As a final test of effects of parasitism on host condition, we ran a repeated measures ANOVA using data from recaptured individuals and using all indices of condition. Specifically, we tested whether a change in body condition between the first and second capture event depended on the change in infection status. Detailed methods and results for this recapture analysis are provided in Appendix A.

Association between parasites and host density

To further examine the association between host population density and parasitism, we averaged parasite and host data at the level of site and trapping interval for a total of 25 observations over three years. We used a mixed model with a built-in temporal autocorrelation function to examine how changes in parasite prevalence and intensity co-varied with host density. Here, we assumed that both parasite prevalence and host density followed a time-decaying covariance process, so that correlations within each variable decreased linearly over time (trapping period) for each site and each year (SUBJECTS) by using the REPEATED option with a first-order autoregressive process. Density was included as a fixed effect, and site and year were random effects. Because the shedding of parasite transmission stages may occur several weeks post-infection, we examined whether host density was associated more strongly with prevalence or intensity from the same sampling interval (T) versus the next 2-week interval (T + 1). Moreover,

because parasitism could itself cause changes in host density, we also compared host density with parasite measures from the previous 2-week time interval (T – 1). Thus, we ran three separate temporal combination analyses for each of three separate parasite measures: cestode prevalence, *Eimeria* prevalence, and cestode intensity (sample sizes for *Eimeria* intensity data were not sufficient to include this variable in analyses). To meet assumptions of normality in this analysis, we used log-transformed host density, log-transformed cestode intensity, and logit-transformed parasite prevalence (Warton & Hui 2011).

To examine spatial effects in the potential relationship between parasite prevalence and host density, we tested whether transmission is localized within a grid as opposed to randomly, as might be the case with indirectly transmitted parasites, using Ripley's K function in the spatstat package 1.14-8 in R (Baddeley & Turner 2005). Detailed methods for this spatial analysis are provided in Supplementary materials.

RESULTS

A total of 472 capture events occurred during the breeding season (June to August) across all three years (2008-2010). Of these, we used only first capture data to determine correlates of infection status (N = 267 animals). No marked animals were captured in multiple sites, indicating parasite transmission between sites was probably rare. Vole density changed by a factor of 30 between trapping intervals within each year (2008, 2009, 2010) and a factor of 20 between years (peak at 2009, low at 2010; Fig 1.1). Mean vole density ranged from 105 to 161 animals per hectare between sites: Site A = 105 voles/ha (range across years 18-174 voles/ha; 95% CI 2008: 115-130 voles/ha; 2009: 164-188 voles/ha; 2010: 15-23 voles/ha); Site B = 161 voles/ha (range across years 12-410 voles/ha; 95% CI 2008: 56-68 voles/ha; 2009: 387-436

voles/ha; 2010: 11-14 voles/ha); Site C = 160 voles/ha (range across years 16-381 voles/ha; 95% CI 2008: 75-91 voles/ha; 2009: 365-400 voles/ha; 2010: 15-17 voles/ha) (Fig 1.1). These density estimates based on field data are consistent with longer term patterns of vole abundance over 14 years (Fig. 1.1 inset; R.J. Smith, unpublished data).

Overall prevalence for *Eimeria* spp. was 53%, with a mean intensity score of 1.7 (± 0.80 S.E.) among the subset of infected animals (Table 1.1). *Eimeria* were moderately aggregated in the population during 2009 (Table 1.2), with a relatively small proportion of voles harboring a large number of oocytes. *Eimeria* prevalence also appeared to differ among sampling locations and fluctuated weakly across trapping intervals and years (Table 1.2; Fig. 1.2). Overall prevalence for cestodes was 24%, with a mean intensity of 263.9 (± 59.7 S.E.) eggs per gram of feces among infected animals (Table 1.1). Cestodes were highly aggregated at the population level, although aggregation was lower in 2009, the year with highest vole density (Table 1.2). Cestode prevalence and intensity did not differ among sampling locations, but changed between years (Table 1.2). We found no evidence for a statistical association between cestode and *Eimeria* presence as binary variables ($N = 267$, $\chi^2 = 2.13$, df = 1, $P = 0.14$). When we focused on data for animals infected with both parasites simultaneously, the intensity of cestodes and *Eimeria* was positively correlated ($N = 29$, $r = 0.51$, $P = 0.005$). Nematode prevalence was 1.2% among voles for 2008-2010. Due to low prevalence of nematodes, we excluded this group from further analysis.

Individual and population-level predictors of infection status

Older animals had a significantly higher probability of cestode and *Eimeria* infection, greater cestode intensity, and higher endoparasite species richness (Table 1.3). Males had significantly

higher cestode prevalence than females (Table 1.3). *Eimeria* intensity was not predicted by attributes of individual hosts, but site was a significant predictor of both *Eimeria* prevalence and intensity (Table 1.3). Parasite species richness also varied among sites (Table 1.3). Seasonal and yearly changes in cestode prevalence were observed with higher prevalence towards the middle of each breeding season (Table 1.3; Fig. 1.2) and in 2009 (Table 1.3). Cestode intensity was also greater in 2009 and was positively correlated with host density (Table 1.3). There was an interaction between host age and density such that the positive relationship between cestode intensity and host density was stronger among sub-adults than adults (Table 1.3). *Eimeria* intensity also increased with host density (Table 1.3). Analyses restricted to the subset of adults with reproductive data showed that cestode intensity was greatest in pregnant and lactating females and in scrotal males, and lowest for non-reproductive adults (Table 1.4). Site, density, and year were again significant predictors of cestode intensity in this reduced data set. *Eimeria* prevalence and intensity, and endoparasite species richness also varied by site and trapping interval (Table 1.4).

Parasitism and host body condition

All host condition indices were significantly positively correlated (adjusted R^2 based on linear regressions ranged from 0.57-0.97). For analyses that included data on animals from all age classes in the same model, three out of four indices of condition (Ri, BMI, and SMI) varied significantly between age classes for each of the three GLMs run with (i) endoparasite species richness, (ii) parasite presence/absence, and (iii) parasite intensity. For example, analysis of Ri in relation to endoparasite richness showed that condition measures were greater for adults than sub-adults or juveniles (Age effect: $\chi^2 = 73.98$; $P = 0.001$) and the same result was seen when

comparing Ri to cestode and *Eimeria* presence (Age effect: $\chi^2 = 78.29$; $P = 0.001$) and intensity (Age effect: $\chi^2 = 11.49$; $P = 0.001$). For each of these analyses, condition measures scaled separately for adults, sub-adults, and juveniles. Fitted models tested using Fulton's Index performed no better than the intercept-only model. No measure of parasitism was significantly associated with any of the four condition indices for this full data set.

For analyses of body condition restricted to adults ($N = 70$), we report results from residual condition measure Ri only because analysis of other condition measures produced similar results. Body condition was significantly associated with both site ($\chi^2 = 10.17$, $P = 0.01$) and host density ($\chi^2 = 4.5$, $P = 0.03$), but not with cestode or *Eimeria* presence/absence. For a separate analysis restricted to infected animals only that included cestode and *Eimeria* intensity as predictor variables (N=14), log cestode intensity was positively associated with adult condition ($\chi^2 =17.33$, $P = 0.0001$). Adult condition also depended on reproductive status ($\chi^2 =19.73$, $P = 0.001$), site ($\chi^2 =34.41$, $P = 0.001$), and trapping interval ($\chi^2 =14.14$, $P = 0.001$). For the GLM including endoparasite species richness, both site and increasing density ($\chi^2 =4.55$, $P = 0.04$) were significant variables.

Host density and population-level parasite measures

We found that both cestode prevalence and intensity were positively associated with vole population density, and *Eimeria* prevalence was negatively associated with vole density, using generalized linear models to examine average values across site-sampling interval combinations. Our models assumed that correlations in parasite and density measures decayed linearly with increasing time between sampling intervals (treating sampling interval within year as a repeated measure, with the same time-decaying covariance structure for all sites and years). For cestodes,

the effect of host density on parasite infection was significant for the T+1 scenario (with host density at time T associated with parasite prevalence at time T+1; $F_{1,6}$=52.78, b=0.384, *P*=0.0001, Fig. 1.3) but not for the T or T-1 scenarios. A separate model showed that average cestode intensity at time T+1 was also significantly positively associated with vole population density at time T ($F_{1,13}$=11.54, b= 0.90, *P*=0.021; both T and T-1 scenarios showed no significant association between cestode intensity and host density). In contrast to the cestode results, *Eimeria* prevalence at time T+1 was significantly negatively associated with vole population density ($F_{1,11}$=21.76, b= -0.63, *P*=0.001; both T and T-1 scenarios were NS). For each of these analyses, there were no significant associations between measures of infection and site or year. Finally, using Ripley's K summary statistic we did not find evidence of focal transmission of cestodes or *Eimeria* for any site or year.

DISCUSSION

Overall, our results showed that montane vole abundance changed dramatically within and between years, but provided only limited support for an association between host population dynamics and parasitism. Evidence in support of an association between cestodes and *M. montanus* population cycles (see Tompkins *et al.* 2002 for a general review on this topic) includes delayed density-dependence in cestode recruitment, with parasites appearing to track host density with a two-week time lag. Our results also showed that cestode aggregation was weaker during the year (2009) with the highest host density and parasite prevalence, and that reproductively active adults had greater cestode intensity, opening the door for cestodes to negatively affect host reproduction. *Eimeria* intensity increased with host density in one analysis, but a separate analysis showed that *Eimeria* prevalence at time T+1 decreased with host

density, suggesting that either the mortality of infected animals is higher, or that *Eimeria* transmission is lower, when host density is high. Importantly, we did not find evidence for negative effects of parasitism by either cestodes or *Eimeria* on host body condition as a proxy for host fitness.

Support for regulatory and destabilizing effects of parasites on host populations has been limited to a relatively small number of intensive field studies, including work on Soay sheep, red grouse, and mice (Gulland 1992; Hudson *et al.* 1998; Pedersen & Greives 2008). One factor these systems share in common is evidence that the individual- and population-level impacts of parasites increase directly with host population size. Despite these oft-cited examples, many field studies have shown no association between parasite prevalence and host density. For example, no or negative relationships between host density and the prevalence of intestinal parasites were found in fossorial water voles (Deter *et al.* 2006), bank voles (Haukisalmi & Henttonen 1990), wood mice (Montgomery & Montgomery 1988) and deer mice (Theis & Schwab 1992). Moreover, Fenton *et al.* (2002) showed in a meta-analysis across experimental studies that the relationship between parasite transmission rate and host density could be either positive or negative, with negative relationships between host density possibly due to the spatial structuring of hosts.

It is important to note that there are multiple factors that could complicate the relationship between host abundance and parasite transmission and impacts. In particular, many cestodes have indirect life cycles that rely on the presence of one or more intermediate hosts. In a comparative study looking across 44 mammalian species and three orders of nematodes, Arneberg (2001) found that host population density was positively correlated with parasite prevalence only for parasites with direct life cycles. The cestodes observed in our study are

known to use free-living oribatid mites as their intermediate hosts (Rausch & Tiner 1949). These mites are likely to be abundant in vole habitats (St. John *et al.* 2006) and as no clustering of prevalence was observed in the spatial analysis, they might not be a limiting step in parasite transmission. In fact, cestode intensity in our study increased as vole density increased, and parasitism peaked just as vole abundance started to decline, consistent with the idea that cestode prevalence and intensity can track host density.

We were surprised to find no evidence for a positive relationship between *Eimeria* prevalence and host density, given that most coccidia are directly transmitted (without intermediate hosts) and have short generation times. It is possible that *Eimeria* transmission depends more strongly on local host density at very small spatial scales as opposed to total host density (Deter *et al.* 2006). Consistent with this idea, site influenced the probability of *Eimeria* infection at the individual level, with specific sampling locations having consistently higher prevalence, indicating that landscape heterogeneities could influence parasite abundance (reviewed in Hess *et al.* 2002; Salvador *et al.* 2011). Moreover, the relationship between *Eimeria* and host density may be confounded by host age; specifically, prevalence could be diluted by uninfected newborns entering the population of continuously susceptible sub-adults and adults, explaining why *Eimeria* prevalence appears to decrease following increases in host density (Figure 2). *Eimeria* intensity was positively correlated with host density in 2009, which may suggest lowered resistance to parasite reproduction at high host densities. Indeed, if the mortality of infected hosts increased with greater intensity of infection, this might cause the negative relationship between *Eimeria* prevalence and host density observed here.

In our study, host body condition did not appear to decline during periods of high host density, which has been shown in other species where parasites exacerbate host declines

(Gulland 1992; Pedersen & Greives 2008). In particular, a negative relationship between population density and host condition is expected when increased crowding leads to resource shortages (Begon *et al.* 2006), which can cause chronic stress (Christian 1950), increased susceptibility to parasites (Beldomenico *et al.* 2008), and population crashes after periods of high density (Charbonnel *et al.* 2008). The lack of evidence for declines in host condition observed here could indicate that voles experienced no shortage of resources or social stress during their high density phase, as might occur if other factors (such as predation) kept density well below the carrying capacity of the environment. We also found no changes in age structure or sex ratio in relation to host population density, and average condition remained relatively constant during all years and across all trapping intervals.

Importantly, we found little support for negative effects of parasites on individual host condition. In general, the negative effects of gastrointestinal helminths are thought to be mediated through the host's energy balance, where there is increased metabolic demand from competition for host resources and from the host mounting defensive responses (Holmes 1995). Lack of negative effects of intestinal parasites on condition or breeding status have also been observed in water voles (Deter *et al.* 2006), common voles (Laakkonen *et al.* 1998), bank voles (Tenora *et al.* 1979), snowshoe hare (Keith *et al.* 1985) and other studies (reviewed in Irvine 2006). Surprisingly, we found a positive association between cestode intensity and adult vole condition in this study. Similar associations have been observed in other host-parasite systems, and could be caused by differences in parasite exposure in relation to foraging or other behaviors. For example, Halvorsen (1986) found that heavy, dominant reindeer were more heavily infected with gastrointestinal nematodes, likely due to greater access to contaminated pasture and a higher rate of nematode ingestion with increased food intake, and a review of

laboratory studies investigating effects of small mammal host diet on parasite biology by Crompton (1987) found that cestode establishment, growth, and reproduction can be affected by host nutritional status. In this study montane voles in better condition could have foraged more effectively, leading to greater exposure to cestode infection and more nutritional resources to support parasite establishment. Additionally, heavier animals with greater condition measures were more likely to be in breeding condition, and thus could have traded-off reproduction against immune defenses, making them vulnerable to chronic infections (Perrin *et al.* 1996; Schwanz 2008). It is also important to note that the condition indices measured here might not be a reliable proxy of individual fitness. As such, other methods such as parentage analysis (Gooderham & Schulte-Hostedde 2011), recapture survival estimates and experimental manipulation of parasite loads in the field may be more effective in identifying links between host fitness and parasitism.

Both the probability of transmission and immunological susceptibility to parasites are important determinants of infection and each of these factors can depend crucially on individual host traits such as age and sex. Our study showed that cestode prevalence was greater in males than in females, likely due to androgens (testosterone) suppressing immune response (Folstad & Karter 1992a) or to behavioral differences between the sexes that could cause differential exposure (Moore & Wilson 2002). Moreover, cestode prevalence was greater in adults, probably due to increased foraging outside the nest in adults and constant exposure to parasites throughout life. On the other hand, *Eimeria* prevalence was high for all age classes and for both sexes, suggesting high transmission efficiency in *Eimeria* (both within and outside of the nest (Jannett 1978)). High prevalence of *Eimeria* in adults further suggests that animals are susceptible to infection following repeated exposures. Moreover, our finding of relatively high prevalence of

both cestode and *Eimeria* parasites in adult voles indicates that parasites are probably not eliminated by the host's immune response, as might be inferred from an age-infection relationship where prevalence or intensity first increased and then decreased with host age (Cattadori *et al.* 2005).

A recent review by Tompkins *et al.* (2011) highlighted that most of the available evidence of parasite impacts on regularly fluctuating host populations depends heavily on other factors also being in play, especially changes in resource abundance and external environmental factors. A common theme appears to be that parasites can affect the rate and magnitude of host population crashes, but that the ultimate cause of host population fluctuations depends on other variables. For example, in the study of Pedersen, Greives (2008), acorn masting was proposed as the main driver of wild mouse population cycles, but parasites appeared to accelerate the population crashes. Holmes (1995) argued that the real significance of disease in altering wildlife population dynamics is likely to be through its interactions with nutrition, predation, and their synergy. For montane voles, although parasites were not documented to directly lower host condition indices, they may still contribute to population dynamics indirectly, if, for example, they cause increased susceptibility to predation, as has been suggested in other host-parasite interactions (e.g. Murray *et al.* 1998; Møller & Nielsen 2007).

Montane vole populations are a useful natural system to investigate the potential impacts of parasitism because their rapid fluctuations in host density can provide a backdrop to examine population level host-parasite interactions. As such, this study adds to the growing body of work that considers the conditions under which parasites affect host dynamics, and the types of parasites most likely to do so. Our study showed that greater parasite loads (cestode intensity and intestinal parasite species richness) were harbored by larger, older, and higher condition

animals, and that one parasite type (cestodes) appeared to positively track changes in host density whereas *Eimeria* prevalence was negatively associated with host density. We found no evidence that parasites negatively affect host body condition or reproductive status. While a large number of studies on host-pathogen systems found little evidence that parasites impact host condition or population dynamics (Tenora *et al.* 1979; Keith *et al.* 1985; Laakkonen *et al.* 1998; Deter *et al.* 2006; Irvine 2006), observational association studies cannot clearly demonstrate that parasites have no effects on their hosts, but rather can suggest fruitful hypotheses to test with experimental studies. A key challenge for ecologists studying the population biology of animal-parasite interactions is to undertake careful analysis of the sub-lethal impacts of infection, such as through experimental manipulation of parasite loads, before concluding that parasites have minimal influence on host population cycles (Irvine 2006). As such, a growing number of treatment studies are revealing the detrimental impacts of parasites previously thought to be benign. Another outstanding challenge is for ecologists to identify generalities that might reduce the importance of parasites as regulating forces on natural populations.

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TABLES

Table 1.1. Average parasite prevalence, species richness (by taxonomic group), and parasite intensity for all montane voles *(M. montanus*) captured in the 3 year time series (*N* = 238). Mean species number and mean intensity were calculated using positive animals only. *Eimeria* intensity was based on categorical scoring as described in Methods text. NA stands for not applicable.

	Prevalence %	Spp # ± (S.E.)	Intensity ± (S.E.)
All intestinal parasites	62.2	1.51 (0.07)	NA
Coccidia	52.9	1.39 (0.06)	1.7 (0.80)
Eimeria A	45.2		1.7 (0.08)
Eimeria B	8.7		2.3 (0.19)
Eimeria C	8.3		2.4 (0.16)
Eimeria D	8.3		1.9 (0.17)
Eimeria E	2.2		1.75 (0.25)
Cestodes	24.4	0.25 (0.03)	263.9 (59.7)
Cestode A	23.7		298.2 (66.5)
Cestode B	2.8		54.3 (36.8)
Nematode	1.2	NA	NA

Table 1.2. Parasite parameters measured separately for each sampling location and year.

Species	Site	*N*	Prevalence %	Mean intensity	(SE)	Mean abundance	(SE)	K^a	D^b
Cestode									
sp.	Site A	64	21.9	206.9	76.1	45.3	19.4	0.048	0.901
	Site B	90	21.1	326.5	154.9	68.9	34.9	0.030	0.947
	Site C	113	25.7	229.6	55.2	58.9	16.9	0.047	0.900
	2008	47	12.8	153.2	67.5	19.6	16.9	0.038	0.947
	2009	214	26.2	265.2	61.6	69.4	17.9	0.047	0.907
	2010	6	0.0	NA	NA	0	NA	NA	NA
Eimeria									
sp.	Site A	64	53.1	90.8	8.8	86.90	9.4	0.482	0.640
	Site B	90	68.9	94.6	5.0	91.85	5.5	0.030	0.947
	Site C	113	40.7	88.6	3.9	36.30	5.0	0.088	0.858
	2008	47	46.8	NA	NA	NA	NA	NA	NA
	2009	214	55.6	92.4	3.9	63.80	63.8	0.215	0.746
	2010	6	16.7	10.0	0.0	9.35	9.4	NA	0.714

a. *K* is the corrected aggregation index. b. *D* is Poulin's (1993) Index of Discrepancy which measures the disparity between observed and uniform distributions.

Table 1.3. Results of generalized linear models (N=230) using data for all animals (juveniles, sub-adults and adults) showing the effect of host variables (age and sex), population variables (site and density), design variables (trapping interval and year) and their interaction on (i) the probability of cestode infection, (ii) cestode morphotype A intensity, (iii) the probability of *Eimeria* infection, (iv) *Eimeria* morphotype A intensity, and (v) endoparasite species richness. Only results for the final simplified models are given (Full model: dependent variable = Age + Sex + Site + Density + Trap interval + Year + Age*Density + Age*Sex + Age*Trap interval + Sex*Density + Sex*Trap interval). Test statistics are Wald χ^2 for binary (0/1) response variables (probability of cestode and *Eimeria* infection) and likelihood ratio χ^2 tests for other dependent variables.

Dependent Variables		Cestode Infection (0/1)		Cestode A Intensity (N=52)		*Eimeria* Infection (0/1)		*Eimeria* A Intensity (N=145)		Endoparasite Species Richness	
Independent Variables		Df	χ^2	Df	χ^2	Df	χ^2	Df	χ^2	Df	χ^2
Host Variables	Age	2	11.5**	1	13.4***	2	8.9**	.	.	2	9.3**
	Sex	1	4.2*	.	.	.	.	.	.	.	.
Population Variables	Site	.	.	.	.	2	27.1***	2	67.3***	2	13.9***
	Density	.	.	1	6.6**	.	.	1	8.2**	.	.
Design Variables	Trapping interval	1	7.7**	.	.	.	.	.	.	.	.
	Year	2	11.4**	1	8.1**	.	.	.	.	2	4.62
Interactions	Age*Density	.	.	2	11.8***	.	.	.	.	.	.

*p<0.05, **p<0.01, ***p<0.001

Table 1.4. Results of generalized linear models on a subset of adult animals with reproductive status recorded (N=70) showing the effect of host variables (sex anintervald reproductive number), population variables (site and density), design variables (trapping interval and year) and their interaction on (i) the probability of cestode infection, (ii) cestode morphotype A intensity, (iii) the probability of *Eimeria* infection, (iv) *Eimeria* morphotype A intensity, and (v) endoparasite species richness. Only results for the final simplified models are given (Full model: dependent variable = Sex + Repro# + Site + Density + Trap interval + Year + Sex*Density + Sex*Trap interval + Repro#*Density + Repro#*Sex + Repro#*Trap period). Test statistics are Wald χ^2 for binary (0/1) response variables (probability of cestode and *Eimeria* infection) and likelihood ratio χ^2 tests for other dependent variables.

	Dependent Variables	Cestode Prevalence		Cestode A Intensity (N=19)		*Eimeria* Prevalence		*Eimeria* A Intensity (N=38)		Endoparasite Species Richness	
Independent Variables		Df	χ^2	Df	χ^2	Df	χ^2	Df	χ^2	Df	χ^2
Host Variables	Sex	1	4.4*	1	6.4**	.	.	.	.	.	.
	Reproductive No.	.	.	3	27.7***	.	.	.	.	.	.
Population Variables	Site	.	.	2	23.8***	2	15.3***	2	22.4***	2	12.6**
	Density	.	.	1	19.9***	.	.	.	.	.	.
Design Variables	Trapping interval	.	.	.	.	1	4.4*	.	.	1	3.80
	Year	.	.	1	32.8***	.	.	.	.	.	.

*p<0.05, **p<0.01, ***p<0.001

FIGURE LEGENDS

Fig. 1.1. Intra-annual and multi-annual population dynamics of montane voles (*Microtus montanus*) near the RMBL in central Colorado. The bottom panel displays population density (voles/ha) by site (A, B, C) for three years (2008-2010), during the breeding season from early June to early August. The inset displays yearly *M. montanus* density recorded at Site A from 1997 to 2010 (R.J. Smith, unpublished data).

Fig. 1.2. The multi-year dynamics of cestode and *Eimeria* prevalence (diamonds on dashed and grey line, respectively) and cestode intensity (squares on dotted line) plotted alongside montane vole (*M. montanus*) log transformed density (vole density, solid line). Note log scale on y-axis.

Fig. 1.3. The correlation between logit transformed cestode prevalence two weeks later (T+1) and montane vole (*M. montanus*) density at time T averaged for 3 sites for 5 trapping intervals per each of 3 years (2008-2010; *N*=18). Prevalence was not possible to record for all trapping intervals due to missing fecal samples.

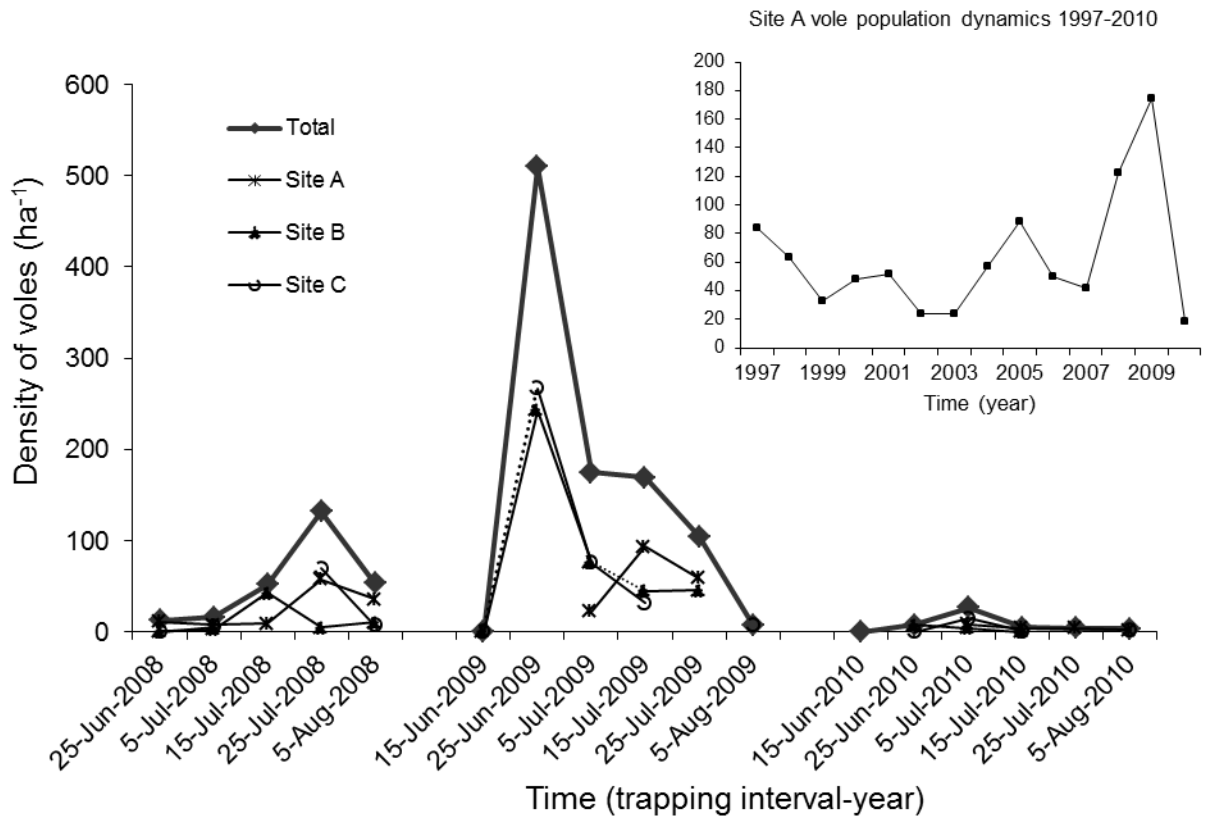
Site A vole population dynamics 1997-2010
Time (year)
Total
Site A
Site B
Site C
Density of voles (ha-1)
Time (trapping interval-year)
25-Jun-2008
5-Jul-2008
15-Jul-2008
25-Jul-2008
5-Aug-2008
15-Jun-2009
25-Jun-2009
5-Jul-2009
15-Jul-2009
25-Jul-2009
5-Aug-2009
15-Jun-2010
25-Jun-2010
5-Jul-2010
15-Jul-2010
25-Jul-2010
5-Aug-2010

Figure 1.1.

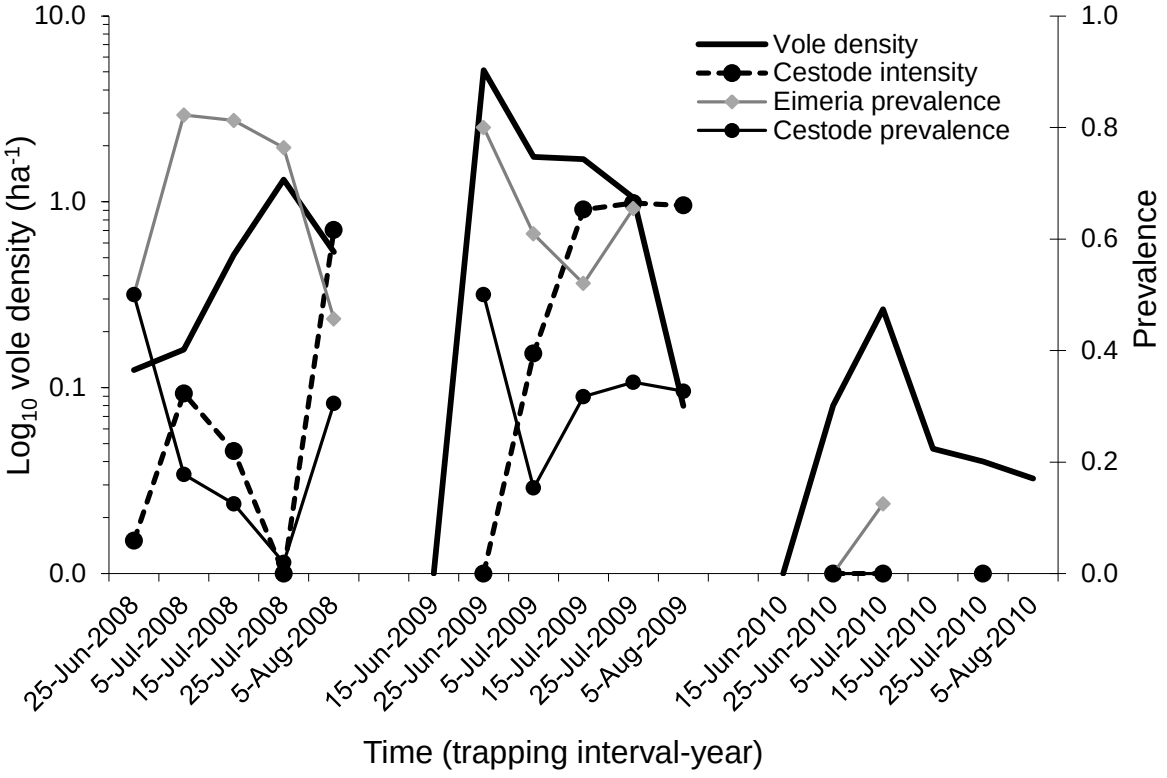
10.0
1.0
0.1
0.0
Log10 vole density (ha-1)
1.0
0.8
0.6
0.4
0.2
0.0
Prevalence
Vole density
Cestode intensity
Eimeria prevalence
Cestode prevalence
25-Jun-2008
5-Jul-2008
15-Jul-2008
25-Jul-2008
5-Aug-2008
15-Jun-2009
25-Jun-2009
5-Jul-2009
15-Jul-2009
25-Jul-2009
5-Aug-2009
15-Jun-2010
25-Jun-2010
5-Jul-2010
15-Jul-2010
25-Jul-2010
5-Aug-2010
Time (trapping interval-year)

Figure 1.2.

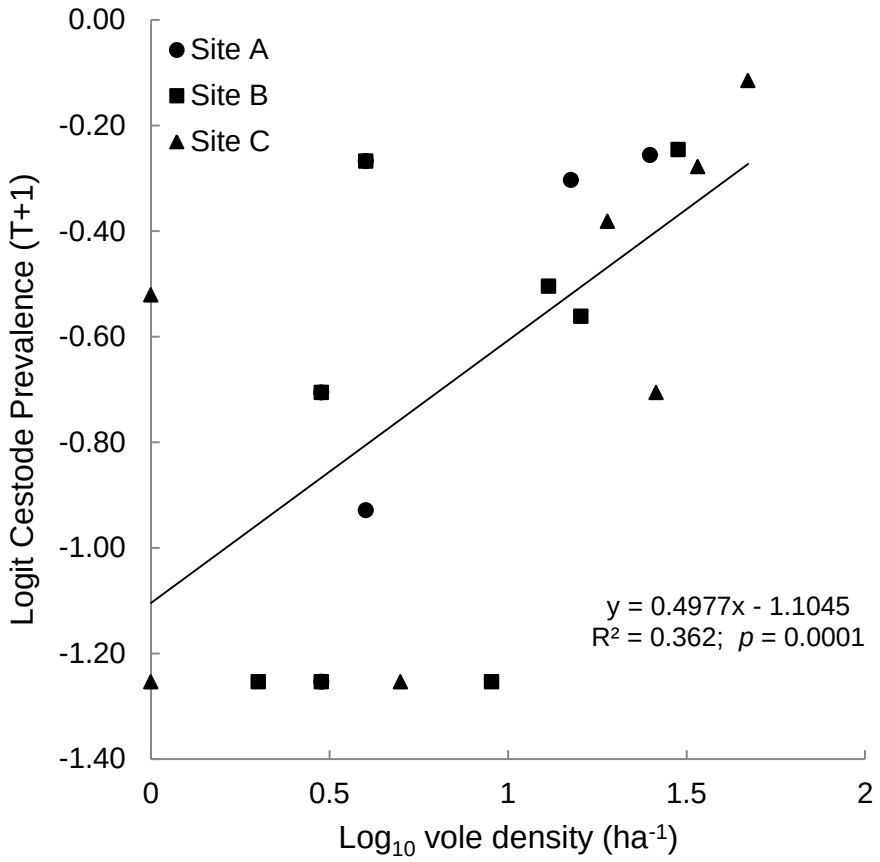
0.00
-0.20
-0.40
-0.60
-0.80
-1.00
-1.20
-1.40
Logit Cestode Prevalence (T+1)
Site A
Site B
Site C
y = 0.4977x - 1.1045
R² = 0.362; p = 0.0001
0
0.5
1
1.5
2
Log10 vole density (ha-1)

Figure 1.3.

CHAPTER 3

DUPLICATION AND POPULATION DYNAMICS SHAPE HISTORIC PATTERNS OF SELECTION AND GENETIC VARIATION AT THE MHC IN RODENTS[1]

[1] J.C. Winternitz and J.P. Wares. Submitted to *Molecular Ecology*, 10/11/2012.

ABSTRACT

Genetic variation at the MHC is vitally important for wildlife populations to respond to pathogen threats. Because natural populations can fluctuate greatly in size, a key issue concerns how population cycles and bottlenecks that could reduce genetic diversity will influence MHC genes. Using 454 sequencing, we characterized genetic diversity at the DRB Class II locus in montane voles (*Microtus montanus*), a North American rodent that regularly undergoes high amplitude fluctuations in population size. We tested for evidence of historic balancing selection, recombination, and gene duplication to identify mechanisms maintaining allelic diversity. Counter to our expectations, we found strong evidence of purifying selection acting on the DRB locus in montane voles. We speculate that the interplay between population fluctuations and gene duplication might be responsible for the weak evidence of historic balancing selection and strong evidence of purifying selection detected. To further explore this idea, we conducted a phylogenetically controlled comparative analysis across 16 rodent species with varying demographic histories and MHC duplication events. Based on generalized linear model-averaging, we found evidence that the number of duplicated loci was positively related to allelic diversity and strength of purifying selection at the DRB locus. Our analyses also revealed that species with a tendency to exhibit cyclic population dynamics also had greater values of Tajima's D, an indicator of balancing selection. This study highlights the need to consider demographic history and genetic structure alongside patterns of natural selection to understand resulting patterns of genetic variation at the MHC.

Key words: Major Histocompatibility Complex, *Microtus montanus*, balancing selection, purifying selection, population dynamics, gene duplication

INTRODUCTION

Genetic variation provides the potential for natural populations to adapt to environmental changes, including those caused by global climate change, habitat alteration, and novel pathogens. However, adaptive genetic variation buffering wild populations against abiotic and biotic threats is itself vulnerable to fluctuations in population size and population connectivity (Saccheri *et al.* 1998; Hess *et al.* 2002; Altizer *et al.* 2003a). A major goal for evolutionary ecology and conservation biology is to understand the impacts of population dynamics on adaptive evolution and genetic diversity (Thompson 1998; Spielman *et al.* 2004b). The major histocompatability complex (MHC), in particular, is a gene region that is vitally important for immune defense in vertebrates (Klein 1986). This region contains the most diverse set of coding genes in vertebrates, with most species examined to date showing high levels of allelic diversity and heterozygosity (Edwards & Hedrick 1998; Knapp 2005). Even among species with small population sizes or low genetic diversity across neutral markers, MHC genes have been shown to exhibit surprisingly high levels of variation (Hedrick *et al.* 2000b; Aguilar *et al.* 2004; Hedrick & Hurt 2012). How this variation persists in the face of population declines or fluctuations is an interesting question, with evidence suggesting that a combination of pathogen-mediated selection and mate choice for specific or dissimilar alleles maintains high diversity at the MHC (Apanius *et al.* 1997; Knapp 2005; Milinski 2006; Spurgin & Richardson 2010).

Glycoproteins encoded by MHC genes bind to foreign antigens and present them to T-cells, initiating the immune response (Klein 1986). There are two major groups of MHC genes: Class I recognizes and presents peptides from intracellular pathogens and Class II binds and displays peptides from extracellular pathogens (Hughes & Yeager 1998). In particular, the Class II DRB locus has been the subject of many non-model animal studies as it harbors extensive

allelic diversity (Bernatchez & Landry 2003). Baseline genetic variation at the DRB locus is thought to be generated primarily by gene duplication via a birth and death process (Nei *et al.* 1997) or by recombination of alleles (Parham & Ohta 1996). Variation at the DRB locus has been associated with parasite resistance in a variety of animals, including fish, ungulates, rodents, primates, and carnivores (Paterson *et al.* 1998; Wegner *et al.* 2003a; Schad *et al.* 2005; Kloch *et al.* 2010; Srithayakumar *et al.* 2011). Exon 2 of the DRB in particular encodes the functionally important antigen binding sites (ABS) recognized by macrophages and antibodies. Thus, proteins encoded by exon 2 bind to unique peptides derived from pathogens, and the genes encoding them can be subjected to intense positive selection, whereby novel types that confer resistance to a specific pathogen can sweep across a population, as evidenced by a greater ratio of amino acid-changing nonsynonymous substitutions to synonymous substitutions at these codons (Hughes & Yeager 1998). In addition to strong molecular signatures of positive selection at the ABS, the persistence of alleles across speciation events (trans-species polymorphisms) provides further evidence that historic balancing selection has shaped the evolution of the DRB locus (Garrigan & Hedrick 2003). Specifically, alleles conferring fitness benefits are retained in populations even as the new populations adapt to novel conditions and become reproductively isolated from each other.

Low genetic diversity at the MHC has been suggested to cause increased susceptibility to infection for some species (e.g. Tasmanian devils (Siddle *et al.* 2007); amphibians (Savage & Zamudio 2011); cheetah (O'Brien & Evermann 1988b)), which could present a serious problem for endangered species with small fragmented populations, where DRB diversity has been diminished (Marsden *et al.* 2009; reviewed in Radwan *et al.* 2010). However, even in the face of prolonged population bottlenecks, some populations have retained surprisingly high MHC

diversity, as reported for San Nicolas Island foxes (*Urocyon littoralis*) (Aguilar *et al.* 2004), water voles (*Arvicola amphibius*, previously *A. terrestris*) (Oliver & Piertney 2012a) and wild African buffalo (*Syncerus caffer caffer*) (Wenink *et al.* 1998). Species that undergo large fluctuations in population size or regular population cycles are particularly interesting to investigate in terms of MHC diversity, as their effective population sizes are dominated by low-density periods; thus, these species may harbor relatively less allelic diversity over time as rare alleles are lost through genetic drift (Hartl & Clark 1997). On the other hand, high MHC diversity could be readily maintained in fluctuating populations if population size rebounds quickly following declines, if balancing selection is strong enough, and if immigration of individuals from neighboring sites introduces novel alleles.

Here, we examined the pattern of MHC diversity in montane voles (*Microtus montanus*), which inhabit alpine grassy meadows of North America ranging from Colorado to Utah (Sera & Early 2003). Voles from the Arvicolinae subfamily (to which montane voles belong) represent an ideal system in which to investigate MHC diversity, as they tend to undergo dramatic population cycles every three to seven years (Krebs 1996; Stenseth 1999). Montane voles in particular undergo high-amplitude and frequent population cycles, peaking in abundance every three to four years (Pinter 1986; R. Smith unpublished data; Winternitz *et al.* 2012). They also have a diversity of parasites (Winternitz *et al.* 2012) and a promiscuous mating system, thus potentially enabling selection to maintain high MHC diversity through mate choice and parasite interactions. Previous studies of allelic diversity at the DRB in other vole species have shown a mix of results (Oliver & Piertney 2006; Axtner & Sommer 2007). Both the MHC Class II DQA and DRB locus appear to have been duplicated within Arvicolinae, but the timing of this is currently unresolved (Bryja *et al.* 2006; Axtner & Sommer 2007). It has been suggested that the

combination of large population sizes, short generation times, and duplicated genes for many cyclic rodents can maintain MHC polymorphisms despite low effective population sizes and population fluctuations. In addition, selection from numerous parasites (Timm 1985) and MHC-based mating preferences (Radwan *et al.* 2008) could oppose the effects of genetic drift on MHC diversity in cyclic rodents.

In this study, we first characterized variation at the MHC Class II DRB locus for *M. montanus* to test for evidence of historic balancing selection acting on the DRB locus. Importantly, this represents the first exploration of MHC Class II diversity for a New World arvicoline species. We also undertook a comparative analysis to examine evolution of the DRB locus across rodent species, with the goal of investigating the influence of population dynamics (i.e. stable, cyclic, or bottlenecked populations) and gene duplication on patterns of allelic diversity and signals of selection. We predicted that rodent species that experience population fluctuations or bottlenecks would harbor lower total nucleotide diversity at the MHC and would show weaker signals of balancing selection on the DRB locus if they are exposed to high degrees of genetic drift. We also predicted that gene duplication at the MHC would correlate with greater allelic divergence and stronger evidence for purifying selection, as alleles from duplicated loci can become specialized to produce products with unique and specific functions (Nei & Rooney 2005). Our ultimate goal was to better understand how species that routinely experience high degrees of genetic drift can maintain immunogenetic diversity over evolutionary timescales.

MATERIALS AND METHODS

Study location and field sampling

Voles were trapped for two consecutive years (2008 – 2009) at four replicate sites less than 5 km from the Rocky Mountain Biological Laboratory, located in the Upper East River Valley, Colorado, U.S.A. (39°N, 107°W). The four trapping sites were comprised of grassy meadows and separated by a minimum of 0.5km at approximately 2900m elevation. A total of 284 voles were captured using Longworth live traps for four or five consecutive days per site every two weeks throughout the breeding season (Jun 15 – Aug 15). Animals were uniquely identified with numbered eartags (National Brand Tag Company), and sex and age were recorded. Full trapping methods are described in Winternitz *et al.* (2012). To obtain tissue samples for genetic analysis, a 2mm tail tip was collected from non-juveniles and stored in 95% ethanol at 5°C after briefly anesthetizing the animals with isoflurane gas.

Tagged primer design, amplification, and 454 sequencing

Our genetic investigation focused on the MHC class II *DRB* gene exon 2 because it has previously been shown to contain most of the functionally important antigen binding sites (ABS) and is, therefore, the most likely candidate for detecting balancing selection acting on MHC class II genes (Hughes & Yeager 1998). In other words, because these sites determine the range of pathogen proteins that can be recognized and displayed to T-cells, leading to the activation of the adaptive immune system, different pathogens should favor the maintenance of different alleles that control their recognition by the host's immune response. We isolated and amplified the DRB gene for all 284 individuals using polymerase chain reaction (PCR) and direct Next Generation (454) sequencing. Samples from 20 individuals (~10%) were cloned and classically Sanger sequenced to validate 454 sequencing results and to establish preliminary allelic data to inform our 454 sequencing protocol. Genomic DNA was extracted using the PureGene DNA isolation kit (Gentra Systems), following the manufacturer's protocol. We used oligonucleotide

forward primer JS1 (5'-AGTGTCATTTCTACAACGGGACG-3') and reverse primer JS2 (5'-GATCCCGTAGTTGTGTCTGCA-3') described by Schad *et al.* (2004). These primers were previously successful in amplifying the DRB locus for numerous rodent species (Froeschke & Sommer 2005; Harf & Sommer 2005; Meyer-Lucht & Sommer 2005; Oliver & Piertney 2006); specifically, they amplified a 171 bp fragment of the second exon of the DRB gene that includes part of the functional antigen-binding site (ABS). This primer system has also been successful in amplifying sequences from multiple loci in rodents when the DRB locus has been duplicated (Galan *et al.* 2010).

All PCRs were performed in a reaction volume of 20 µl, each containing 40–100 ng of DNA, 0.5 mM of each primer (Invitrogen), 4ul of 5X reaction buffer, 2ul of 2.5mM MgCl2, 2ul of a mix of 10 mM deoxyribonucleotide triphosphates, and 0.2ul of (5u/ul) GoTaq®Flexi DNA Polymerase (Promega M8295). Thermocycling was carried out on an Eppendorf Mastercycler® ep with an initial denaturation step at 96°C for 120s followed by 30 cycles of denaturation at 94°C for 30s, annealing at 57°C for 30s , elongation at 72°C for 60s, and a final extension at 72°C for 10 min. PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen K4500-01) and transformed plasmids into One Shot®Top10 Chemically Competent *E. coli*. Transformed cells were grown on X-gal coated LB plates with 50ug/ml ampicillin overnight, and recombinant clones were detected by white/blue screening. Six to ten colonies containing inserts were randomly selected per individual and sequenced using M13 primers. Sequencing reactions were performed with the Big Dye 3.1 sequencing kit (ABI) and their products were separated on an ABI 3730xl 96-capillary DNA Analyzer. Sequences were checked and aligned using Geneious Pro v5.5 (Kearse *et al.* 2012).

To assign 454 sequencing reads to specific individuals, 9-bp tags were used to create 18 forward and 18 reverse 5' tagged primers that resulted in 324 unique JS1-tagged and JS2-tagged primer pairs. These 9-bp sequences (created at http://faircloth-lab.github.com/edittag/) were developed to have an edit distance of five, whereby five mutations are required for one tag to transform into another sequence (Faircloth & Glenn 2011). We visually assessed band intensities on agarose gels to verify that approximately equimolar quantities of PCR products were obtained with the 36 different tags in all combinations. Individual PCR products were concentrated and normalized using the SequalPrep™ Normalization Plate Kit (Invitrogen A10510-01). 10ul of sample from each individual was pooled and sequenced as a single 454 Titanium run at the Georgia Genomics Facility, Athens, Georgia. To further visualize and analyze sequencing data and assign reads to individuals, we used the SESAME software (Meglécz *et al.* 2011).

MHC genotyping and allele validation

Preliminary analysis based on cloning and Sanger sequencing results revealed 1-4 distinct alleles per individual, indicating that the DRB locus in *M. montanus* has undergone at least one duplication event. Based on these findings, we calculated the minimum coverage necessary to obtain at least three copies of each allele at 0.999 probability using the method of Galan *et al.* (2010). The analysis indicated that coverage of 46 reads per individual was sufficient for accurately genotyping individuals (amplicons) with a duplicated gene in a diploid species (Galan *et al.* 2010).

In addition to sufficient coverage, reliable genotyping of individuals requires that true alleles be distinguished from artifacts that can arise from PCR amplification errors and 454

sequencing errors. Point mutations due to DNA polymerase errors in PCR and sequencing reactions, and indels (insertion or deletion less than 3bp) are common errors (Margulies *et al.* 2005; Huse *et al.* 2007) and can be identified if they cause frame shift mutations or if they produce shortened or elongated alleles of relatively low frequency within amplicon reads. Finally, chimeras, produced by in-vitro recombination of true alleles during PCR, are difficult to address because they may look similar to true in-vivo recombinants and may occur in a relatively high number of reads (Longeri *et al.* 2002; Galan *et al.* 2010). Since artificial chimeras must always co-occur with parental alleles, they should be identified by examining all sequence variants present in an individual (Zagalska-Neubauer *et al.* 2010). Procedures for artifact filtering and data validation can be found in Appendix B.

STATISTICAL ANALYSIS

Sequence Polymorphism and Testing for Recombination

Sequences were aligned using Clustal W in MEGA 5.05 (Tamura *et al.* 2011). MEGA 5.05 was employed to calculate allele mean genetic distance (*d*) based on nucleotide divergence according to the Kimura two-parameter distance. The Kimura two-parameter distance (Kimura 1980) was also applied to construct a neighbor-joining phylogenetic tree of the 21 alleles in MEGA 5.05 based on the shared sequence sections of all alleles (Saitou & Nei 1987). We conducted a bootstrap analysis with 5,000 replicates to assess reliability of branching within the tree. This was also performed considering only non-antigen binding sites, as ABS sites may be more similar to each other due to positive selection for similar amino acids changes. Another phylogenetic tree of DRB alleles was constructed within a Bayesian framework with MrBayes 3.2 (Ronquist *et al.* 2012). The likelihood settings of the model corresponded to the parameter values estimated from the data, as there was no one model with posterior probability > 0.05 after

sampling across the entire general time reversible (GTR) model space. We set priors to default values. Four Metropolis-coupled Markov chains (three of them 'heated', temperature = 0.10) were run for 10^6 generations and sampled every 100 generations. The first 25% of trees were discarded as burn-in, resulting in a total of 15002 sampled trees. To calculate the posterior probability of each bipartition, the majority-rule consensus tree was computed from these 15002 sampled trees.

Sequence polymorphism was analyzed using DnaSP v5 (Librado & Rozas 2009). Tests for recombination events within alleles were conducted with the program GENECONV (Sawyer 1989) and MaxChi2 (Maynard Smith 1992), which search for unusual substitution patterns, and RDP (Martin & Rybicki 2000) which uses a phylogenetic tree to detect anomalous regions of the alignment. These programs are implemented in the RDP3 program (Martin *et al.* 2010). These methods performed well in an assessment of fourteen recombination detection methods (Posada 2002). The default parameters were used for GENCONV and RDP, and the variable window size with a fraction of 0.10 variable sites per window was selected for MaxChi2 to reduce the risk of false negatives (see RDP3 manual). We also used GARD (Pond *et al.* 2006), which uses a genetic algorithm to search a multiple-sequence alignment for putative recombination break points, on the web-based server datamonkey (http://www.datamonkey.org).

Molecular Tests of Balancing Selection

We tested if positive selection had been historically operating on the montane vole DRB exon 2 using two approaches, first comparing relative rates of non-synonymous (dN) and synonymous (dS) substitutions across the gene (Hughes & Nei 1988), and second comparing likelihoods of codon-based models that assume positive selection or neutrality (Nielsen & Yang 1998). First,

MEGA 5.05 was used to calculate the relative rates of dN and dS according to the method of Nei, Gojobori (1986) with Jukes, Cantor (1969) correction for multiple hits for all alleles. The substitution rates were calculated separately for non-antigen-binding sites (nonABS) and 15 ABS defined by Brown *et al.* (1993). A codon-based two-tailed Z test of selection was performed on ABS, nonABS, and all codons to determine if dN≠dS. One-tailed Z tests were performed to identify the direction of selection, positive or negative (dN>dS or dN<dS). Because there was evidence of duplication at the DRB locus, we also tested substitution rates for ABS and nonABS sites separately for each putative locus.

We next determined which codon sites were under diversifying positive or negative selection by comparing the congruence of three codon-based models of sequence evolution using the server datamonkey. The three different codon-based maximum likelihood methods, SLAC (Single Likelihood Ancestor Counting), FEL (Fixed Effects Likelihood) and the less conservative REL (Random Effects Likelihood), can be used to estimate the dN/dS ratio at every codon in the alignment. These methods of detecting amino acid sites under selection have been shown to perform as well or better than the M8 model in PAML (Kosakovsky Pond & Frost 2005), and we considered a codon to be evolving under selection when it was identified as such by at least two methods (Kosakovsky Pond & Frost 2005). We also employed MEME (mixed effects model evolution) which combines fixed effects at the level of a site with random effects at the level of branches, in effect allowing some branches to be under positive selection while others are under negative selection. This method is most appropriate to detect episodic diversifying selection affecting individual codon sites (Kosakovsky Pond *et al.* 2011), which may be expected to occur with the introduction of novel parasite species or genotypes.

Phylogenetic Tests of Balancing Selection across Rodent Species

To further examine whether historic balancing selection has been operating on the *M. montanus* DRB locus, we constructed phylogenetic trees to identify the position of *M. montanus* DRB alleles relative to those of other rodent species and to test for trans-species persistence of particular DRB alleles. We used NCBI BLAST searches in Geneious v5.5 to identify and extract DRB exon 2 sequences in 15 rodent species available and 2 non-rodent outgroups (tree shrew, *Tupaia belangeri* and white-tailed deer, *Odocoileus virginianus*; see next section and Table S2.1 for the complete dataset). We constructed a neighbor-joining phylogenetic tree based on nucleotide divergence according to the Kimura two-parameter distance using bootstrap analysis with 5,000 replicates in MEGA 5.05. Three alleles were selected randomly from each rodent species. The NJ tree was based on the shared sequence section (171bp) of all alleles and rooted with the tree shrew (Genbank ref# GU825729) and white-tailed deer (Genbank ref# AF082161).

Comparing Patterns of Selection and MHC Diversity across Rodents

Signatures of selection may be weaker in species with populations subject to high degrees of genetic drift, such as species that have undergone population bottlenecks (Miller & Lambert 2004; Ejsmond & Radwan 2011), or in cyclic species with frequent prolonged low density periods (Oliver *et al.* 2009a). Selection may also differ among species due to gene copy number, whereby duplicated genes may be specialized for specific biological functions, and thus experience stronger negative selection than single loci (Jarvi *et al.* 2004; Axtner & Sommer 2007; Burri *et al.* 2010). To test for patterns of selection across rodent species with varying population dynamics and gene copy numbers, we collected sequence information on montane voles and the 15 additional rodent species with data available for the DRB locus using Geneious

and GenBank. Overall, MHC data comprised 27 studies representing 601 alleles from 5565 individual animals and 16 species. We aligned alleles using Clustal W in MEGA 5.05 and removed all duplicate alleles, pseudogenes, and alleles with insertions or deletions, as these may be nonfunctional. We then trimmed alleles to 171bp (codons 22-78) for comparable results across species, and assigned ABS at 15 codon sites based on Brown *et al.* (1993). We also compiled neutral cytochrome B sequences to control for demographic effects and for which Genbank data was available, comprising 436 haplotypes from nine species. These sequences were compiled in the same way as the MHC data for comparison, whereby we only included unique haplotypes.

For all 16 rodent species we compiled five dependent variables to quantify MHC diversity and indicators of selection: dN/dS at ABS and a measure analogous to Tajima's D (indicators of balancing selection), dS-dN at non-ABS sites (indicator of purifying selection), average nucleotide divergence (π) and number of alleles (indicators of genetic diversity) Additional details on these variables are provided in Table 2.4. We used a measure analogous to Tajima's D (pseudo-Tajima's D) not as a population statistic but as a means of calculating how much phylogenetic structure there was per amount of allelic (haplotype) diversity. We were concerned with the patterns of signals, not with the statistical significance of the signals themselves.

To examine the influence of species-specific population dynamics and gene copy number on the strength of selection and MHC diversity, we assigned each species to a categorical measure of population dynamics using three levels: stable, multiannual cycles, or bottlenecked. We delineated species to these categories based on published studies of their population ecology derived from systematic literature searches on Web of Science using the search terms "species

binomial name and pseudonyms" and "pop*" or "cyclic" or "bottleneck". Species assigned to multiannual cycles were those that fluctuated in abundance by a factor of more than 2 at a minimum of every 2 years (Table S2.1). Species assigned to the bottlenecked category were those that underwent a reduction in population size of greater than 80% or that could be observed in the molecular history of the species (Table S2.1). If a species was found to experience multiannual cycles or bottlenecks in one population, we classified the entire species by that category. Species for which studies of population size or abundance over time reported no evidence of multiannual cycles or bottlenecks were assigned to the 'stable' category. A minimum of two studies per rodent species detailing population dynamics was required to classify species. Genetic variables, including the number of DRB loci per species, were compiled from the studies that provided sequence information. These were also found with systematic literature searches on Web of Science using the search terms "species binomial name and pseudonyms" and "MHC" and "class II". We then restricted our search to studies focusing on exon 2 of the DRB gene.

For each species in the analysis, we compiled data on several additional variables that could influence measures of MHC diversity and evolution. First, to control for the effect of sampling effort on estimated genetic diversity, we recorded the number of individuals sampled per study for each species. Second, body mass is known to scale with many life-history traits, including population size, reproductive rate, and evolutionary rate (Martin & Palumbi 1993), and thus was included as a covariate. Body mass (g) data were extracted from a previously-published database of mammalian traits (PanTHERIA; Jones *et al.* 2009). Third, effective population size (Ne) can impact genetic diversity by affecting the realized mutation rate, strength of selection, and the amount of genetic drift experienced by a population (Hartl & Clark 1997);

further, Ne has been shown to correlate positively with measures of genetic diversity (Frankham *et al.* 2002). As we could find Ne estimates for only six species in the literature (Sommer *et al.* 2002b; Zheng *et al.* 2003; Galbreath & Cook 2004; Milishnikov 2004; Busch *et al.* 2007; Winternitz unpublished data), we instead used census population size as a correlate of effective population size (Møller *et al.* 2008; Garamszegi & Nunn 2011), with the caveat that the ratio of Ne/N is approximately 0.1 (Frankham 1995). Population size for each species was estimated by multiplying average population density (individuals per km^2) from the PanTHERIA database by the species geographic range size (km^2) extracted from spatial data provided by the 2010 IUCN Red List (http://www.iucnredlist.org/technical-documents/spatial-data#mammals), following a similar approach taken by previous comparative analyses (Nunn *et al.* 2003, Nunn *et al.* 2005). Our full comparative dataset can be found in Table S2.1. Variables were Log-transformed or square root arc-sin transformed (dS rate data; Sokal & Rohlf 1995) when necessary to meet normality assumptions.

We tested for effects of population dynamics and gene duplication on signatures of selection and MHC diversity. Model selection was performed using both GLM analysis and phylogenetic least squares (PGLS) regression analyses to control for effects of phylogeny. Our initial full models to explain pseudo-Tajima's D, dN/dS, dS-dN, log number of alleles and π included the following species trait predictor variables: population dynamics, number of DRB loci, log body mass (g), log population size, and log sample size. For the GLM analysis, initial models were simplified using an exhaustive search based on AICc in the *glmulti* package (Calcagno & de Mazancourt 2010) in R (Team 2012). Models with strong support (AICc weights within 4 units of the lowest AICc value; Burnham & Anderson 2002) and significant *P*-values after correcting for multiple hypotheses testing using the False Discovery Rate

(implemented in the program QVALUE; Storey 2002) were retained in the confidence set shown in Tables S2.3-S2.4. The PGLS regression was conducted using the *caper* package in R (Orme 2011) using Pagel's λ statistic to account for phylogenetic non-independence in the predictor and response variables. Rodent phylogeny was constructed using information from the mammalian supertree (Bininda-Emonds *et al.* 2007) and polytomies were made binary for PGLS using the *multi2di* function in the *Ape* R package (Paradis *et al.* 2004). We tested for phylogenetic signal on each predictor variable as well as on dS at ABS and nonABS using two methods: Blomberg's *K* and Pagel's λ. Blomberg's *K* (Blomberg *et al.* 2003), was computed using the *picante* package (Kembel *et al.* 2010) in R; Blomberg's *K* describes phylogenetic signal of continuous traits, where $K = 1$ indicates a trait is evolving under Brownian motion (stochastic evolution) and $K < 1$ indicates a trait has less phylogenetic signal than expected. Pagel's λ (Pagel 1992) tests for phylogenetic signal through a variance-covariance structuring of the trait data with the species tree, and returns a value of λ that describes the phylogenetic signal of the data. When $\lambda = 0$, the tree is star-shaped and all trait values are independent. When $\lambda = 1$, the original tree best explains the phylogenetic structure of the data (Freckleton *et al.* 2002).

RESULTS

Allele Validation

Amplification barcodes were identified in 30,697 reads, of which 21,072 (69%) were assigned to 261 out of an initial 284 samples corresponding to an average of 80.7 (SD = 88.0) reads per individual. Reads were not assigned to 23 individuals due to degraded DNA. Based on the criteria of a minimum of 54 reads for reliable genotyping (see Appendix B), 127 individuals were retained and final genotypes were based on 17,874 reads (58% of the initial number), with the mean coverage at this stage of 140.7 reads (SD = 93.3, range 54-526). Originally we

identified 82 putative alleles, but 61 were removed from the analysis because they were classified as artifacts or deemed potentially not functional (following criteria described in Materials and Methods). We detected a total of 21 DRB alleles among the 127 individual montane voles with sufficient read coverage. Sequences of all alleles were deposited in the Sequence Read Archive (Accession Numbers XXXXX) following the nomenclature of Klein *et al.* (1990) and labeled Mimo-DRB*01 through Mimo-DRB*21. BLAST search confirmed the homology to other rodent *DRB* sequences for all alleles (99 to 91% concordance).

The mean number of alleles per individual was 2.19 (SD = 0.67), and the maximum number of alleles in a single individual was four, suggesting that the MHC DRB locus in montane voles is duplicated. We found no evidence for genetic recombination or gene conversion events between aligned sequences and/or ancestral relicts of such events using GENECONV, MaxChi2, RPD, or GARD. Results comparing alleles detected with cloning and 454 sequencing (14/20 individuals had sufficient clones and read coverage for genotyping) indicated 74% congruence between genotypes determined by the two methods. Not all alleles detected using 454 sequencing were detected with cloning, as only 6-10 colonies per individual were selected, resulting in a 47-76% probability of selecting every allele at least twice. However, all alleles detected in at least two individual PCRs with cloning were also detected in those same individuals using 454 sequencing.

Allelic diversity and clustering

There were 67 variable sites out of 171 total sites, with a total of 82 mutations. The nucleotide diversity across all sequences (π, average number of substitutions per site between sequences) was 0.13299 (± 0.016 SD). The average number of nucleotide differences between sequences

was 22.72 with mean pairwise nucleotide distances of 0.185. The average number of AA differences between sequences was 6.5 and mean pairwise AA distance computed for all sites ranged from 0 to 0.726, with the average 0.294.

Alleles clustered into three main groups based on nucleotide divergence using Kimura two-parameter distances considering all sites, only non-antigen binding sites, and using a Bayesian approach considering all sites (Fig. 2.1). Alleles Mimo-DRB*05, Mimo-DRB*07, and Mimo-DRB*15 formed a non-monophyletic group. As allele number per individual (1-4 alleles) indicated a duplicated locus, the phylogenetic clustering of alleles into four main clusters does not unambiguously represent specific loci. Based on the evidence of two putative loci, we combined the four clusters into two main groups based on shared attributes of classical DRB alleles, though we acknowledge these are not strictly monophyletic. This was done by grouping Cluster 1 into Group 1, a strongly supported monophyletic clade found in the majority of individuals (see below), and grouping everything else into the non-monophyletic Group 2. We also present summary statistics for the individual clusters (Cluster 1-4), as these may be biologically relevant groups (e.g. recently diverged alleles).

Mean nucleotide and amino acid divergence was smaller for Group 1 than Group 2 (Table 2.1). Similarly, Cluster 1 had the lowest nucleotide and amino acid divergence, followed by Cluster 2 and Cluster 3, with Cluster 4 having the greatest divergence (Fig. 2.2). Of the 127 individuals that provided reliable genotype data based on read number, 60% had alleles from both Group 1 and 2. Six percent of individuals had no alleles from Group 1 and 34% had no alleles from Group 2, indicating that either a PCR artifact such as allelic dropout (where primers fail to amplify specific alleles) is present or that montane voles vary in their gene copy number. It is possible that more than two DRB loci are present in *M. montanus* and specific loci amplify

more effectively. However, this would indicate extreme allelic dropout, as a maximum of four alleles were found per individual. It is more likely that montane voles display gene copy number variation, as is found in other species (carnivores, Bowen *et al.* 2004; primates, Bontrop 2006; rodents, Kloch *et al.* 2010).

Molecular evidence of balancing selection

Neither nucleotide nor amino acid pairwise differences were higher at ABS (10.5±1.7 and 4.8±0.9) than at nonABS (12.2±1.8 and 6.5±1.3), indicating that measures of selection were not necessarily stronger for sites involved in antigen recognition. The ratio of dN/dS at both the ABS and nonABS was less than 1, indicating no support for balancing selection (Table 2.2). Considering only nonABS codons (N=42) and all codon sites (N=57) the ratio of dN/dS was significantly less than 1, indicating purifying selection in non-directed Z tests ($\omega \neq 1$) (nonABS, P = 0.03; All sites, P = 0.02; Table 2.2). When considering all sites (N=57) both Groups 1 and 2 indicated purifying selection based on the directed Z test ($\omega < 1$) (Group 1, Z =1.829, P = 0.035; Group 2, Z =2.127, P =0.018). The codon-based maximum likelihood methods also detected signatures of purifying selection at a greater frequency than positive selection. Only 1 codon (codon 26) was identified as positively selected (posterior probability >95%) based on congruence with two or more methods (FEL and MEME, Table 2.3). In contrast, based on results from MEME, SLAC, FEL, and REL, 3 codons were identified as undergoing episodic diversifying selection (codons 26,30,57) and 8 were identified as undergoing purifying selection at nonABS sites (codons 29,36,42,43,49,54,58,62; Table 2.4).

Phylogenetic Evidence of Balancing Selection

To compare *M. montanus* DRB alleles to those of other rodents and to examine evolution in MHC allelic lineages, we constructed a phylogenetic tree of 16 rodent species using DRB sequence data (Fig. 2.3). *M. montanus* alleles clustered among alleles from its nearest relatives, the bank vole (*Myodes glareolus*) and root vole (*Microtus oeconomus*). Alleles in Group 1 (Cluster 1) formed a monophyletic clade and clustered with *M. glareolus* while alleles in Group 2 (Cluster 2-4) were dispersed among alleles of both *M. glareolus* and *M. oeconomus*. Cluster 2 (Mimo-DRB*03, Mimo-DRB*19, Mimo-DRB*21) and Cluster 3 (Mimo-DRB*08, Mimo-DRB*12, Mimo-DRB*16, Mimo-DRB*20) formed monophyletic clades, but Cluster 3 included a *M. oeconomus* sequence. As observed from the neighbor joining allele tree of *M. montanus* (Fig. 2.3), Cluster 4 (Mimo-DRB*05, Mimo-DRB*07, Mimo-DRB*18) was not monophyletic and its sequences were dispersed between those of montane and bank voles.

Across all rodents, there was evidence of trans-species evolution at the DRB, indicating that ancestral alleles with important functions are retained in descendant species (Takahata 1990), although similar patterns could arise from convergent evolution. Alleles from some distantly related species, including tuco tucos (*Ctenomys talarum*), the Malagasy giant rat (*Hypogeomys antimena*), the banner-tailed kangaroo rat (*Dipodomys spectabilis*), and the Eurasian beaver (*Castor fiber*) formed monophyletic clades, while other alleles were shared among close relatives (ex. *Spermophilus citellus* and *S. suslicus*, *Apodemus flavicollis* and *A. sylvaticus*) (Fig. 2.3). Some rodent DRB alleles were widely dispersed across multiple species (*Gerbillurus paeba*, *Rhabdomys pumilio*, *Rattus rattus*, *Peromyscus maniculatus*, *A. flavicollis*, *A. sylvaticus*, and *Arvicola terrestris*), indicating that balancing selection may have preserved alleles across speciation events.

Predictors of Selection and MHC Diversity across Rodents

Rates of nonsynonymous (dN) and synonymous substitutions (dS) across 601 DRB exon 2 sequences from 16 rodent species were associated with categorical measures of population dynamics and the presence of gene duplication. Most rodent species included in this study showed a significantly elevated rate of dN to dS (dN/dS>1) at the functionally important ABS (Fig. 2.4A), consistent with other work showing that historic positive selection acts to increase diversity at these sites (Bernatchez & Landry 2003; Sommer 2005). However, four out of five species with 'cyclic' population dynamics (and potentially lower effective population sizes) did not show evidence of positive selection on MHC based on non-directed Z-tests ($P > 0.05$). Testing for negative selection with directed Z-tests at non-ABS sites across species revealed significantly higher dS than dN in four out of six species with duplicated loci, consistent with expectations that purifying selection may be the norm for duplicated MHC loci (Fig. 2.4B, Table S2.2).

To identify predictors of signals of selection and diversity at the MHC while controlling for shared evolutionary history and other species traits, we performed GLMs and PGLS linear models. The confidence set of models for all five predictor variables (dN/dS at ABS, pseudo-Tajima's D, dS-dN at non-ABS, average nucleotide divergence (π), and number of alleles) had significant intercepts and full model Q values (P-values adjusted by the False Discovery Rate to control for multiple testing) less than 0.05 (Table S2.3), indicating that these models were valid. The confidence sets of models showed no evidence for phylogenetic signal explaining the response variables after applying PGLS methods (Table S2.4), indicating that it was not necessary to account for phylogeny in these analyses. This was generally the case for the predictor variables as well, as most species traits did not show phylogenetic signal using

Blomberg's *K* or Pagel's λ (Table S2.5). However, the presence of gene duplication had a significant λ value (λ = 0.81, *P* = 0.00), indicating that more closely related species were more likely to share duplication status than expected by chance.

Of the five dependent variables we examined in our multivariate models, pseudo-Tajima's D, average nucleotide divergence (π), and negative selection (dS-dN) had models with statistically significant predictor variables (Table S2.6). In particular, pseudo-Tajima's D was significantly greater for MHC than neutral genes and greater for species that underwent multiannual population fluctuations than for species characterized as bottlenecked or stable (Fig. 2.5A), indicating that signals of balancing selection on MHC were stronger for cyclic rodent species. An analysis of variance (ANOVA) on pseudo-Tajima's D yielded significant variation among species by population dynamics ($F_{2,15}$= 6.58, *P* =0.01), and a post hoc Tukey test further showed that cyclic species had significantly greater pseudo-Tajima's D than stable and bottlenecked species at $P < 0.05$; (Fig. 2.5A). When considering effects of gene duplication on measures of selection and genetic diversity across rodent species, both nucleotide diversity and the strength of negative (purifying) selection increased with the number of DRB loci (our measure of MHC gene duplication; Fig. 2.5B). No other predictor variables were significantly associated with measures of genetic diversity or selection at the MHC.

DISCUSSION

We found high levels of genetic diversity at the DRB locus in montane voles (21 alleles detected in 127 individuals; close to the average of the 16 rodent species from 31 populations used in this study based on a log-allele to log-sample size regression: ($y = 0.207x + 0.74$; $R^2 = 0.093$)). Because this species undergoes high amplitude population fluctuations that could lead to

diversity loss through genetic drift, we further tested for evidence of historic balancing selection to explain the maintenance of this high diversity. Our results showed that the DRB in montane voles lacks molecular signals of balancing selection based on dN/dS ratios, but does show evidence of balancing selection based on the trans-species persistence of alleles. In other words, because montane vole alleles also appear across multiple rodent species, this implies these alleles have persisted longer than neutral polymorphisms (Takahata & Nei 1990) due to balancing selection retaining adaptive alleles. Analysis of 650 DRB sequences across all 16 rodent species studied to date supports the conservation of alleles across species, a pattern that diverges from the DQA (a different MHC II gene) which does not show clear evidence for trans-species polymorphism in rodents (Pfau *et al.* 1999). Whether this difference in allelic preservation is due to biological function or architecture of the specific genes remains to be determined. We also found no evidence of recombination at the DRB in montane voles, in accord with results from six other mammal species (Furlong & Yang 2008), indicating that recombination has not made significant contributions to DRB variation. However, recombination was found at the DQA locus in three Old World vole species (Bryja *et al.* 2006), highlighting that different mechanisms could be responsible for generating high diversity in different MHC loci, despite their similar functions and close proximity to each other.

We found strong evidence for purifying selection operating on the DRB locus of *M. montanus* using multiple codon-based likelihood methods. This result runs counter to evidence for balancing selection that appears to be a rule for DRB evolution in other mammal species (Bernatchez & Landry 2003). In montane voles, purifying selection appears to be acting at eight non-ABS sites (codons 29, 36, 42, 43, 49, 54, 58, and 62) as detected by the codon-based likelihood methods used here. Importantly, two non-ABS sites (codon 36 and 54) also showed

signals of purifying selection in six other mammal species (Furlong & Yang 2008), raising the question of why purifying selection might operate on the MHC. One answer might be that conserved regions that experience purifying selection are important for the basic structural elements of the antigen binding sites, beyond the more variable sites that recognize specific epitopes. Purifying selection might also occur when duplicated loci develop specific roles for resistance to specific pathogens or mate choice and undergo reduced evolutionary rates of selection (Jordan *et al.* 2004). A third explanation could be that species subject to strong genetic drift experience the loss of rare alleles from newly duplicated loci, thus resulting in apparent negative selection.

Our comparative analysis examined whether other rodent species show weak molecular evidence of balancing selection and strong evidence of purifying selection at the MHC, similar to *M. montanus*. We found some evidence that cyclic species (those that undergo regular population fluctuations, such as montane voles) may experience weaker positive selection than non-cyclic species. On the other hand, other studies on rodents showed that high variance in population size does not negatively affect genetic diversity at neutral markers (Berthier *et al.* 2006). In fact, some previous studies found high neutral genetic diversity in periodically fluctuating rodent populations and concluded that increased gene flow through movement of individuals with novel genotypes during the low abundance phase (when population structure is more fluid) could counter the force of genetic drift (Ims & Andreassen 2005; Aars *et al.* 2006), pointing to the importance of metapopulation dynamics. Indeed, high amplitude fluctuations in abundance might cause an increase in genetic diversity if dispersal is negative density-dependent and if alleles invade adjacent populations during the low phase in population cycles (Ehrich *et al.* 2009).

Species that undergo severe and/or repeated population bottlenecks could experience different evolutionary forces than those that undergo more regular population cycles. In fact, cyclic populations tend to have relatively large population sizes and are characterized by interconnected metapopulations with frequent opportunities for gene flow (Berthier *et al.* 2006). In contrast, bottlenecked populations tend to be isolated and without opportunity for rescue effects to replenish lost alleles. In our comparative analysis, relatively few species were categorized as bottlenecked and this small sample size limited the power of GLMs to detect impacts of bottlenecks on the strength of selection or genetic diversity. An earlier meta-analysis focusing on bottleneck effects found that both neutral and non-neutral diversity were lower in bottlenecked species, and further showed that two measures of MHC diversity (heterozygosity and allelic diversity) were reduced 15% more than neutral genetic diversity, possibly due to the combined effects of genetic drift and negative frequency-dependent selection acting to imbalance the frequency of alleles (Sutton *et al.* 2011).

Our comparative analysis provided strong support for an association between gene duplication and greater measures of purifying selection and allelic diversity at the DRB. This result is most consistent with the hypothesis that gene duplication allows diverse alleles to take on specific roles and purifying selection acts to maintain functional divergence between alleles on different copies of the locus (Hughes & Friedman 2004; Axtner & Sommer 2007). Other studies have shown that duplicated DRB loci can be nonfunctional (Axtner & Sommer 2007; Oppelt *et al.* 2010) but their RNA products may still be expressed (Zagalska-Neubauer *et al.* 2010). Therefore, the functional significance of alleles from duplicated MHC loci should be confirmed using observational or experimental associations with parasite infection status. As multiple MHC loci have been reported from different taxonomic groups (fish, Reusch *et al.*

2004; mammals, Bryja *et al.* 2006; birds, Zagalska-Neubauer *et al.* 2010), and these loci could potentially contribute to increasing allelic diversity vital for immune function, it is important to consider the potential causes behind MHC gene copy number variation. At present, it remains an open question as to what species traits, which might include life history traits, population size and MHC architecture, are most strongly correlated with MHC diversity and gene duplication.

Our analysis showed that species with demographic histories characterized by population fluctuations (both cyclic and bottlenecked species) show higher values of pseudo-Tajima's D (Garrigan & Hedrick 2003). These results suggest that fluctuating populations have greater balancing selection, a conclusion that is supported by the difference in pseudo-Tajima's D estimates between adaptive MHC genes and neutral mitochondrial cytochrome B genes. It is important to note that tests of selection that rely on allele frequencies or heterozygosity are subject to the confounding effect of demographic processes on small populations, which can mimic the effect of selection by removing rare alleles (and genotypes), inflating the number of heterozygotes and balancing allele frequencies. Thus, we echo recommendations made by others to compare patterns of variation at adaptive genes with those of neutral markers, as both will be impacted by population dynamics, and to look for significant departures from adjusted neutral expectations (Bernatchez & Landry 2003; Piertney & Oliver 2006; Bryja *et al.* 2007).

In summary, our study provides empirical support for gene duplication as a mechanism that could maintain genetic diversity at the MHC in montane voles that undergo population fluctuations, and corroborates this result using evidence from multiple rodent species in a phylogenetically controlled analysis. More generally, purifying selection acting on duplicated loci could cause the high divergence between alleles observed among species with duplicated DRB genes. In addition, we found evidence that population demographics influence the signal

of historic balancing selection, causing reduced positive selection but increased balancing selection in cyclic rodents. These results have implications for tests of selection, as patterns of natural selection in wild populations are not independent of demographic history.

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TABLES

Table 2.1. Summary of sequence and allelic diversity divided by group and cluster for all 21 *Microtus montanus* DRB alleles. Nuc. refers to nucleotide, AA refers to amino acid.

Assemblage	Mean nuc. distance (SE)	Mean AA distance (SE)	Total allele count (%)	No. animals with alleles
Group 1	0.02 (0.01)	0.02 (0.01)	179 (63.7%)	119
Group 2	0.32 (0.05)	0.67 (0.15)	102 (36.3%)	84
Cluster 1	0.02 (0.01)	0.02 (0.01)	179 (63.7%)	119
Cluster 2	0.02 (0.01)	0.02 (0.02)	6 (2.1%)	6
Cluster 3	0.02 (0.01)	0.04 (0.02)	24 (8.5%)	25
Cluster 4	0.10 (0.02)	0.2 (0.07)	72 (25.6%)	65
All	0.19 (0.03)	0.29 (0.07)	281	127

Table 2.2. Non-synonymous (dN) and synonymous (dS) substitutions (± S.E. from 5000 bootstraps) as well as their ratio in ABS and non-ABS based on sites defined by Brown et al. (1993). *M. montanus* alleles are assembled into 2 groups (shaded gray) and 4 clusters based on phylogenetic clustering.

Region/Assembly	dN	dS	dN/dS	P(w≠1)	P(w<1)
ABS N=15					
Group 1	0	0.067±0.058	0.000	0.250	0.125
Group 2	0.246±0.084	0.340±0.178	0.722	0.488	0.247
Cluster 1	0	0.067±0.058	0.000	0.238	0.125
Cluster 2	0	0.047±0.052	0.000	0.376	0.186
Cluster 3	0	0	NA	1.000	1.000
Cluster 4	0.225±0.077	0.276±0.170	0.817	0.763	0.382
All	0.311±0.118	0.407±0.167	0.765	0.585	0.291
Non-ABS N=42					
Group 1	0.009±0.005	0.043±0.025	0.214	0.193	0.097
Group 2	0.108±0.025	0.310±0.104	0.349	0.073	**0.034**
Cluster 1	0.009±0.005	0.043±0.025	0.214	0.202	0.097
Cluster 2	0.012±0.009	0.039±0.032	0.303	0.406	0.202
Cluster 3	0.020±0.011	0.026±0.027	0.782	0.809	0.404
Cluster 4	0.045±0.021	0.065±0.038	0.682	0.583	0.290
All	0.084±0.020	0.237±0.064	0.353	0.065	**0.030**
All sites N=57					
Group 1	0.007±0.003	0.049±0.023	0.138	0.076	**0.035**
Group 2	0.19±0.026	0.317±0.085	0.440	**0.036**	**0.018**
Cluster 1	0.007±0.003	0.049±0.023	0.138	0.076	**0.040**
Cluster 2	0.009±0.006	0.042±0.025	0.213	0.223	0.114
Cluster 3	0.015±0.009	0.018±0.019	0.850	0.864	0.432
Cluster 4	0.087±0.024	0.117±0.047	0.738	0.490	0.247
All	0.131±0.026	0.276±0.060	0.474	0.039	**0.020**

Abbreviations: ABS, antigen-binding sites; N, number of codons in each category; P, probability ($\alpha<0.05$) that dNand dS are different ($w\neq1$) and that dN<dS ($w<1$) using a Z-test. Significant P-values are in bold.

Table 2.3. Codons under selection based on significance determined from at least two of three different codon-based maximum likelihood methods: Single Likelihood Ancestor Counting, S; Fixed Effects Likelihood, F; and the less conservative Random Effects Likelihood, R. Episodic selection is based on significant results from mixed effects model evolution, M. Significance was determined with $\alpha < 0.05$ or Bayes Factor > 50, respective to the likelihood model.

Type of Selection	Codon	Models (S,F,R,M)
Episodic Diversifying	26	M
	30*	M
	57	M
Positive	26	F,M
Negative	29	F,R
	36	F,R
	42	S,F,R
	43	S,F,R
	49	S,F,R
	54	F,R
	58	S,F,R
	62	F,R

*Denotes ABS site based on Brown et al. (1993).

Table 2.4. Dependent variables used in the comparative analysis representing MHC diversity and indicators of selection.

Dependent variable	Indicates	Interpretation	Prediction
dN/dS at ABS	Historic balancing selection	High dN/dS indicates positive selection at sites that recognize pathogens	Higher in species with strong balancing selection; reduced in species with lower effective population size
Tajima's D*	Relatively recent balancing selection	D>0 (equal frequency of alleles) indicates balancing selection[a] or a recent population reduction[b] D<0 (excess of rare alleles) indicates purifying selection or population expansion[c]	Higher D in species undergoing strong balancing selection, or in species with recent reductions in population sizes
dS-dN at non-ABS	Purifying selection	High dS-dN indicates greater rates of silent substitutions that preserve amino acid configurations	Higher in species with duplicated loci
Nucleotide diversity (π)	Genetic diversity	Extent of variation between sequences	Higher in species with duplicated loci; higher in species with larger effective population size
Number of alleles	Genetic diversity	Number of unique sequences	Higher in species with duplicated loci; higher in species with larger effective population size

* Tajima's D is a population-level estimate of selection; however, most studies sampled multiple populations and did not specify private alleles, so the final dataset represents Tajima's D calculated at the species level. To test the robustness of our conclusions, separate estimates of Tajima's D were calculated per study as a proxy for population when species data included multiple studies.

[a] Garrigan and Hedrick 2003

[b] Maruyama and Fuerst 1985

[c] Maruyama and Fuerst 1984

FIGURE LEGENDS

Figure 2.1. Neighbor-joining (NJ) phylogeny and Bayesian estimated posterior probabilities for all 21 *M. montanus* (Mimo-DRB) alleles using a tree shrew (*Tupaia belangeri*) sequence as an outgroup (GenBank accession no. GU825729). We constructed the NJ phylogeny with the Kimura-two parameter nucleotide distance considering all sites. The *inset* is the NJ phylogeny considering only non antigen binding sites. Node numbers indicate bootstrap support ≥ 50 (5,000 replicates) and numbers in parentheses indicate Bayesian posterior probabilities above 80%. *Scale bar* indicates the genetic distance. Alleles are partitioned hierarchically into two groups, and three monophyletic clusters and a fourth group, representing two to four putative loci.

Figure 2.2. Alignment of 21 identified amino acid sequences of the partial DRB exon 2 of the montane vole, *Microtus montanus* (Mimo-DRB). A *dot* indicates congruence with the amino acid sequence of Mimo-DRB*01. Sequences are organized hierarchically into putative loci by group (Group 1 and 2) and cluster (Cluster 1-4). Gray shading denotes antigen binding sites (ABS) according to Brown et al. (1993).

Figure 2.3. Trans-species polymorphism of the MHC class II DRB gene in 16 rodent species. The DRB tree on the right is a neighbor-joining phylogeny of all 21 *M. montanus* DRB alleles (in black) compared with 45 DRB sequences from 15 different rodent species, using a tree shrew (*Tupaia belangeri*) and white-tailed deer sequence (*Odocoiles virginianus*) as outgroups. GenBank ascension numbers are given after each species name. *M. montanus* alleles (Mimo-

DRB colored black) cluster among its nearest relatives, the bank vole (*Myodes glareolus*) and root vole (*Microtus oeconomus*). Alleles in Group 1 (Cluster 1) cluster with *M. glareolus* while alleles in Group 2 (Cluster 2-4) are dispersed among alleles of both *M. glareolus* and *M. oeconomus*. The species tree on the left was derived from the mammal supertree (Bininda-Emonds *et al.* 2007) and depicts the evolutionary divergence between species, with the *scale bar* indicating Myr. Numbers at the tips indicate the putative number of DRB loci for each species. *M. montanus* is boxed. The *scale bar* indicates genetic distance in units of nucleotide substitutions per site.

Figure 2.4. Evidence of balancing selection at the antigen binding sites (ABS) (Brown et al. 1993) and negative selection at non-ABS across 16 rodent species and *Tupaia*. A) The ratio of non-synonymous to synonymous substitutions per site (d_N/d_S) was calculated at non-ABS (open bar) and ABS (filled bar) sites separately for each species. Significant departures from neutrality (dN/dS) = 1 at the ABS were determined with codon based Z-tests using the Nei-Gojobori method with Jukes-Cantor correction in Mega 5.05. Variance was computed using the bootstrap method (1,000 replicates). *Asterisks* denote significance at $P < 0.05$ (See Table S2 for *P* values). *Dotted line* indicates neutrality (dN/dS) = 1 and open circles indicates species with cyclic population dynamics. B) Evidence of purifying selection at non-ABS across 16 rodent species and *Tupaia*. We performed codon-based Z-tests as above. The test statistic (dS-dN) was calculated for non-ABS (open bar) and ABS (filled bar) sites separately for each species. *Astericks* denote significance at $P < 0.05$ and open triangles indicate species with duplicated DRB genes (See Table S2.2 for *P* values).

Figure 2.5. Interspecific trait effects on signals of selection and nucleotide diversity at the DRB in 16 rodent species. A) Relationship between population dynamics categorized as stable, cyclic, and bottlenecked, and pseudo-Tajima's D, an indicator of historic selection and past population changes. Pseudo-Tajima's D was calculated at the species level by combining all MHC DRB sequences ('MHC Total', in gray), and at the 'population' level by calculating pseudo-Tajima's D from MHC sequences obtained in each study and taking the mean per species. Pseudo-Tajima's D was also calculated for cytochrome B sequences for the nine species with data available. *N* indicates sample size and *error bars* indicate 95% confidence intervals. B) Relationship between the number of DRB loci per species and average nucleotide divergence (π, black diamonds) and strength of purifying selection (dS-dN at non antigen binding sites, open squares). For the secondary y-axis, zero indicates equal rates of synonymous and nonsynonymous substitutions, while positive values indicate negative selection for amino-acid changing substitutions. The black dotted line is the linear best fit for π, and the gray dotted line is the linear best fit for 'purifying selection'.

FIGURES

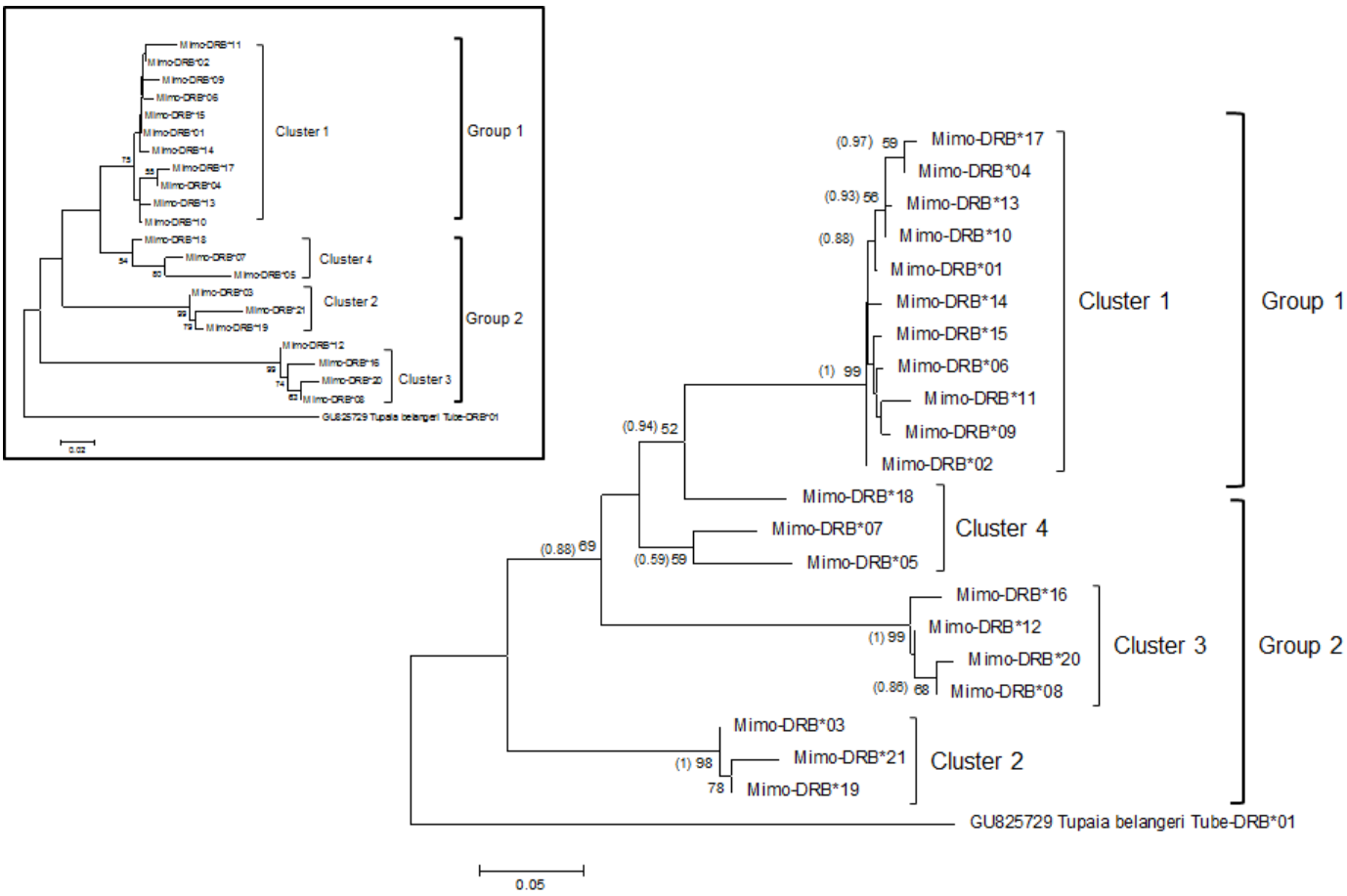

Figure 2.1.

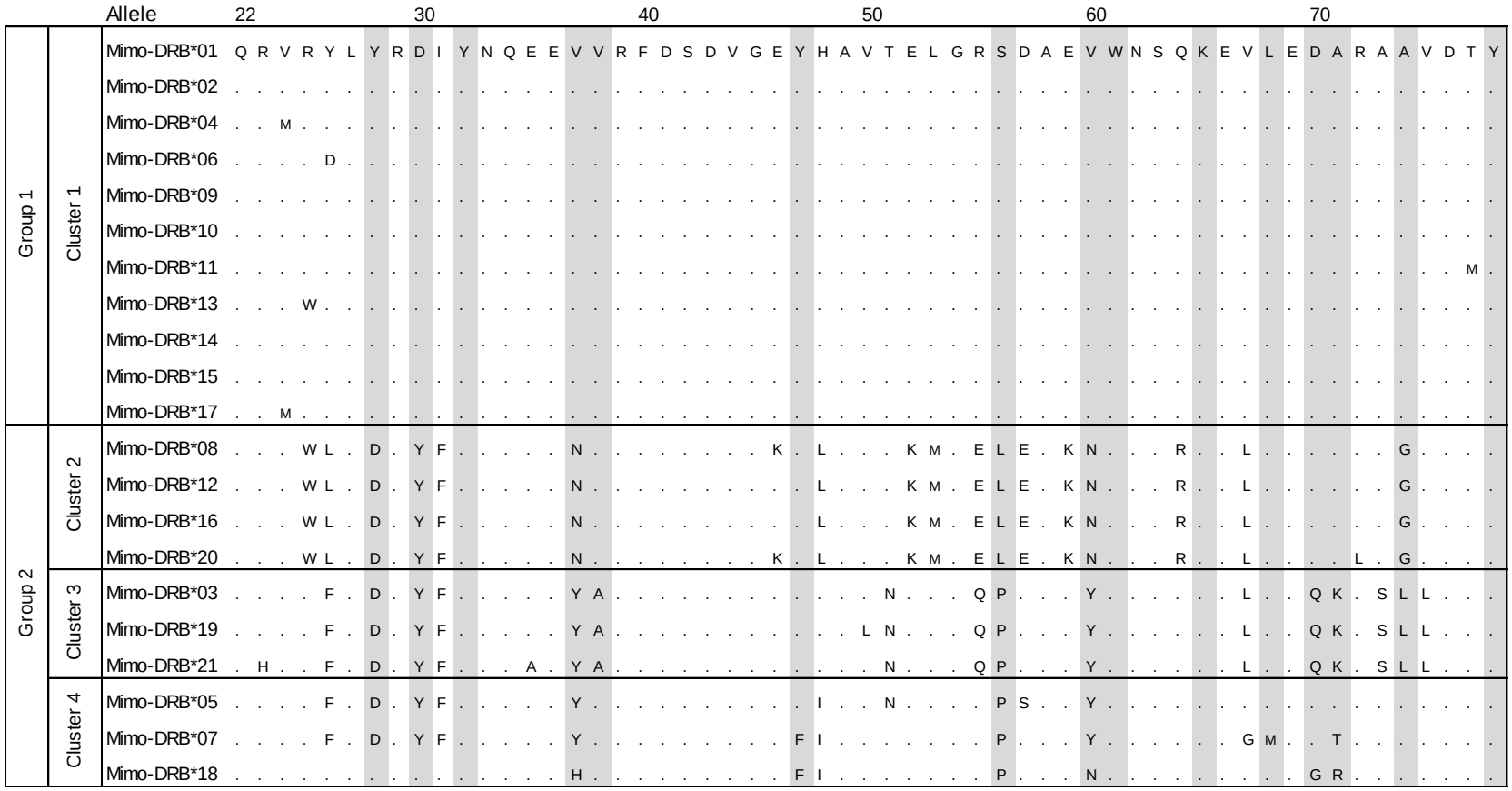
Allele
22
30
40
50
60
70
Group 1
Cluster 1
Mimo-DRB*01 Q R V R Y L Y R D I Y N Q E E V V R F D S D V G E Y H A V T E L G R S D A E V W N S Q K E V L E D A R A A V D T Y
Mimo-DRB*02
Mimo-DRB*04 M
Mimo-DRB*06 D
Mimo-DRB*09
Mimo-DRB*10
Mimo-DRB*11 M
Mimo-DRB*13 W
Mimo-DRB*14
Mimo-DRB*15
Mimo-DRB*17 M
Group 2
Cluster 2
Mimo-DRB*08 W L D Y F N K L K M E L E K N R L G
Mimo-DRB*12 W L D Y F N L K M E L E K N R L G
Mimo-DRB*16 W L D Y F N L K M E L E K N R L G
Mimo-DRB*20 W L D Y F N K L K M E L E K N R L L G
Cluster 3
Mimo-DRB*03 F D Y F Y A N Q P Y L Q K S L L
Mimo-DRB*19 F D Y F Y A L N Q P Y L Q K S L L
Mimo-DRB*21 H F D Y F A Y A N Q P Y L Q K S L L
Cluster 4
Mimo-DRB*05 F D Y F Y I N P S Y
Mimo-DRB*07 F D Y F Y F I P Y G M T
Mimo-DRB*18 H F I P N G R

Figure 2.2.

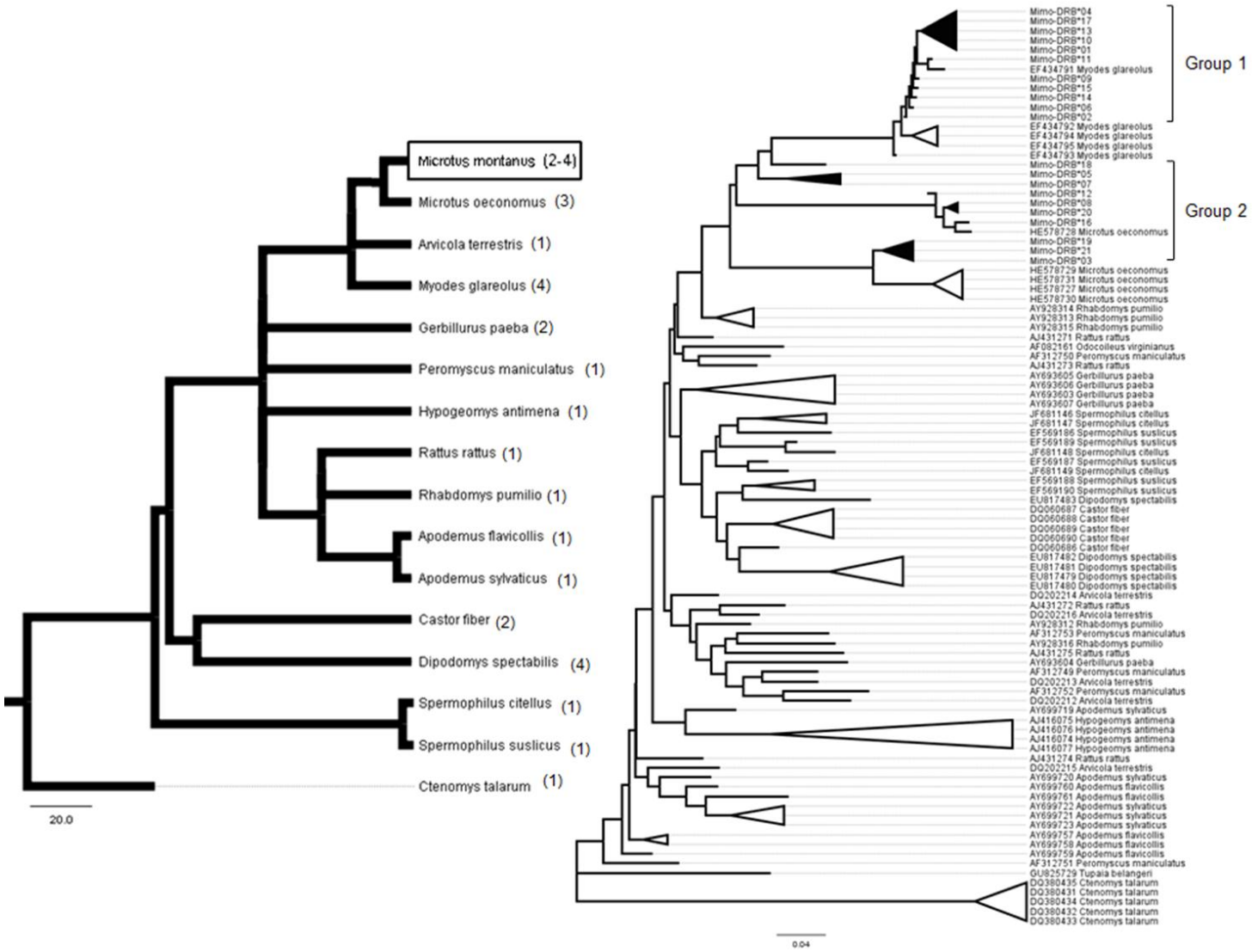
Microtus montanus (2-4)
Microtus oeconomus (3)
Arvicola terrestris (1)
Myodes glareolus (4)
Gerbillurus paeba (2)
Peromyscus maniculatus (1)
Hypogeomys antimena (1)
Rattus rattus (1)
Rhabdomys pumilio (1)
Apodemus flavicollis (1)
Apodemus sylvaticus (1)
Castor fiber (2)
Dipodomys spectabilis (4)
Spermophilus citellus (1)
Spermophilus suslicus (1)
Ctenomys talarum (1)
20.0
Group 1
Group 2
0.04

Figure 2.3.

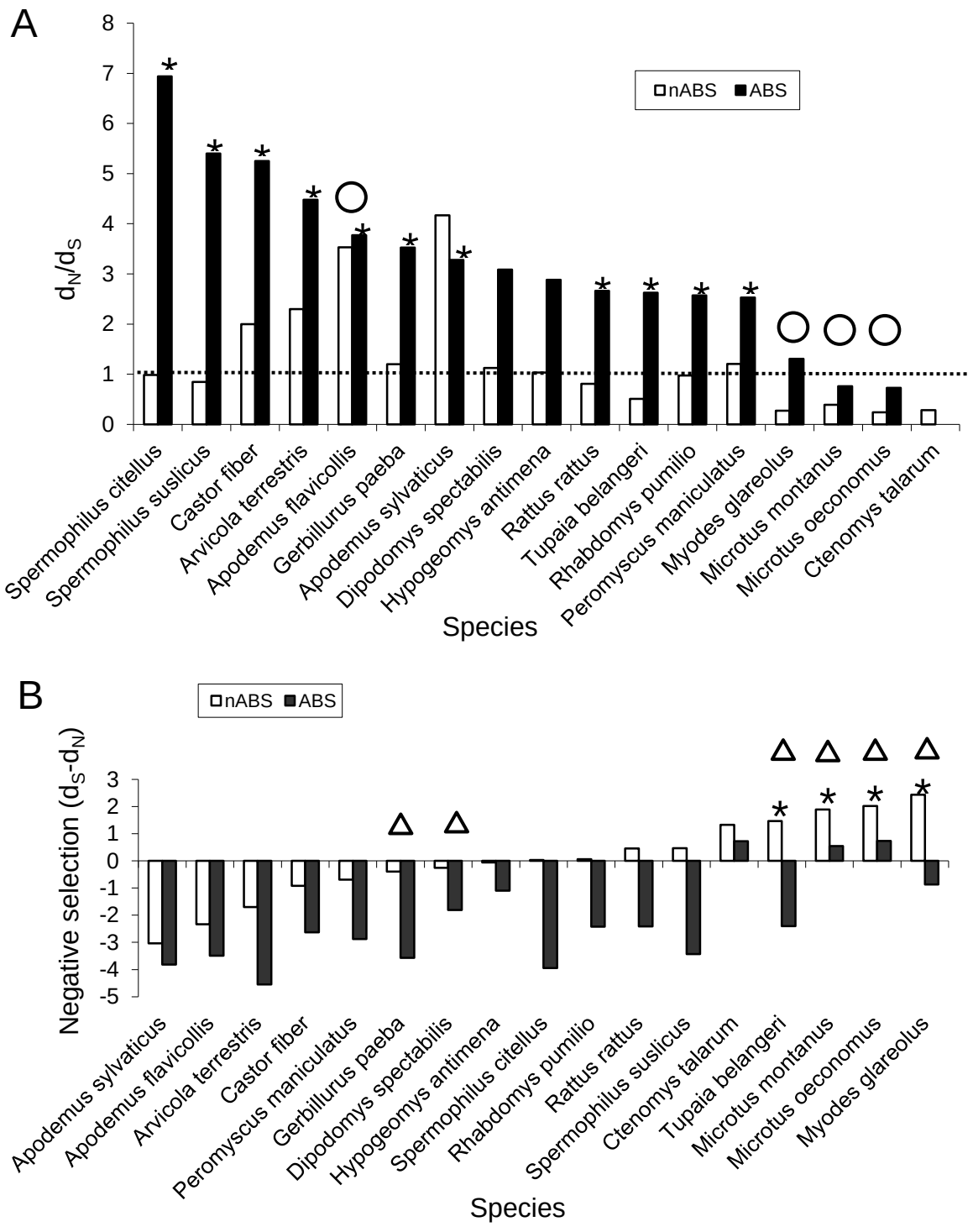
A
nABS
ABS
d_N/d_S
Spermophilus citellus
Spermophilus suslicus
Castor fiber
Arvicola terrestris
Apodemus flavicollis
Gerbillurus paeba
Apodemus sylvaticus
Dipodomys spectabilis
Hypogeomys antimena
Rattus rattus
Tupaia belangeri
Rhabdomys pumilio
Peromyscus maniculatus
Myodes glareolus
Microtus montanus
Microtus oeconomus
Ctenomys talarum
Species
B
nABS
ABS
Negative selection (d_S-d_N)
Apodemus sylvaticus
Apodemus flavicollis
Arvicola terrestris
Castor fiber
Peromyscus maniculatus
Gerbillurus paeba
Dipodomys spectabilis
Hypogeomys antimena
Spermophilus citellus
Rhabdomys pumilio
Rattus rattus
Spermophilus suslicus
Ctenomys talarum
Tupaia belangeri
Microtus montanus
Microtus oeconomus
Myodes glareolus
Species

Figure 2.4.

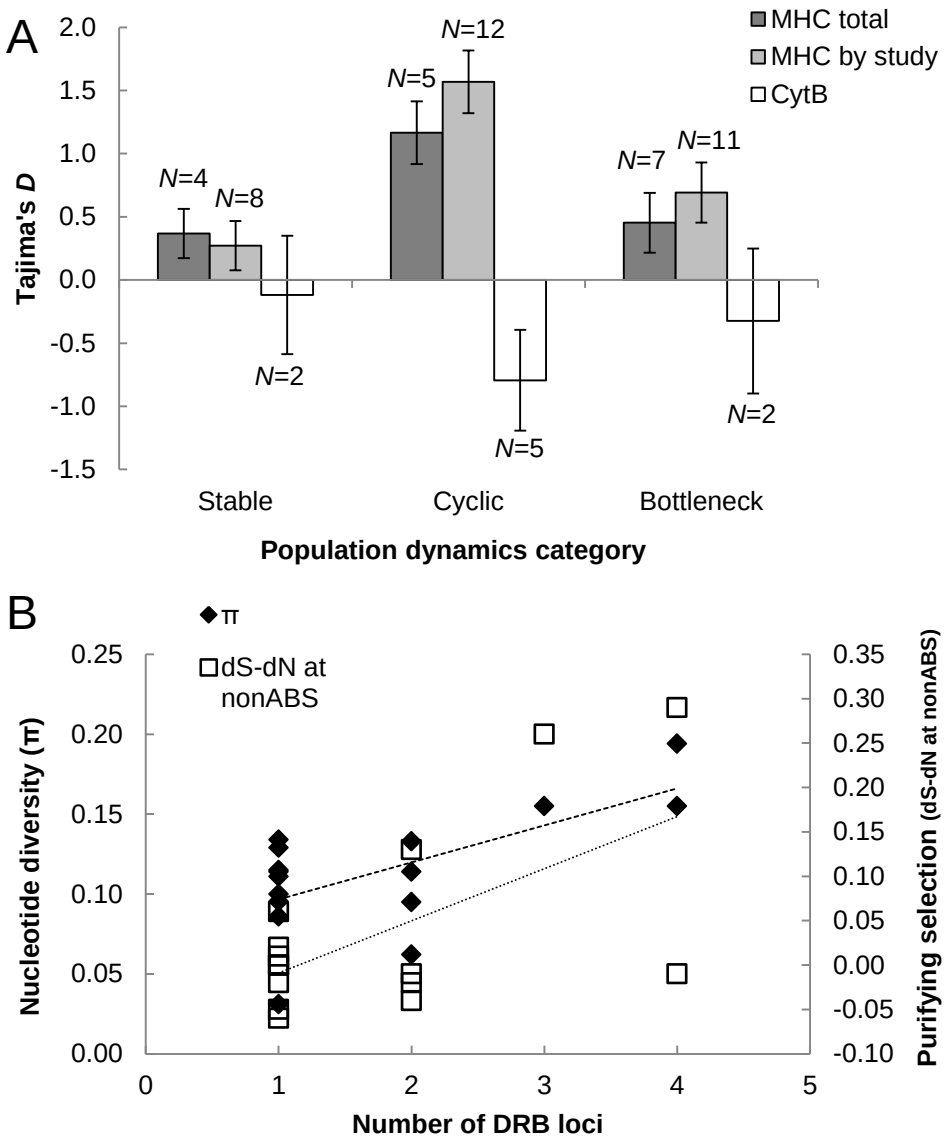
A
MHC total
MHC by study
CytB
Tajima's D
2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
N=4
N=8
N=2
N=5
N=12
N=5
N=7
N=11
N=2
Stable
Cyclic
Bottleneck
Population dynamics category
B
π
dS-dN at nonABS
Nucleotide diversity (π)
Purifying selection (dS-dN at nonABS)
0.25
0.20
0.15
0.10
0.05
0.00
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.05
-0.10
0
1
2
3
4
5
Number of DRB loci

Figure 2.5.

CHAPTER 4

WILD VOLES MAINTAIN HIGH NEUTRAL AND MHC DIVERSITY INDEPENDENTLY OF PARASITISM[1]

[1] J.C. Winternitz and S. Altizer. To be submitted to the *Journal of Evolutionary Ecology*.

ABSTRACT

Understanding the selective forces that maintain genetic diversity is a major focus of evolutionary biology. In particular, the high variability of genes of the Major Histocompatibility Complex (MHC), which plays a prominent role in vertebrate immunity, is thought to arise from a combination of parasite-mediated and sexual selection. An outstanding question involves the degree to which balancing selection on the MHC can oppose genetic drift and maintain genetic variation in the face of severe population bottlenecks. To address this question we examined genetic diversity, population structure at neutral and MHC genes, and measures of parasitism in a fluctuating population of montane voles (*Microtus montanus*). We found high measures of neutral and MHC allelic variability, indicating genetic drift has had little impact on this vole population, despite regular population bottlenecks. Next, we tested whether individuals with greater diversity at the MHC experienced lower measures of infection by several gastrointestinal parasites. MHC diversity did not predict overall parasite richness or the presence/absence of two common endoparasites. Two specific MHC alleles predicted *Eimeria* intensity and cestode load, but no other associations between specific alleles and infection measures were detected. Measures of neutral genetic diversity based on microsatellites were positively associated with host body condition, total parasite richness and cestode intensity. One explanation for greater parasite loads among hosts with greater genome-wide diversity is that these individuals might better tolerate heavy infections than more inbred hosts. Overall, these results suggest that the parasites examined here do not have a strong role maintaining the high observed MHC variation in cyclic wild voles, and other potential mechanisms of selection such as mate choice should be considered.

Key words: Major Histocompatibility Complex, host-parasite relationship, balancing selection, *Microtus montanu*s, cestodes, *Eimeria*, microsatellites

INTRODUCTION

Genetic diversity is a key determinant of individual fitness and also influences the adaptive potential of populations in response to environmental change (Thompson 1998; Keller & Waller 2002; Spielman *et al.* 2004b). Understanding how population dynamics impact genetic diversity is a fundamental goal of conservation genetics. In particular, fluctuating populations that undergo periodic bottlenecks can experience short-term losses of neutral genetic diversity that may be recovered quickly through gene flow; however, long-term neutral genetic diversity depends on a combination of interacting factors including genetic drift and population interconnectedness (Saccheri *et al.* 1998; Frankham *et al.* 2002; Hess *et al.* 2002).

Genes under selection are more relevant than neutral markers for predicting the adaptive potential of populations (Reed & Frankham 2001). The Major Histocompatibility Complex (MHC) in particular is well-suited for studies of the adaptive maintenance of genetic variation because of its known immunogenetic function (Klein 1986) and large body of empirical evidence documenting associations with parasites in wild populations (Bernatchez & Landry 2003; Kurtz *et al.* 2004; Sommer 2005; Piertney & Oliver 2006). This region contains the most diverse set of coding genes in vertebrates, with most species examined to date showing high levels of allelic diversity and heterozygosity (Edwards & Hedrick 1998; Knapp 2005). How this variation persists in the face of population declines or fluctuations is an interesting question, with evidence suggesting that a combination of pathogen-mediated selection and mate choice for specific or dissimilar alleles can maintain high diversity at the MHC (Apanius *et al.* 1997; Knapp 2005; Milinski 2006; Spurgin & Richardson 2010) (Reusch *et al.* 2001; Milinski 2006).

The majority of studies assessing MHC variation in nature have documented reduced MHC in bottlenecked populations subject to large and prolonged fluctuations in size (reviewed in

Radwan *et al.* 2010; Sutton *et al.* 2011). In rarer cases, some populations have maintained high MHC diversity despite evidence from neutral diversity suggesting extreme population bottlenecks (Aguilar *et al.* 2004; van Oosterhout *et al.* 2006; Oliver & Piertney 2012b). These findings of high relative MHC diversity have been interpreted as scenarios where strong selection pressure from parasites maintains adaptive variation in the face of genetic drift, but there are few opportunities to verify this suggestion directly. Thus, studies that examine both neutral and MHC diversity in contemporary populations subject to periodic bottlenecks, and that relate these measures to potential selective forces such as parasite infection, are needed to better understand the selective maintenance of genetic diversity.

Here, we examined the pattern of neutral and MHC genetic diversity and evidence for their associations with parasitism in montane voles (*Microtus montanus*) that inhabit alpine grassy meadows of North America ranging from Colorado to Utah (Sera & Early 2003). Voles from the Arvicolinae subfamily (to which montane voles belong) are a useful system to investigate the role of parasites as selective agents maintaining MHC variation over population bottlenecks because they undergo frequent multi-annual population bottlenecks (Krebs 1996; Stenseth 1999) and can be subject to temporally and spatially variable selection pressures (Bryja *et al.* 2007; Oliver *et al.* 2009a). Montane voles in particular undergo high-amplitude and frequent population cycles, peaking in abundance every three to four years (Pinter 1986; R. Smith unpublished data). They also have a diversity of parasites (Winternitz *et al.* 2012) and a promiscuous mating system, thus providing the opportunity for parasite-mediated selection and sexual selection to influence MHC variability. High allelic diversity and high divergence between alleles have been recorded at the MHC class II DRB locus in montane voles (Winternitz and Wares unpublished data). This previous study found evidence for historic balancing

selection through trans-species persistence of alleles (Garrigan & Hedrick 2003), but weak evidence of positive selection at the functionally important antigen binding sites (ABS) (Hughes & Yeager 1998), and strong evidence for purifying selection maintaining allelic divergence.

The goal of our current study was to characterize population genetic structure in montane voles using neutral microsatellite markers and to compare these patterns with evidence of population structure based on the MHC Class II DRB locus to look for contemporary evidence of balancing selection in a naturally fluctuating population. In addition, we tested whether parasites could serve as potential agents of selection to maintain high MHC variation by focusing on several species of gastrointestinal parasites shown to be common in wild montane voles (Winternitz et al. 2012). We expected to find negative associations between measures of MHC diversity at the individual level and measures of parasitism, consistent with the idea that genetic diversity at genes encoding antigen binding sites allows hosts to recognize and defend against a greater diversity of pathogens (e.g. Wegner *et al.* 2003b; de Bellocq *et al.* 2008). We also tested for associations between parasite infection and specific MHC alleles, as previous work showed that particular alleles might confer protection against specific parasites (e.g. Westerdahl *et al.* 2005; Tollenaere *et al.* 2008; Froeschke & Sommer 2012). Finally, we examined associations between neutral genetic diversity, measures of infection, and host body condition, in part because inbreeding and an overall loss of genetic diversity has been linked to increased disease susceptibility and lower fitness in other vertebrate species (e.g. Coltman *et al.* 1999; Acevedo-Whitehouse *et al.* 2003; Spielman *et al.* 2004a).

MATERIALS AND METHODS

Sites and field sampling

Voles were trapped for 3 consecutive years (2008 – 2010) at three replicate sites within 5 km of the Rocky Mountain Biological Laboratory, located in the Upper East River Valley, Colorado, U.S.A. (39°N, 107°W). The three trapping sites (Kettle Ponds 1 (KP1), Kettle Ponds 2 (KP2), and Research Meadow (Rmed)) were comprised of grassy meadows and separated by a minimum of 0.5km at approximately 2900m elevation. A total of 262 voles were captured using Longworth live traps 4-5 consecutive days per site every two weeks throughout the breeding season (Jun 15 – Aug 15). No marked animals were captured in multiple sites. Vole density changed by a factor of 30 between trapping intervals within each year (2008, 2009, 2010) and a factor of 20 between years (peak at 2009, low at 2010). Mean vole density also differed among sites: KP1 = 105 voles/ha (range 18–174 voles/ha/yr); KP2 = 161 voles/ha (range 12–410 voles/ha/yr); Rmed = 160 voles/ha (16–381 voles/ha/yr), with a full analysis of vole density provided in Winternitz et al. (2012). Only data from 2008 and 2009 are included in this study as insufficient samples were obtained in 2010 owing to low density. Animals were uniquely identified with numbered ear tags (National Brand Tag Company) and sex, age, body mass (g) and length (mm) were recorded. A 2mm tail tip was collected from non-juveniles and stored in 95% ethanol at 5°C after anesthetizing the animals with isoflurane gas for 1 min. Fecal samples were stored in 10% formalin.

Parasitism measures

Methods for parasite identification and quantification are detailed in Winternitz *et al.* (2012). Briefly, we used salt flotation using sodium nitrate solution (specific gravity 1.2-1.5) to isolate intestinal parasites eggs (Dryden *et al.* 2005), and parasite oocytes and eggs were quantified per gram of feces to determine the presence of infection and to estimate parasite intensity. Fecal egg

counts are commonly used in longitudinal studies and can provide a valuable non-invasive approximation of worm burden (Brenner 1970; Keymer & Hiorns 1986; Scott 1988; Ferrari *et al.* 2004; Froeschke & Sommer 2005; Harf & Sommer 2005).

Morphological measurements were used to identify three major taxonomic groups of intestinal parasites: coccidia (*Eimeria,* (Levine & Ivens 1965)), cestodes and nematodes. Although the fitness consequences of parasite infection have never been assessed in montane voles, these parasites are known to reduce host fitness in numerous vertebrate species through reducing body condition, survival, susceptibility to predation and reproductive success (Wiger 1977; Scott & Lewis 1987; Scott 1988; Fuller & Blaustein 1996; Vorisek 1998; Holmstad *et al.* 2005; Lello *et al.* 2005; Hakkarainen *et al.* 2006; Turner *et al.* 2011), thus, creating the potential for them to be agents of selection for montane voles. We identified five *Eimeria* morphospecies in montane voles following sporulation. We identified two species of cestodes with similar transmission (ingestion of infected intermediate hosts (orabatid mites) from the environment), and so data on both cestodes were combined for most analyses. We also observed eggs of one nematode species as *Syphacia spp.* at 2% prevalence.

Multiple measures of infection status were examined for individual voles: parasite species richness, and for cestodes and *Eimeria*, the presence/absence and intensity of infection (combining data for all morphospecies). Because multiple parasite species could infect any single host, we summed the number of intestinal parasite species (counting separately each morphospecies of *Eimeria*, cestodes and nematodes, for a maximum of 8 species) as one measure of parasitism. Cestode intensity was estimated as the number of oocytes/eggs per gram of feces, and *Eimeria* intensity was scored categorically on a scale of 0-3 per scan of the entire coverslip (1 = 1-10 oocysts, 2 = 11-100 oocysts, and 3 = > 100 oocysts). For analyses considering

associations between genetic variability and parasitism, we included non-infected individuals in analyses of load, as we were interested in the genetic predictors of entire scale of infection from 0 to maximum intensity.

Microsatellite genotyping

Six microsatellite loci were used to estimate the amount of neutral genetic variation in populations (Table S3.1): *Mar076* (HEX), *Ma68* (FAM), *Ma88* (NED), *Msmoe02* (NED), *AV13* (HEX), and *Ma54* (FAM). The names in parentheses refer to the fluorescent dye used to label the forward primer for each locus. Microsatellite loci were amplified in two separate multiplex 20 μL reactions containing 40–100 ng of DNA, 0.5 mM of each primer (Invitrogen), 4ul of 5X reaction buffer, 2ul of 2.5mM MgCl2, 2ul of a mix of 10 mM deoxyribonucleotide triphosphates, and 0.2ul of (5u/ul) GoTaq®Flexi DNA Polymerase (Promega M8295). Loci *Mar076*, *Ma68*, and Ma88 were amplified in multiplex A and loci *Msmoe02*, *AV13*, and *Ma54* were amplified in multiplex panel B. Thermocycling was carried out on an Eppendorf Mastercycler® ep with the same cycling scheme for both panels: an initial denaturation step at 95°C for 2 min followed by 30 cycles of denaturation at 94°C for 30s, annealing at 57°C for 90s, elongation at 72°C for 60s, and a final extension at 60°C for 30 min. The PCR products were electrophoresed at the Georgia Genomics Facility on an Applied Biosystems 3730xl 96-capillary DNA Analyzer. Genemarker v2.4.0 was used for genotyping individuals.

MHC genotyping

Our genetic investigation focused on the MHC class II *DRB* gene exon 2 because it has previously been shown to contain most of the functionally important antigen binding sites (ABS)

and is, therefore, the most likely candidate for detecting balancing selection acting on MHC class II genes (Hughes & Yeager 1998). The methods used to identify MHC genetic diversity in montane voles are described in detail elsewhere (Winternitz and Wares unpublished). Briefly, we extracted genomic DNA using the PureGene DNA isolation kit (Gentra Systems), following the manufacturer's protocol. We used oligonucleotide forward primer JS1 and reverse primer JS2 described by Schad *et al.* (2004) which amplified a 171 bp fragment of the second exon of the DRB gene that includes part of the functional antigen-binding site (ABS). This primer system has been successful in amplifying sequences from multiple loci in rodents when the DRB locus has been duplicated (Galan *et al.* 2010). To assign 454 sequencing reads to specific individuals, 9-bp tags were used to create 18 forward and 18 reverse 5' tagged primers that resulted in 189 unique JS1-tagged and JS2-tagged primer pairs. These 9-bp sequences (created at http://faircloth-lab.github.com/edittag/) were developed to have an edit distance of five, whereby five mutations are required for one tag to transform into another sequence (Faircloth & Glenn 2011). All PCRs were performed in a reaction volume of 20 µl, each containing 40–100 ng of DNA, 0.5 mM of each primer (Invitrogen), 4ul of 5X reaction buffer, 2ul of 2.5mM MgCl2, 2ul of a mix of 10 mM deoxyribonucleotide triphosphates, and 0.2ul of (5u/ul) GoTaq®Flexi DNA Polymerase (Promega M8295). Thermocycling was carried out on an Eppendorf Mastercycler® ep with an initial denaturation step at 96°C for 120s followed by 30 cycles of denaturation at 94°C for 30s, annealing at 57°C for 30s, elongation at 72°C for 60s, and a final extension at 72°C for 10 min. Individual PCR products were concentrated and normalized using the SequalPrep™ Normalization Plate Kit (Invitrogen A10510-01). 10ul of sample from each individual was pooled and sequenced as a single 454 Titanium run at the

Georgia Genomics Facility, Athens, Georgia. To further visualize and analyze sequencing data and assign reads to individuals, we used the SESAME software (Meglécz *et al.* 2011).

Preliminary analysis (Winternitz and Wares unpublished) revealed up to 4 distinct alleles per individual, indicating that the DRB locus in *M. montanus* has undergone at least one duplication event. Based on these findings, we calculated the minimum coverage necessary to obtain at least three copies of each allele at 0.999 probability using the method of Galan *et al.* (2010). This analysis and empirical verification indicated that coverage of 54 reads per individual was sufficient for accurately genotyping individuals (amplicons) with a duplicated gene in a diploid species (Galan *et al.* 2010) Full procedures for artifact filtering and data validation can be found in Winternitz and Wares (unpublished).

STATISTICAL ANALYSIS

Estimates of neutral genetic variation

We tested whether our microsatellite loci were amplifying evenly, were unlinked, and met neutrality assumptions. Quantification of genotyping error rate across the 6 microsatellite loci was conducted by re-genotyping 10-18 samples per locus. The presence of null alleles, allelic stuttering, and large allele dropout was tested using MicroChecker (Van oosterhout *et al.* 2004). Genotypic linkage disequilibria between all pairs of loci was tested within each population by exact tests using Markov chain methods in GENEPOP version 4.0.10 (Raymond & Rousset 1995b; Rousset 2008) and corrections for multiple tests were performed using the FDR approach using the program QVALUE (Storey 2002).

Intrapopulation genetic variation was estimated based on the mean number of alleles per locus (A), observed heterozygosity (H_o), expected heterozygosity (H_e; (unbiased estimates, Nei

1978), and intrapopulation fixation index (Fis), calculated using the program GenAlEx (Peakall & Smouse 2006). To compare allelic richness at different sites in 2008 and 2009, we used the rarefaction procedure implemented in FSTAT 2.9.3.2 (Goudet 2001) and estimated allelic richness for the smallest number of individuals typed for a locus in a sample (5 for each site by year, 20 for all sites combined by year). The conformance of the allele frequencies with Hardy–Weinberg expectations for all loci at all sites was tested by applying the exact tests of Guo and Thompson (1992) in GENEPOP. Significant *P*-values were determined by correcting for multiple hypotheses testing using the FDR approach.

Global differences in genotype frequencies between years and sites were assessed with Markov chain Monte Carlo approximations of Fisher exact tests (Raymond & Rousset 1995a) calculated in Arlequin ver. 3.5 (Excoffier 2005; Excoffier & Lischer 2010). The standardized measure of genetic differentiation G'_{ST} (Hedrick 2005) per locus was calculated in SMOGD v1.2.5 (Crawford 2009). The genetic structure of populations and years was investigated by an analysis of variance framework (Weir & Cockerham 1984) using the Analysis of Molecular Variance (AMOVA) approach used in Arlequin, with significance ($P<0.05$) determined using 10,000 permutations. The number of migrants (N_m) between sites per year, between sites pooling years, and across years was estimated using private alleles (Barton & Slatkin 1986) and correcting for sample size in GENEPOP.

Estimates of MHC variation

MHC class II DRB exon 2 variability in *M. montanus* was calculated for all sites by year, for each site, and for each year. The average number of alleles observed, allelic diversity (π) and average number of bp differences between alleles (K) were calculated in DnaSP v.5.10 (Librado

& Rozas 2009). Expected heterozygosity (H_e) and deviation from Hardy–Weinberg expectations were estimated using the software Arlequin using allele frequency data. The genetic structure of populations and years at the MHC was investigated using the AMOVA approach used in Arlequin, with significance ($P<0.05$) determined using 10,000 permutations. Observed heterozygosty (H_o) was calculated based on the assumption of a duplicated DRB locus by categorizing individuals with 1-2 alleles as homozygous and with 3-4 alleles as heterozygous (following Winternitz and Wares unpublished ms). To identify the functional diversity across alleles, we delineated alleles to supertypes based on five z-scores (hydrophobicity, steric bulk, polarity, and 2 electronic effects) that characterize the amino acid sequences (Sandberg *et al.* 1998). A matrix of the 5 z-scores per allele was constructed and hierarchical clustering by z-scores was performed in SPSS v20 using the average linkage (between groups) method and Euclidean distance measurement (Doytchinova & Flower 2005; Schwensow *et al.* 2007). The average number of supertypes observed was calculated per site and year.

Pairwise F_{ST} values for the multiple DRB loci of MHC were calculated using Arlequin 3.5 by entering the allele sequences and number of individuals with that allele in each population as haplotype data. Differences in the allele frequencies between populations were assessed with Markov chain Monte Carlo approximations of Fisher exact tests (Raymond & Rousset 1995a) in Arlequin 3.5.

Analysis of contemporary selection- comparison with neutral markers

Under balancing selection, MHC genes should show lower among-population divergence measures than that observed for neutral markers. We used the Average Percent Difference

(APD) of MHC and microsatellite loci between pairs of individuals within populations (Miller & Lambert 2004; Miller *et al.* 2010), and describe the methods further in Appendix C.

Individual genetic predictors of infection

Because previous work identified weak evidence of historic balancing selection and strong molecular evidence of purifying selection at the DRB locus in montane voles (Winternitz and Wares unpublished), we tested whether MHC genotypes were associated with parasite infection status. To control for background levels of genome wide diversity we calculated individual heterozygosity as the number of heterozygous loci out of all 6 typed microsatellite loci (Coltman *et al.* 1999). As a second estimate of neutral genetic diversity, we calculated the mean squared genetic distance (d^2) between microsatellite alleles due to stepwise mutation following Coulson *et al.* (1998). Individual genetic variability at the MHC was calculated in several ways to identify different components potentially influencing parasite infection status. For each individual, we calculated the presence-absence of 21 Mimo-DRB alleles. We also recorded the total number of alleles, the number of alleles based on unique amino acid sequences, the presence-absence of five MHC supertypes, the total number of supertypes, the pairwise divergence between alleles calculated in Mega 5 (Tamura *et al.* 2011), and the observed heterozygosity (1-2, homozygote; 3-4, heterozygote). Count variables were log-transformed when necessary to meet normality assumptions.

Prior to analyses, we tested for independence of the genetic predictor variables. Neutral variables (individual heterozygosity and d^2) and continuous MHC variables (listed above) were not significantly correlated based on Spearman's Rank test (R ranges from -0.02-0.04, all $P>0.05$). However, all continuous MHC variables were significantly correlated (R ranges from

0.35-0.89, P<0.001). Therefore, we ran all analyses with only one MHC predictor variable at a time. As an exploratory analysis to reduce the number of potential tests, we employed model simplification testing for associations between the parasite response variables (presence-absence of *Eimeria* and cestodes, *Eimeria* load, cestode intensity, and endoparasite species richness) and the binomial predictor variables of specific alleles and specific supertypes (Full model for alleles: parasite measure = Mimo-DRB*01 + Mimo-DRB*02 + … + Mimo-DRB*21. Full model for supertypes: parasite measure = supertype 1 + supertype 2 + ... + supertype 5). Predictors of parasite presence-absence were identified using Generalized Linear Models (GLMs) fitted to binomial error structures with logit link functions. GLMs for *Eimeria* load and endoparasite species richness were fitted with poisson error structures with loglinear link functions. GLMs for log (cestode intensity) (considering only positive individuals) were fitted with linear error structures and identity link functions. We used an exhaustive search based on AICc in the *glmulti* package (Calcagno & de Mazancourt 2010) in R (Team 2012) to identify the best models. Variables from models with strong support (AICc weights within 4 units of the lowest AICc value; Burnham & Anderson 2002) and significant *P*-values after correcting for multiple hypotheses testing using the FDR approach were retained in the subsequent analyses.

We tested for the influence of neutral genetic diversity measures (individual heterozygosity and mean d^2) on parasite infection with the prediction that higher individual heterozygosity and microsatellite divergence would relate to lower prevalence and intensity measures, indicating hybrid vigor (Coulson et al. 1998). We tested for associations between MHC adaptive immune genes and parasite infection measures with the following predictions: i) the presence of specific alleles and supertypes would be related to high or low parasite prevalence and load (consistent with negative frequency dependent selection); ii) heterozygotes

would have lower parasite burdens (consistent with overdominant selection); iii) greater numbers of alleles would be negatively or nonlinearly associated with parasite burden (indicating overdominant selection or intermediate allele hypothesis; (Wegner *et al.* 2003a); iv) greater allelic divergence and a higher number of supertypes per individual would be negatively correlated with parasite burden (again indicating overdominant selection). We included the following ecological variables and host characteristics, as these were previously shown to predict infection status (Winternitz *et al.* 2012): age, sex, body condition (based on the residuals from a ln mass-ln length regression), site, capture period, year, and log host density per ha at the site and time of capture.

Neutral heterozygosity and genetic diversity have been shown to associate with host fitness measures (Coulson *et al.* 1998; Coltman & Slate 2003), and variation in (MHC) immune genes can influence host condition via distribution of host resources and costs of immune activation (Zuk & Stoehr 2002; Kurtz *et al.* 2006). We tested for neutral and immune gene diversity associations with body condition in wild voles, using the above mentioned predictor variables and condition as the response variable using GLMs with a linear identity link.

RESULTS

Neutral genetic variation

A total of 139 individuals were successfully genotyped for the six microsatellite loci. The genotyping error rate was estimated to be 0.06% per reaction and 0.01% per locus (Table S3.2). No null alleles were detected across the loci, and there was no evidence of linkage disequilibrium or departure from Hardy Weinberg Equilibrium after FDR corrections. All relationships between loci testing for linkage disequilibrium were not significant after correction for multiple

tests. Summary statistics were calculated for the six microsatellite loci by site and by year (Table 3.1). Allelic richness and heterozygosity (observed and expected) were high across all sites in each year, and the fixation index F_{is} did not indicate levels of inbreeding. The site Research Meadow (Rmed) was out of Hardy Weinberg equilibrium when pooling samples by year, together with the year 2009 and the metapopulation overall (after FDR corrections). In these cases, observed heterozygosity was lower than expected.

There was no evidence of subpopulation structure between years or sites based on AMOVA tests, and 99% of the variation in allelic variation was partitioned within individuals (Table S3.3). Estimates of migration between subpopulations using private alleles and corrected for sample size were high (e.g. 18 migrants between sites per year). Exact tests of sample differentiation based on genotype frequencies also showed no evidence of population structure when performed for each site by year ($P = 0.12$) or for sites across both years combined ($P = 0.43$). Corrected G_{ST}' ranged from 0.08-0.22 across the six loci (Table S3.4).

MHC diversity

A total of 21 DRB alleles were detected from 123 individuals that qualified as being reliably genotyped at the MHC DRB locus, having a sufficient minimum coverage of 54 reads (mean 140.7, SD = 93.3, range 54-526)**.** The frequencies of the alleles and supertypes in the three sampled populations for 2008 and 2009 are shown in Table 3.2, Fig. 3.1, and Table S3.5. Each site had private alleles, and all alleles were represented in the peak density year (2009, 21 alleles) but not the lower density year (2008, 10 alleles). Similarly, only four out of five supertypes were represented in 2008, while all were present in 2009. Specific allele and supertype frequencies were similar among the three sites and over time (Fig. 3.1). Expected heterozygosity estimates

using allele frequency data were high (Table 3.2), but observed heterozygosity estimates (using binomial classification) were less than 0.5, possibly reflecting variable copy number in montane voles, as has been observed in other rodents (Kloch *et al.* 2010). Similar to the microsatellite results, there was no evidence for population structure using exact tests by year (P = 0.46) or population (P = 0.08). In this case, most variation in MHC allele frequencies was attributed to the combination of year by population (P = 0.06; Table S3.6). There was weak evidence for genetic drift based on APD correlations between MHC and neutral markers, but overall genetic drift did not appear to be a significant factor affecting montane vole functional or neutral diversity in the years studied (see Appendix C for more detail).

Associations between parasitism and neutral genetic diversity

We tested whether estimates of neutral genetic diversity were correlated with each other or with MHC genetic diversity estimates. Mean microsatellite heterozygosity was not significantly associated with d^2 (Spearman's R = 0.142, P = 0.12), and these microsatellite variables were not correlated with the four measures of MHC diversity (Spearman's R ranges from -0.079 – 0.142, P > 0.05 for all pairwise correlations). We then tested for associations between neutral genetic diversity and measures of parasitism. Mean microsatellite heterozygosity was positively associated with endoparasite species richness (Table 3.3, Fig. 3.2A). Neutral divergence, d^2, was positively correlated with cestode intensity among infected individuals (Fig. 3.2b; Table 3.3). No other measures of parasite infection (including the presence/absence of *Eimeria* and cestodes and *Eimeria* intensity) were significantly associated with the two measures of microsatellite diversity.

Associations between MHC diversity, specific alleles and parasitism

Three measures of MHC diversity, the number of MHC alleles, the number of MHC supertypes and the divergence between alleles carried by an individual were positively related to cestode load (Table 3.3; Table S3.8). The presence/absence of *Eimeria* and cestodes were not associated with MHC diversity measures, nor was total parasite richness. When looking at associations between the presence or absence of specific MHC alleles and measures of infection, we found that the presence of Mimo-DRB*06 (at 10% frequency within the population) was positively related to *Eimeria* intensity, and that the presence of Mimo-DRB*07 (at 20% frequency within the population) was negatively related to cestode intensity (Table 3.3, Fig. 3.3). Specific MHC alleles were not significantly associated with the presence/absence of *Eimeria* and cestodes. Individual characteristics (sex, condition) and ecological variables (site, trapping period, year) included as covariates based on previously-demonstrated associations with parasitism in montane voles (Winternitz *et al.* 2012) showed relationships similar to previously-published work.

Body condition and genetic diversity

We examined whether vole body condition, as a proxy for host fitness, was related to measures of genetic diversity. Vole condition was positively associated with microsatellite divergence d^2, but not with other measures of microsatellite or MHC diversity (Table 3.4). The presence of Mimo-DRB*14 was associated with poorer body condition, but this allele was only found in two adults with condition scores available (out of a total of 97 animals).

DISCUSSION

The montane vole population studied here showed relatively high levels of MHC diversity at the DRB locus and across neutral microsatellite loci, despite ecological evidence that these animals undergo repeated high amplitude population fluctuations (summarized in Winternitz et al. 2012). We found no evidence for genetic sub-structuring among sampling locations or years based on neutral markers or based on MHC allele frequencies. Fixation indices for both markers were low or zero, and genetic variability was mostly captured within individuals (microsatellites), and by site per year (MHC), indicating that genetic drift and population sub-structuring was weak or absent in this population. Although our study found evidence for private DRB alleles by site, differences in private alleles were eroded during the high density year (2009), most likely due to movement between sites. Thus, migration across populations and between years could produce a "rescue effect" that replenishes allelic diversity during peak density years with high gene flow (Berthier *et al.* 2006). Differences in the frequency of specific MHC alleles were similar across sampling locations and over 2 years, whereby common alleles remained common, and rare alleles remained rare. This timeframe relates to approximately six generations of voles; thus, a longer time series would be useful in determining whether allele frequencies vary over time, particularly in relation to parasite infection. For example, a study on nine wild populations of guppies (*Poecilia reticulate)* over two years (8-12 generations) found that five populations experienced a greater change in population structure at MHC class II loci than at neutral markers, likely mediated by temporally variable parasite pressure (Fraser *et al.* 2010).

We expected to find negative associations between MHC allelic diversity measures and parasitism, especially if heterozygous individuals enjoy a selective advantage over homozygous individuals because they can combat a broader spectrum of parasites (e.g., Doherty and

Zinkernagel, 1975). Counter to our expectations, this study showed that measures of high MHC diversity were generally not associated with parasite infections. Our analyses of specific alleles that might associate with parasite infection showed that Mimo-DRB*07 predicted low cestode intensity, but another allele, Mimo-DRB*06, was associated with greater *Eimeria* intensity. These associations could by caused by negative frequency-dependent associations with specific parasites (Doherty & Zinkernagel 1975), or fluctuating selection, where temporal and/or geographic variation in parasites can select for different sets of MHC alleles at different times and locations (Hill 1991). Exploring the mechanism of selection operating on specific MHC requires longer sampling timescales to observe temporal changes in allele-parasite frequencies and to ask whether the direction of associations are consistent over time (Spurgin & Richardson 2010).

High levels of neutral genetic diversity based on microsatellite markers in fluctuating voles can be attributed to large effective population size and high rates of migration between sampling locations (Berthier *et al.* 2006). Parasites have also been shown to be negatively associated with neutral genetic diversity, possibly through an excess of deleterious mutations in more inbred individuals or by overdominance operating on parasite resistance in more heterozygous individuals (Coltman *et al.* 1999; Acevedo-Whitehouse *et al.* 2005; Rijks *et al.* 2008). We asked whether parasites might be implicated as agents mediating neutral diversity, and found that greater neutral diversity related to both higher parasite load (cestode intensity and endoparasite species richness) and higher body condition, while simultaneously controlling for other variables (sex, condition, trapping period, year, and site). Other studies have found lower neutral genetic diversity to be correlated with higher parasite infection (Coltman *et al.* 1999; Acevedo-Whitehouse *et al.* 2003), as well as showing no relationship (Schwensow *et al.* 2007).

One explanation for the positive associations between genetic diversity and parasitism found in our study could be that animals with greater genome-wide diversity might better tolerate heavy infections than more inbred hosts. This explanation assumes that parasites are indeed harmful, and that the parasites kill off hosts with low genetic diversity. In support of this idea, work on song sparrows, New Zealand robins, Soay sheep and other species showed that that inbred individuals tended to have lower overall fitness and survival, particularly during population crashes when resources were limiting and parasite infections were common (Keller *et al.* 1994; Coltman *et al.* 1999; Keller & Waller 2002; Jamieson *et al.* 2007). An alternative hypothesis is that parasites have little impact on host fitness, but genetically diverse hosts survive longer than their less diverse counterparts due to other ecological pressures independent of parasitism affecting host fitness. In this case, genetically diverse individuals that survive longer could simply accumulate more parasites over time. In support of this idea, a previous analysis on montane voles (Winternitz et al. 2012) showed that animals with greater cestode loads also had higher measures of body condition, possibly because heavier animals ingested more food and, as a result, incidentally consumed more parasite infectious stages. These same animals could have better survival and might be exposed to parasites over longer timescales. With this in mind, it would be useful to re-examine the importance of neutral genetic diversity for parasite infection during ecologically stressful conditions or for younger individuals which might then accumulate infections over time (Coltman *et al.* 1999; Rijks *et al.* 2008).

Overall, our results showed evidence for a genetically diverse wild rodent population that maintains neutral and adaptive genetic diversity over the course of repeated high amplitude fluctuations. Alleles are likely exchanged between vole subpopulations during periods of high density when migration supports gene flow. Surprisingly, most measures of infection reported

here did not depend on measures of MHC or neutral genetic diversity; two exceptions were that cestode load and endoparsite richness were positively associated with neutral and MHC genetic diversity. We suggested two exclusive hypotheses for the positive association between genetic diversity and infection measures: 1) the 'harmful parasite' hypothesis, whereby parasites kill off the hosts with low genetic diversity and accumulate in genetically diverse hosts that can tolerate or mediate parasite effects on fitness. The alternative hypothesis is 2) the 'benign parasite' hypothesis, whereby parasites have little impact on hosts, but genetically diverse hosts survive longer than their less diverse cohort due to other ecological pressures independent of parasitism affecting host fitness. It is important to note that our study did not quantify all parasite types, and other parasites such as blood borne protozoa are known to negatively affect fitness in other vole populations (Watkins *et al.* 1991; Smith *et al.* 2005). Additionally, other work showed that ectoparasitic arthropods (fleas, ticks and lice) were negatively associated with MHC variability in other wild populations (Oliver *et al.* 2009b; Schad *et al.* 2012).

We also point out that other selective pressures could limit the potential for parasites to build up within the population, thus lowering their impacts on vole fitness and reducing their role as agents of selection. Such pressures may come from predators, which are known to have high impacts on vole population sizes (Korpimäki *et al.* 2002), and might pre-empt parasites from acting as a significant selective force (Hass 1989). This idea is consistent with previous results of low positive selection at functionally important MHC antigen binding sites (Winternitz and Wares unpublished). High levels of purifying selection at non-ABS sites remains to be fully explained, but may be due to population dynamics that remove rare novel variants via genetic drift during the low phase of population cycles, thus resulting in apparent negative selection. Finally, mate choice for specific alleles or genotypes is another possible explanation for evidence

of purifying selection at the MHC in *M. montanus*, and the effect of mate choice in maintaining MHC diversity has been well established in other vertebrates (reviewed in Penn & Potts 1999; Milinski 2006; Havlicek & Roberts 2009). Thus, future work should consider other factors, such as predation and mate choice, when investigating mechanisms influencing MHC variation in wild montane voles and other vertebrates.

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TABLES

Table 3.1. Estimates of microsatellite genetic diversity for each sampling site by year, including sample sizes (N), average number of alleles observed (A), allelic richness corrected for sample sizes of 5 individuals per site/year, 20 individuals per metapopulation, and 35 individuals per site (R), observed heterozygosity (H_o), unbiased estimates of heterozygosity (H_e), intrapopulation fixation indices (F_{is}), and probability of rejection of Hardy-Weinberg equilibrium (significant probabilities after correcting for FDR are in bold). Abbreviations for sampling locations are given in Methods text.

Level	Category	N	A	R	H_o	H_e	F_{is}	HW(p)
2008								
	KP1	6	6.17	5.69	0.861	0.856	-0.093	0.592
	KP2	6	6.33	5.99	0.911	0.846	-0.199	0.741
	Rmed	11	8.67	6.29	0.881	0.885	-0.048	0.502
	Metapopulation	23	7.06	10.29	0.884	0.863	-0.114	0.430
2009								
	KP1	32	11.00	6.19	0.866	0.865	-0.002	0.327
	KP2	38	11.67	5.96	0.837	0.856	0.021	0.081
	Rmed	45	13.17	6.41	0.846	0.877	0.036	0.032
	Metapopulation	115	11.94	10.83	0.850	0.866	0.018	**0.008**
Site								
	KP1	38	11.50	11.42	0.866	0.879	0.001	0.283
	KP2	44	12.33	11.71	0.846	0.865	0.008	0.156
	Rmed	56	14.00	12.97	0.852	0.889	0.032	**0.017**
	Overall	138	12.61	12.20	0.855	0.878	0.014	**0.015**

Table 3.2. MHC class II DRB exon 2 variability in *M. montanus* for each sampling site and year. Average number of alleles observed (A), average number of allele supertypes ($A_{supertypes}$), number of private alleles, ($A_{private}$), observed heterozygosity using binomial classification (H_o), expected heterozygosity (H_e), nucleotide diversity (π), and number of pairwise basepair changes (K).

Level	N	A	$A_{supertypes}$	$A_{private}$	Ho	He	Pi	K
All	123	21	5	0	0.293	0.845	0.133	22.700
Category								
Subpopulation								
KP1	32	14	5	1	0.250	0.804	0.089	15.133
KP2	42	15	5	3	0.310	0.862	0.079	13.540
Rmed	49	15	5	3	0.306	0.867	0.096	16.424
Year								
2008	17	9	4	0	0.176	0.826	0.076	12.991
2009	106	21	5	11	0.311	0.857	0.090	15.329

Table 3.3. Results of generalized linear models showing the effect of genetic variables, host variables, population variables, and design variables on (i) the probability of *Eimeria* infection, (ii) the probability of cestode infection, (iii) *Eimeria* morphotype A intensity, (iv) cestode morphotype A intensity (excluding uninfected animals), and (v) endoparasite species richness. MHC predictor variables were run in separate models and we only show the results for MHC number of alleles and specific alleles, as the results from the other models are qualitatively similar (See Table S3.8).

	Dependent	Eimeria presence N=96		Cestode presence N=96		Eimeria intensity N=90		Cestode intensity N=25 infected		Endoparasite species richness N=96	
Independent		df	χ^2	df	χ^2	df	χ^2	df	χ^2	df	χ^2
Genetic variables	Microsatellite heterozygosity	.	.	.	.	.	.	.	.	1	4.855*
	Microsatellite d^2	.	.	.	.	.	.	.	.	.	.
	MHC number of alleles	.	.	.	.	.	.	.	.	.	.
	Specific Mimo alleles	.	.	.	.	1 (Mimo*06)	4.167*	1 (Mimo*07)	7.760**	.	.
Host variables	Age	.	.	2	4.613	.	.	.	.	.	.
	Sex	.	.	.	.	.	.	.	.	.	.
	Condition	.	.	.	.	.	.	1	7.132**	.	.
Population variables	Site	2	6.836*	.	.	2	10.303**	.	.	.	.
	Density	.	.	.	.	.	.	.	.	.	.
Design variables	Trap period	.	.	.	.	.	.	.	.	.	.
	Year	1	3.024	.	.	.	.	.	.	.	.

Note: Only the results for final simplified models are given (full model: dependent variable = microsatellite heterozygosity + microsatellite d^2 + MHC variable + specific MHC alleles + age + sex + condition + site + host denisty + trap period + year). Test statistics are Wald χ^2 for binary (0 or 1) response variables (probability of *Eimeria* and cestode infection) and likelihood ratio χ^2 tests for other dependent variables. *, P<0.05; **, P<0.01; ***, P<0.001.

Table 3.4. Results of generalized linear models showing the effect of genetic variables, host variables, population variables, and design variables on the condition of montane voles (*Microtus montanus*). MHC predictor variables were run in separate models and we only show the results for MHC number of alleles and specific alleles, as the results from the other models are qualitatively similar.

	Dependent	Condition N=97		Condition N=97 for specific Alleles	
Independent		df	χ^2	df	χ^2
Genetic variables	Microsatellite heterozygosity	.	.	.	.
	Microsatellite d^2	1	4.095*	1	3.948*
	MHC number of alleles	.	.	.	.
	MHC heterozygosity	.	.	.	.
	MHC divergence	.	.	.	.
	MHC number of supertypes	.	.	.	.
	Specific alleles (Mimo*14)	.	.	1	7.032**
Host variables	Age	2	55.362***	2	57.469***
	Sex	1	3.295	.	.
Population variables	Site	.	.	.	.
	Density	.	.	.	.
Design variables	Trap period	.	.	.	.
	Year	.	.	.	.

Note: Only the results for final simplified models are given (full model: dependent variable = microsatellite heterozygosity + microsatellite d^2 + MHC variable + specific MHC alleles + age + sex + condition + site + host denisty + trap period + year). Test statistics are likelihood ratio χ^2 tests for dependent variables. *, P<0.05; **, P<0.01; ***, P<0.001.

FIGURE LEGENDS

Figure 3.1. Allele frequencies of MHC class II DRB alleles for *M. montanus* (Mimo-DRB) across sampling locations (A) and years (B). MHC supertype frequencies by year are shown in the inset of Figure 1B. Abbreviations: KP1, Kettle Ponds 1; KP2, Kettle Ponds 2; Rmed, Research Meadow.

Figure 3.2. Relationship between (A) endoparasite species richness and average microsatellite heterozygosity, and (B) Log cestode intensity and d^2 in *M. montanus*. Lines are the linear best-fit for visualization.

Figure 3.3. Infection intensity and specific MHC Mimo-DRB allele associations in *M. montanus*. (A) *Eimeria* intensity is significantly associated with the presence of Mimo-DRB*06 (A), and Log cestode intensity (infected animals only) is significantly associated with the absence of Mimo-DRB*07 (B).

FIGURES

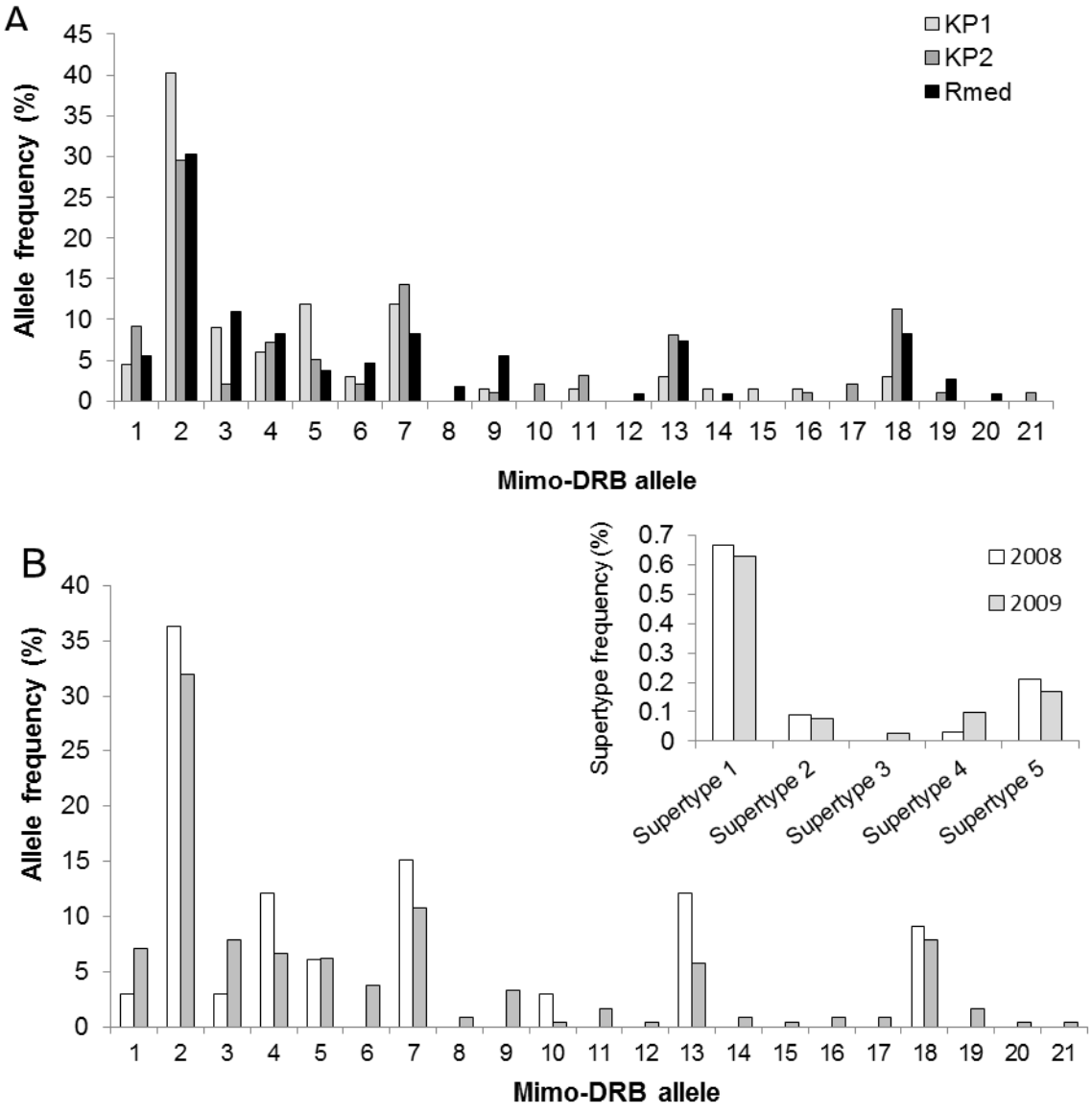

Figure 3.1.

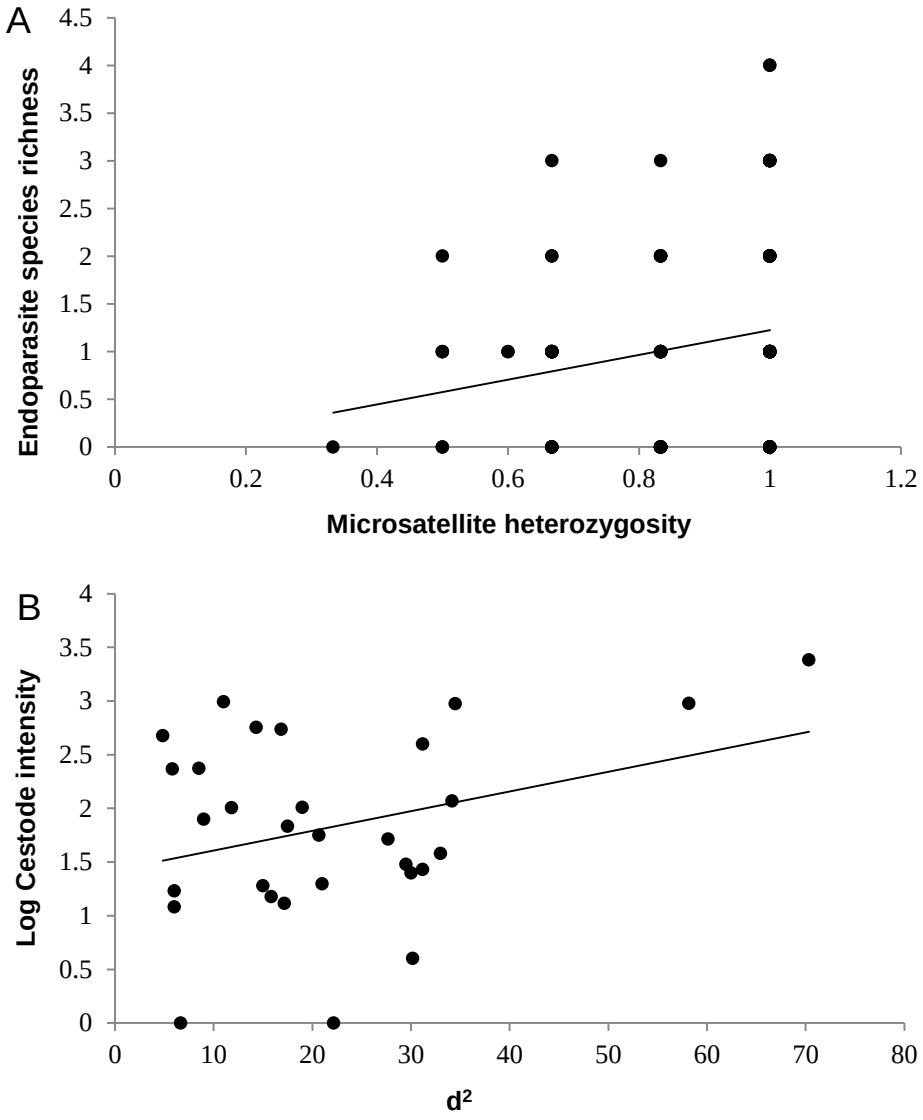
A
Endoparasite species richness
Microsatellite heterozygosity
B
Log Cestode intensity
d²

Figure 3.2.

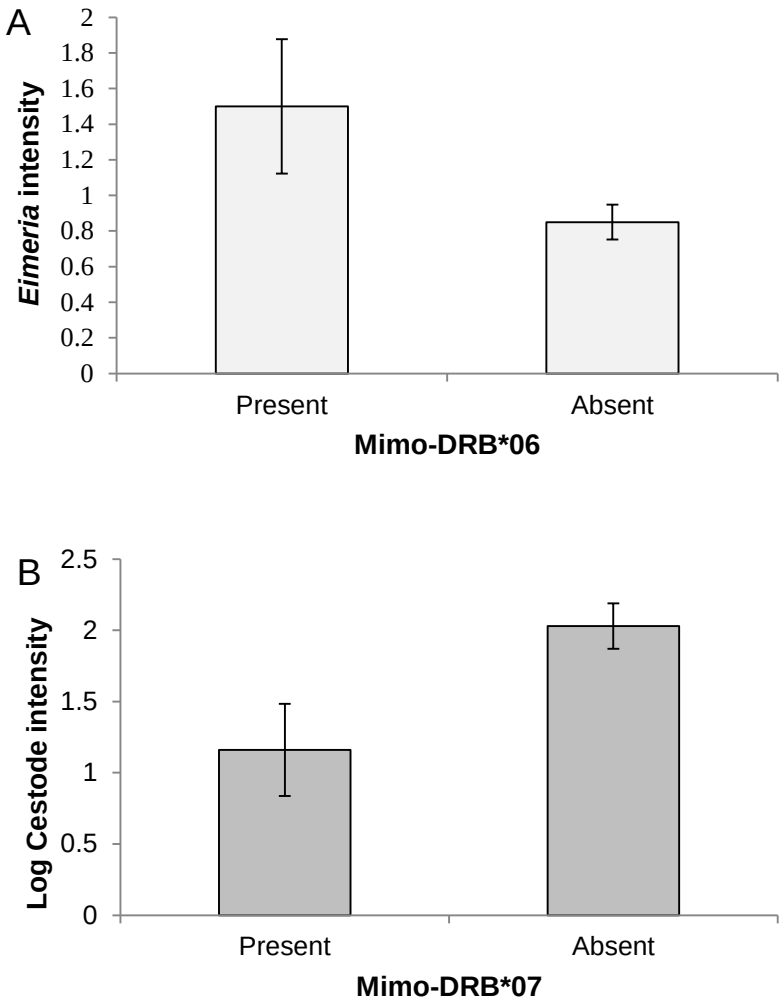
A
Eimeria intensity
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Present
Absent
Mimo-DRB*06
B
Log Cestode intensity
2.5
2
1.5
1
0.5
0
Present
Absent
Mimo-DRB*07

Figure 3.3.

CHAPTER 5

SEXUAL SELECTION AND PARASITISM EXPLAIN FUNCTIONAL VARIATION OF THE MHC ACROSS MAMMALS[1]

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ABSTRACT

Diversity at the major histocompatibility complex (MHC), vitally important for vertebrate immune defense, varies widely across species. Parasites have been identified as a major evolutionary force driving MHC polymorphisms across species, but sexual selection and disassortative mating is another likely mechanism. MHC-based mating preferences have been observed for multiple species including humans, but the generality of mate choice as a driver of MHC polymorphism in the wild is debated. In reality, both parasite-mediated selection and sexual selection may act in concert in wild populations. To investigate potential contributions of parasitism and sexual selection in explaining among-species variation in MHC diversity, we used comparative methods to examine measures of MHC diversity across 115 mammal species, including carnivores, chiroptera, primates, rodents, and ungulates. Specifically, we tested whether parasite species richness and relative testes size (as an indicator of sexual selection) were correlated with two measures of MHC class II DRB diversity: allelic richness and nucleotide diversity. Controlling for mammal phylogeny, neutral mutation rate and confounding ecological variables (i.e. population size, body mass, and sampling effort), we found that parasite species richness was positively correlated with MHC nucleotide diversity for rodents and ungulates, and negatively correlated for carnivores. In contrast, relative testes size was positively correlated with MHC nucleotide diversity for all taxa combined. Mammal taxonomic group was the strongest predictor of MHC allelic richness, with ungulates having lower diversity in general. This study provides support for both parasite-mediated selection and sexual selection in shaping variation in functional MHC polymorphism across a broad suite of mammals.

Key words: Major Histocompatibility Complex, sexual selection, parasite-mediated selection, balancing selection, phylogenetic comparative methods.

INTRODUCTION

A significant fraction of the mammal genome is dedicated to immune defense, and these immune genes are well known for their high genetic variability (Chinwalla *et al.* 2002; Trowsdale & Parham 2004). Parasites have long been viewed as a major selective force in shaping host genetic diversity, especially for genes of the immune system that interact directly with a multitude of pathogens (Sommer 2005); (Barreiro & Quintana-Murci 2010)and for which the rate of adaptive evolution has been shown to be high (Huang *et al.* 2004; Tonteri *et al.* 2010). Mate preferences and sexual selection can also influence variation across immune genes; in particular, the "good genes" hypothesis for resistance to parasites has been invoked to explain why some animals hold mating preferences when they receive no direct benefits from being choosy (Hamilton & Zuk 1982). Thus, the direct effects of parasite infections on host fitness, combined with sexual selection for mates that harbor greater parasite resistance and/or confer beneficial genes to progeny, are the two most likely forces shaping immunogenetic diversity in animals.

The major histocompatibility complex (MHC) is an ideal candidate for investigating the determinants of diversity at immune genes because it plays a crucial role in immune defense for virtually all vertebrates and it has been shown to mediate mate preference in a variety of species, including humans (Potts & Wakeland 1990; Hedrick 1994; Bernatchez & Landry 2003). The MHC encodes glycoproteins which bind to foreign antigens and present them to T-cells, initiating an immune response (Klein 1986). There are two major groups of MHC genes: Class I responds to intracellular pathogens and Class II binds and displays peptides from extracellular pathogens (Hughes & Yeager 1998). In particular, the Class II DRB locus has been extensively studied because of its great allelic diversity, and has been shown to be associated with parasite

resistance in numerous animal populations (Bernatchez & Landry 2003). The DRB exon 2 region encodes the functionally important antigen binding sites (ABS). These sites are responsible for recognizing peptides derived from pathogens and have been shown to be the positions of intense positive selection with a greater ratio of amino acid-changing nonsynonymous substitutions (dN) to synonymous substitutions (dS) at codons along the sequence (Hughes & Yeager 1998; Garrigan & Hedrick 2003). Because different MHC alleles recognize and bind to specific pathogen proteins, multiple alleles are required to confer resistance to different pathogen strains and species (Klein 1986).

Different vertebrate species show different levels of variation at the MHC (Mikko *et al.* 1999), with some variation explained by differential parasite pressure across species (Garamszegi & Nunn 2011). Nevertheless, past work has shown that even endangered species (that otherwise show extremely low diversity based on selectively neutral loci) display relatively high levels of genotypic diversity at MHC loci (Gutierrez-Espeleta *et al.* 2001; Garrigan & Hedrick 2003; Aguilar *et al.* 2004). In such cases, strong balancing selection (such as that generated by frequency-dependent selection or overdominance associated with host-pathogen conflicts) is needed to maintain the high levels of MHC diversity observed in many species (Bernatchez & Landry 2003; Sommer 2005). The mechanisms underlying among-species variation are less well documented, and this has rarely been studied in a comparative sense (but see Simkova *et al.* 2006; de Bellocq *et al.* 2008; Garamszegi & Nunn 2011).

Theoretical studies have shown that disassortative mating could also preserve allelic diversity across MHC loci (Hedrick 1992), and numerous species, including rodents, fish, birds, and humans have been shown to discern individual genotypes based on olfactory cues (Yamazaki *et al.* 1978; Boyse *et al.* 1987; Potts *et al.* 1994; Carroll *et al.* 2002; Penn 2002;

Milinski *et al.* 2005) and prefer scents of mates with complementary MHC genotypes (Wedekind & Furi 1997; Radwan *et al.* 2008; Cutrera *et al.* 2011). A key challenge facing researchers studying mate choice and MHC is that the focus of selection (nucleotide or allelic diversity) and the strength of selection may be context dependent, such that ecological and demographic factors influence the opportunity for and benefits of being choosey (Roberts 2009). As a result, most studies showing MHC-based mating preferences are based on laboratory or captive experiments, and studies conducted in the wild have shown mixed results (Paterson & Pemberton 1997; Consuegra & Garcia de Leaniz 2008; Schaschl *et al.* 2008; Schwensow *et al.* 2008; Setchell & Huchard 2010; Cutrera *et al.* 2012). Comparative studies that examine variation in MHC across species in relation to relation to estimates of mate choice offer another approach to evaluating the general importance of sexual selection for maintaining MHC variation in natural populations (relative to parasitism and other species traits), although to date, such work has not been undertaken.

Here, we use a cross-species comparative approach to investigate the relative influence of the two proposed main selective forces on MHC polymorphism: parasite-mediated selection and sexual selection. Our analysis focuses on mammals from several orders, as this group has been relatively well studied for MHC variation, parasites and infectious diseases, and behaviors and morphology associated with sexual selection and reproductive skew. Key questions motivating this study were: 1) are measures of allelic and nucleotide diversity elevated in species with higher potential for mate choice? 2) are these genetic measures elevated in species with greater parasite species richness? 3) Is there an association between parasite species richness and degree of sexual selection? To elucidate the general effect of sexual selection on MHC diversity, we classified each mammal species according to mating system (monogamy, polygyny/polygamy,

polyandry/promiscuity) and the intensity of sexual selection (using relative testes size as a proxy for competition among males to produce offspring). We also augmented existing data on parasites and pathogens reported to infect free-living populations to broaden the scope of previously identified correlations between MHC Class II diversity and parasite richness (prior evidence: in rodents (10 species), (de Bellocq *et al.* 2008); in primates (27 species), (Garamszegi & Nunn 2011); in cyprind fish (14 species), (Simkova *et al.* 2006)). We employed comparative phylogenetic techniques to test whether parasites drive immune gene diversity across species, controlling for the potentially confounding effects of host phylogeny and ecological traits. For the first time, we also determine if sexual selection is broadly associated with immunogenetic variation across a range of mammal species.

MATERIALS AND METHODS

MHC data

Sequence data for 115 mammal species involved in this study were compiled from the Genbank using Geneious v.5.6.3. We first performed a preliminary search with the key term "MHC class II DRB" and recovered all sequences for any mammal species. We retained sequences including exon 2 of the DRB locus. In addition, we performed systematic literature searches on Web of Science and Biosis using the previously identified species binomial name and MHC as key terms. Sequences from subspecies were combined at the species level, and all species names were corrected according to Wilson and Reeder (2005) (http://www.bucknell.edu/msw3/). Primate taxonomy followed the nomenclature from the Global Mammal Parasite Database (Nunn & Altizer 2005) and the dataset from Garamszegi, Nunn (2011) to correspond with parasite data. For each species, we recorded the number of animals sampled at the DRB locus to control for

sampling effort because a greater number of alleles tend to be discovered when a higher number of individuals are sequenced.

Sequences were grouped according to Order (Artiodactyl (Ungulate), Carnivora, Cetacea, Chiroptera, Primate, and Rodentia), imported into Mega version 5 (Tamura *et al.* 2011), and aligned by MUSCLE (Edgar 2004). Sequences differed in length; thus to estimate rates of substitution according to a standard sequence length, we trimmed all exon 2 sequences to 171bp (the mean sequence length). We removed pseudogenes and alleles with nucleotide insertions or deletions, as these may represent non-functioning alleles. We also removed DRB6 alleles from the primate order, as this locus is thought to be non-functional (Bodmer *et al.* 1992). We checked for duplicates within species and removed non-unique sequences. Final numbers of sequences were recorded as numbers of alleles per species. For analyses of allelic richness, we used the residuals from a regression analysis of log(number of alleles) on log(number of animals sampled) to control for the effect of uneven sampling effort across species.

Rates of selection for functional variation can also be a biologically important measure of diversity. This is especially true at sites along the sequence that are responsible for binding to foreign peptides (antigen binding sites, ABS (Hughes & Yeager 1998). To compile estimates of substitution rates, we employed the most commonly used Nei and Gojobori (1986) method with Jukes and Cantor (1969) correction for multiple substitutions at the same site in Mega 5 to compute the within species averages for amino-acid changing nonsynonymous substitutions (dN) at 15 ABS based on (Brown *et al.* 1993). We repeated this process for synonymous substitutions (dS) at ABS, which provide a baseline value of neutral substitution rates. The number of alleles varies by allelic lineage (Garamszegi *et al.* 2009) and by the number of duplicated DRB loci (Winternitz and Wares, unpublished). However, as the majority of the species in our dataset are

non-model organisms and because no information is available on the specific DRB lineage or gene copy number, we could not include these variables in our analysis and retain power to test our main predictions. We compiled the number of recorded DRB loci per species for potential post-hoc analyses, and this was available for 61 species overall.

Parasite data

Coevolution between hosts and parasites is thought to generate genetic diversity in both sets of partners. Therefore, hosts exposed to a greater diversity of parasites could experience selection for greater genetic diversity for resistance (de Bellocq *et al.* 2008). In addition, as MHC class II genes recognize extracellular parasites, there may be stronger relationships between certain groups of parasites (those with prominent extracellular stages) and measures of DRB diversity. We compiled parasite richness data for each species using the Global Mammal Parasite Database (www.mammalparasites.org), the most comprehensive collection of published records of parasitic organisms from free-living populations of mammals (Nunn & Altizer 2005). For each host species, we recorded the total number of parasite species, using all records of viruses, bacteria, protozoa, helminths, and arthropod parasites.

Parasite species richness estimates are influenced by research effort (Walther *et al.* 1995), whereby host species that are better studied scientifically have a greater number of parasites reported to infect them. We therefore controlled for uneven sampling effort among mammalian hosts using the number of citations for each host species (and taxonomic variants) from Web of Science as an indicator of scientific effort per host. Following previous studies (Altizer *et al.* 2003b; Garamszegi & Nunn 2011), we used these citation counts over the number of hosts sampled for each parasite, as not all studies published the number of animals sampled, and some studies had highly inflated sample sizes despite testing for only a single parasite. We used the residuals from a regression analysis of log(parasite species richness) against log(citation count) to obtain estimates of relative parasite species richness per mammal species.

Estimates of sexual selection

Mating system, and specifically the potential for female mate choice (as females tend to be the choosier sex (Tregenza & Wedell 2000)), is expected to influence the strength of sexual selection on the MHC. Females in monogamous or polygynous mating systems may be constrained in their choice for mates that can provide direct benefits (e.g. territory, resources, protection) (Clutton-Brock & Parker 1995). In contrast, females in promiscuous or polyandrous mating systems are expected to have greater opportunity to select a mate that contributes indirect (i.e. genetic) benefits. Relative testes size (testes mass/body mass) was used as a quantitative estimate of female promiscuity, and has been shown to be a strong predictor of sexual selection and mating system across mammals (e.g. primates, (Harcourt *et al.* 1981); rodents; (Kenagy & Trombulak 1986); carnivores, (Iossa *et al.* 2008)). We compiled testes mass data and male body mass from the literature (Appendix D). In some instances, only testes length, circumference, or volume measurements were available, and in those cases we converted these data to mass using the method of (Moller 1991; Harcourt *et al.* 1995). We then used the residuals from a regression analysis of log(testes mass) against log(male body mass) to obtain relative testes size per species. Relative testes size did not differ between observed mass and converted mass based on transformations (independent-sample $t_{63} = -0.643$, $P = 0.533$). Mating system was also included as a categorical variable based on the female's perspective of the relative potential number of partners she can choose between with three levels: 1) monogamous, 2) polygynous and/or polygamous, and 3) promiscuous and/or polyandrous. Mating system data was obtained from the literature, and can be found in Appendix D.

Ecological trait data

For each species in the analysis, we also compiled data on several additional variables that could confound analyses of MHC diversity and evolution. First, effective population size (N_e) can impact genetic diversity by affecting the realized mutation rate, strength of selection, and the amount of genetic drift experienced by a population (Hartl & Clark 1997) N_e can also influence parasite richness measures as larger populations can theoretically retain more parasites than smaller populations (Anderson & May 1978; Nunn *et al.* 2003). In addition, species with greater population density may retain more parasites with density-dependent transmission (Arneberg 2002) and species with larger geographic range sizes may encounter a greater diversity of parasites (Roberts 2002). We used census population size as a proxy for effective population size, with the caveat that the ratio of N_e/N is approximately 0.1 on average (Frankham 1995). Population size was estimated by multiplying average population density (individuals per km^2) from the PanTHERIA database (Jones *et al.* 2009) by the species geographic range size (km^2) extracted from spatial data provided by the 2010 IUCN Red List (http://www.iucnredlist.org/technical-documents/spatial-data#mammals). Previous studies have shown that this measure of population size is a significant predictor of N_e (Møller *et al.* 2008; Garamszegi & Nunn 2011), and has also been shown to be a significant predictor of parasite species richness (Nunn et al. 2003). Second, body mass is known to scale with many life-history traits, including population size, reproductive rate, and evolutionary rate (Martin & Palumbi 1993), and thus was included as a covariate. Body mass (g) data were extracted from PanTHERIA (Jones *et al.* 2009), or when not available, the primary literature. Because log body mass and log population size were correlated ($R^2 = 0.388$, $F_{1,89} = 56.345$, $P = 0.0001$), we used the residuals from a regression analysis of log(population size) versus log(body mass) to obtain relative body mass data. Our full comparative dataset can be found in Table S4.1. Variables were log transformed or square root arc-sin transformed (dS rate data; Sokal & Rohlf 1995) when necessary to meet normality assumptions.

Comparative analyses

We tested for significant effects of parasite species richness and estimates of sexual selection on MHC allelic diversity and rates of positive selection at functional sites. Because closely related species are more likely to share genetic and life history traits (Harvey & Pagel 1991), we included phylogenetically controlled comparative methods in our analyses. Model selection was performed using both generalized linear model (GLM) analysis and phylogenetic least squares (PGLS) regression analyses to control for effects of phylogeny. Our initial full models to explain dN at ABS and relative allelic richness included the following selection predictor variables: corrected parasite species richness, relative testes size, and mating system. Because relative testes size and mating system were borderline significantly correlated (R^2=0.044, $F_{1,83}$ = 3.779, P=0.055), we ran separate models with only one sexual selection variable used at a time. Other predictor variables included: taxon, body mass and population size. Models of the rate of dN at ABS also included dS at ABS as a covariate to control for underlying substitution rate. To test for associations between relative testes size (as a response variable) and the predictor variables mating system and corrected parasite species richness, we ran PGLS models controlling for the effects of taxonomic group and male body mass.

For the GLM analysis, initial models were performed using the statistical software SPSS v. 20 and simplified using AICc (Akaike information criteria corrected for smaller sample sizes) following (Crawley 2002). Results are reported as likelihood ratio χ^2 for analyses of the continuous dependent variables (dN at ABS and relative allelic richness). After simplifying models by sequentially removing non-significant terms, we were left with the lowest AICc model which was used in phylogenetically controlled analyses. The PGLS regression was conducted using the *caper* package in R (Orme 2011) using Pagel's λ statistic to account for non-

independence in the predictor and response variables. The phylogeny was constructed using information from the mammalian supertree (Bininda-Emonds *et al.* 2007) and polytomies were made binary for PGLS using the *multi2di* function in the *Ape* R package (Paradis *et al.* 2004). Species in the dataset but missing from the supertree assumed the names of the closest relatives in the supertree (i.e. *Papio cynocephalus* was changed to *Papio hamadryas* and *Zalophus wollebaeki* was changed *to Zalophus californianus*). We tested for phylogenetic signal on each predictor variable as well as on dN at ABS and relative allelic richness using two methods: Blomberg's *K* and Pagel's λ. Blomberg's *K* (Blomberg *et al.* 2003), was computed using the *picante* package (Kembel *et al.* 2010) in R; Blomberg's *K* describes phylogenetic signal of continuous traits, where $K = 1$ indicates a trait is evolving under Brownian motion (stochastic evolution) and $K < 1$ indicates a trait has less phylogenetic signal than expected. Pagel's λ (Pagel 1992) tests for phylogenetic signal through a variance-covariance structuring of the trait data with the species tree, and returns a value of λ that describes the phylogenetic signal of the data. When $\lambda = 0$, the tree is star-shaped and all trait values are independent. When $\lambda = 1$, the original tree best explains the phylogenetic structure of the data (Freckleton *et al.* 2002).

To test the robustness of our results, we ran GLM model comparison using the *glmulti* package (Calcagno & de Mazancourt 2010) in R (Team 2012). Models with strong support (AICc weights within 4 units of the lowest AICc value; Burnham & Anderson 2002) were retained in the confidence sets, and variable estimates for those models are shown in Tables S4.5.

RESULTS

Our final dataset comprised 115 mammal species (26 carnivores; 3, cetaceans; 14, chiropterans; 37, primates; 16, rodents; 19, ungulates), 2454 sequences and 2665 parasite species. We tested

for relationships between estimates of parasite-mediated selection (parasite species richness) and sexual selection (relative testes size and mating system) on the rate of positive selection (dN at ABS) and MHC allelic richness across mammals. We found that parasite species richness, corrected for citation counts, was positively associated with dN at ABS for rodents and ungulates and negatively associated for carnivores (Table 4.1 and Table S4.3, Fig. 4.1A). While the relationship for ungulates was not significant based on $\alpha = 0.05$, the effect size estimate did not cross zero. Predictors of sexual selection were included in these models and we found that the categorical variable mating system was not a significant predictor of positive selection at the DRB locus ($P > 0.05$, 95% CIs of the estimate crossed zero (Table S4.5)). In contrast, relative testes size was positively correlated with dN at ABS across all orders (Table 4.1, Fig. 4.1B). Taxon and the neutral substitution rate (dS at ABS) were also significant predictor variables, but no other life history or ecological traits were significant. These results were the same for the phylogenetically controlled models, as the maximum likelihood value of lambda was estimated to be zero, indicating no phylogenetic signal. All predictor and response variables except relative allelic richness and corrected parasite species richness showed strong phylogenetic signal and were significantly more similar among closer relatives (Table S4.2). The lack of phylogenetic signal in the full model was due to the fact that the variable 'taxon' was included in the model, and this captured most of the phylogenetic signal (values excluding 'Taxon' as a predictor variable ranged from $\lambda = 0.16$ (Relative allelic richness model) to $\lambda = 1$ (dN at ABS models)).

We were also interested to test if a species' allelic richness was related to sexual- or parasite-mediated selection indices. None of the selection variables were significant predictors of allelic richness, and the only ecological or life history trait that was significant was taxonomic

group (Table 4.1). Allelic richness was significantly lower for ungulates than any other taxonomic group (Fig. 4.2A). A post-hoc ANOVA to identify the sources of variation in relative allelic richness across taxa revealed that ungulates also had significantly fewer duplicated DRB loci than other orders, while primates had the most ($F_{1,39} = 8.359$, $P = 0.006$, Fig. 4.2B). In addition to predicting allelic diversity, taxonomic group was also a significant predictor of MHC sequence diversity (dN and dS at ABS), being greatest for bats and primates and lowest for carnivores and ungulates (Fig. 4.2C). Overall, these results are robust when considering the confidence set of models within 4 ΔAICc units from the lowest AICc model (Table S4.5).

DISCUSSION

This is the first study to consider the joint effects of parasite-mediated selection and sexual selection on patterns of MHC diversity across mammals. We found evidence that parasite-mediated selection (represented by corrected parasite species richness) was associated with positive selection at the MHC DRB locus in rodents and ungulates only. Species in these two groups that harbored greater parasite richness also showed higher rates of functionally significant evolutionary change at the MHC. By comparison, greater sexual selection (represented by relative testes size) was related to greater positive selection for mammal species across all orders examined here.

Only a handful of other studies have considered interspecific predictors of MHC polymorphism, and most have focused on the relationship between parasite diversity and MHC allelic richness. In spite of this focus and the extensive intraspecific empirical evidence for MHC-parasite associations (reviewed in Bernatchez & Landry 2003; Spurgin & Richardson 2010), there is surprisingly weak support for parasites driving differences in MHC diversity

between species. One study found allelic richness to be positively correlated with helminth species richness across 10 rodent species (de Bellocq *et al.* 2008), while another study found that the rate of positive selection at ABS (but not allelic richness) was positively related to nematode species richness (but not total species richness) across 27 species of primates (Garamszegi & Nunn 2011). Similarly, we found the rate of positive selection at antigen binding sites was related to greater parasite species richness in rodents and ungulates, but not other orders. Interestingly, there was a negative relationship between parasite species richness and dN at ABS in carnivores. We speculate that carnivores are more threatened than other taxa examined in this study (as evidenced by the ICUN Red List), and may be more vulnerable to genetic drift that concurrently reduces their genetic diversity and increases their susceptibility to parasitism. Many threatened carnivores have depleted MHC diversity (wild dogs, (Marsden *et al.* 2009); Ethiopian wolves, (Kennedy *et al.* 2011); cheetahs, (Castro-Prieto *et al.* 2011)) and have simultaneously experienced declines from infectious diseases such as rabies, canine distemper, and sarcoptic mange (Pedersen et al. 2008)(O'brien & Evermann 1988a). Future studies on interspecific MHC variability could incorporate threat status or include a more precise measure of population size. Another worthwhile direction would be to test for associations between parasite species richness across populations within species, as the total variation between allele number, rates of positive selection, and parasite species richness between populations may be less influenced by sampling effort and life history variation (e.g. Prugnolle *et al.* 2005; Cutrera & Lacey 2006; Dionne *et al.* 2007).

In contrast to the taxon-specific evidence of parasite-mediated selection, we found that relative testes size, as an indicator of sperm competition and intensity of sexual selection operating at the species level, was positively associated with rate of positive evolution at antigen

binding sites across all mammals in our study. Mating system as a categorical predictor was not significantly associated with any measure of MHC diversity, however recent genetic techniques have revealed that social mating system may not be an accurate proxy of genetic mating system (Clutton-Brock & Isvaran 2006). This finding provides evidence that species with high potential for female mate choice tend to have higher MHC nucleotide diversity at functionally important sites. There are several non-exclusive explanations for this result. The first is that species with greater relative testes size (and hence, greater sperm competition (Harcourt *et al.* 1995)) may have faster reproductive rates, increasing the speed of positive selection for new variants. Indeed, Sommer *et al.* (2002a) found higher levels of MHC variation in the fast-reproducing and promiscuous tufted-tailed rat (*Eliurus myoxinus*) compared to the life-time mated monogamous Malagasy jumping rat (*Hypogeomys antimena*) and hypothesized that reproductive rate might constrain MHC polymorphism. A second hypothesis is that greater sperm competition indicates greater promiscuity and increased exposure to sexually or socially transmitted diseases, which may enhance selection on immune defenses (e.g. Nunn *et al.* 2000; MacManes & Lacey 2012). As a third possibility, females with more potential mates might be choosy and by employing pre-copulatory or post-copulatory mating preferences, serve to increase MHC variability. A fourth hypothesis is that relative testes size is correlated with androgen levels (Parapanov *et al.* 2009), which can suppress immune function or mediate male behavior and increase exposure to parasites (Folstad & Karter 1992b; Veiga *et al.* 1998). It is important to note that we found no relationship between parasite species richness and relative testes size or mate choice in an analysis that controlled for body size and phylogeny. Bordes *et al.* (2011) also looked for this relationship across 55 rodent species and found that testes size was positively correlated with

only certain macroparasite groups. This indicates that relative testes size is related to MHC nucleotide variability independently of parasitism.

Because species can have similar life history traits and ecological conditions due to shared evolutionary history, phylogenetic relatedness must be taken into account during comparative analyses (Harvey & Pagel 1991). We were interested to identify taxon-specific relationships behind MHC polymorphism, so we included taxonomic group as a variable of interest. The variable 'taxon' was significant in every model and absorbed most of the variation due to phylogeny, in effect eliminating the phylogenetic signal. Indeed, we found taxonomic category to be an important predictor for MHC allelic and sequence diversity. Specifically, ungulates had significantly lower allelic richness than any other order, which may be due to their overall lower average number of duplicated DRB loci (Fig. 2). Primates on the other hand, had significantly greater allelic richness than ungulates, as well as a higher average number of duplicated DRB loci. Nucleotide diversity (π) is positively associated with the number of duplicated DRB loci in rodents (Winternitz and Wares unpublished) and this may be an important mechanism providing baseline genetic variation. Life history traits or ecological conditions that promote or reduce MHC gene duplication events are therefore of interest for understanding MHC polymorphism in natural populations.

Overall, we found support for both parasite-mediated selection and sexual selection as forces maintaining MHC variation in mammals. While support for parasites as agents of selection was only found for rodents and ungulates, support for sexual selection was sweeping across mammals. Some potential explanations behind this pattern include increased rates of evolution by mating system, greater selection on immune genes by socially or sexually transmitted disease, stronger female mate choice, and reduced immunocompetence in species

with relatively smaller testes for their body mass. The underlying basis for this finding can only be speculative in comparative analyses, but these results encourage more studies on the influence of sexual selection on MHC variability in wild populations.

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TABLES

Table 4.1. Results of generalized linear models using data for all species showing the effect of selection variables (relative testes size and corrected parasite species richness), life history traits (taxon, relative body mass, Log population size, and transformed dS at the antigen binding sites), and their interaction on (i) the rate of dN at antigen binding sites, and (ii) relative allelic richness at the DRB locus. Only results for the final simplified models are given. (i) Full model for dN at ABS: Relative testes size + Corrected PSR + Taxon + Relative body mass + LogPopSize + Transformed dS at ABS + Taxon*Corrected PSR + Taxon*Relative testes size + Taxon*Transformed dS at ABS. (ii) Full model for Relative allelic richness: Relative testes size + Corrected PSR + Taxon + Relative body mass + LogPopSize + Taxon*Corrected PSR + Taxon*Relative testes size. Test statistics are likelihood ratio $\chi 2$ tests for dependent variables.

	Dependent variables			
	dN at ABS (N=66)		Relative allelic richness (N=66)	
Predictor variables	df	χ^2	df	χ^2
Selection variables	.	.	.	.
Relative testes size	1	12.060**	.	.
Corrected PSR	1	3.36	.	.
Life history traits	.	.	.	.
Taxon	4	41.041***	4	11.546*
Relative body mass	.	.	.	.
Log population size	.	.	.	.
Transformed dS at ABS	4	9.331**	.	.
Interactions	.	.	.	.
Taxon*Corrected PSR	1	10.308*	.	.

*P<0.05, **P<0.01, ***P<0.001

FIGURE LEGENDS

Figure 4.1. Associations between parasitism, sexual selection and the rate of non-synonymous substitions (dN) at antigen binding sites (ABS) across taxa. A) Log parasite species richness corrected for sampling effort is significantly associated with the residuals of a dN ~ dS (at ABS) regression for Carnivora (black points, thin solid best-fit line), Rodentia (grey diamond, solid best-fit line), and Ungulates (= Artiodactyla + Perissodactyla ;star, hatched best-fit line) only (N = 72). B) Relationship between relative testes mass and the residuals of a dN~dS regression (at ABS) is significantly positive overall (best-fit line) and positive for all taxa (N = 62).

Figure 4.2. Measures of genetic diversity at the MHC by taxonomic group. A) Relative allelic richness, B) the number of DRB loci, and C) the rate of nonsynonymous substitutions (dN) and synonymous substitutions (dS) at the antigen binding sites (ABS) for carnivora, chiroptera, primates, rodents, and ungulates. Error bars denote 95% confidence intervals.

FIGURES

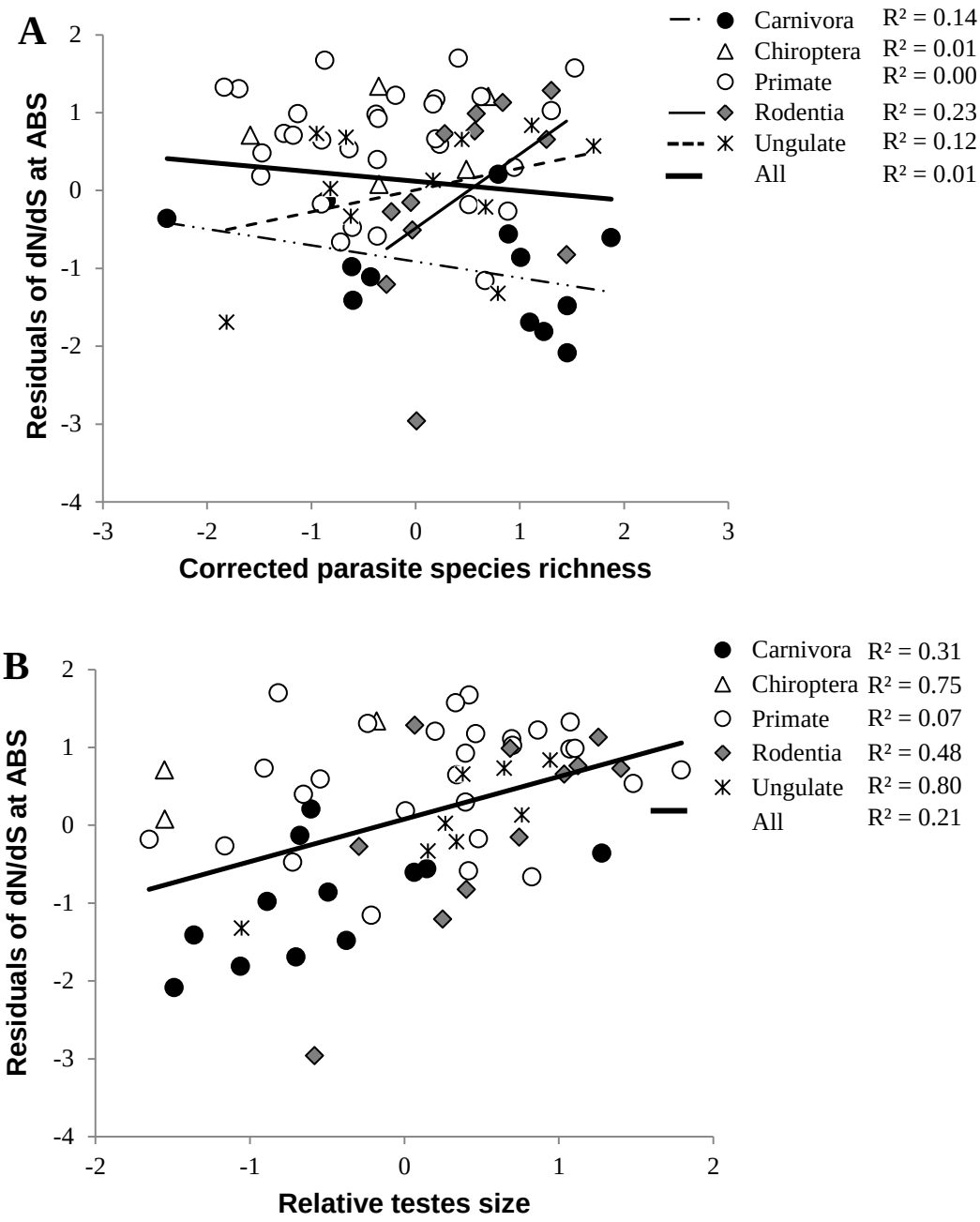

Figure 4.1.

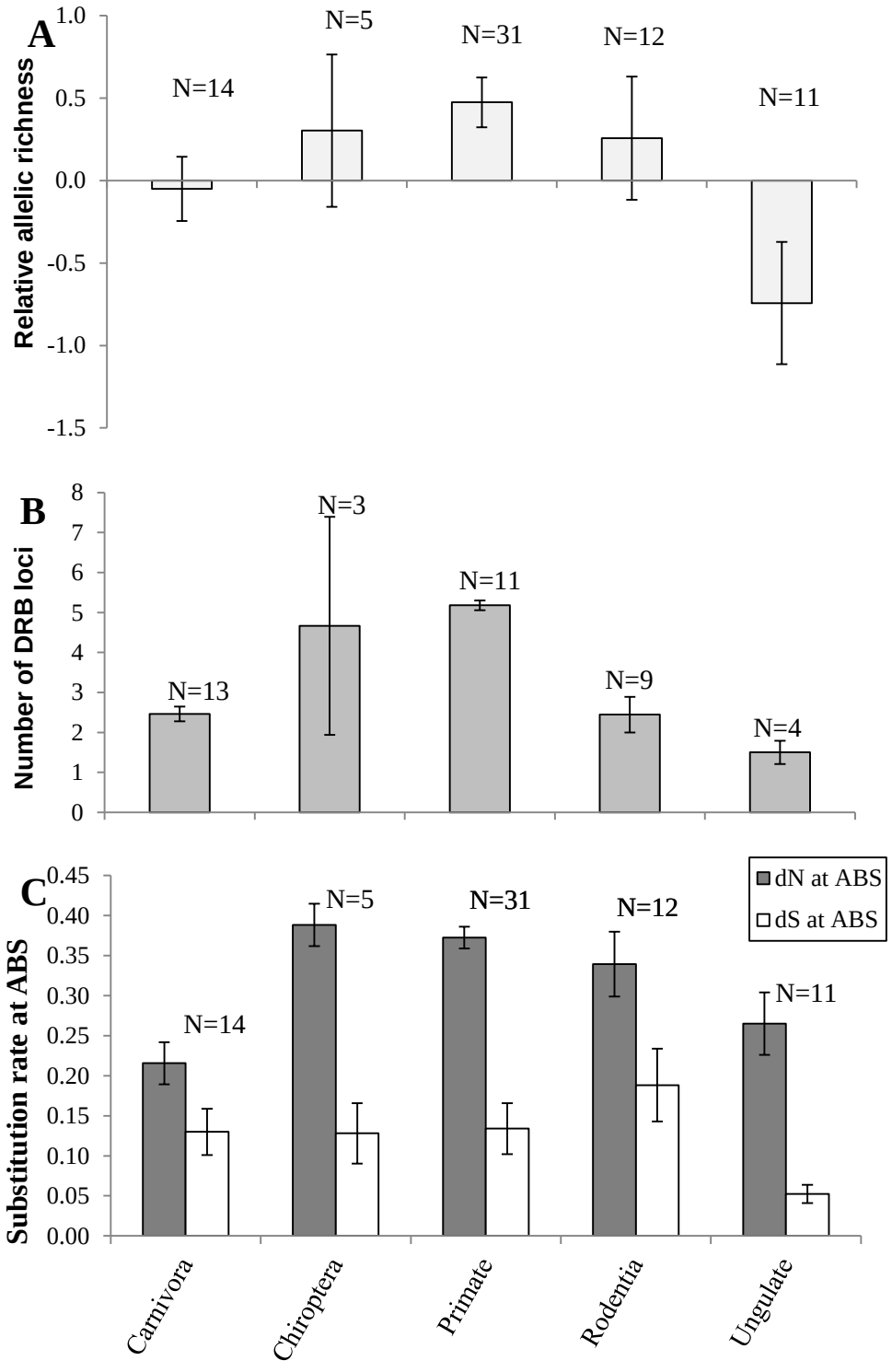
A
Relative allelic richness
1.0
0.5
0.0
-0.5
-1.0
-1.5
N=14
N=5
N=31
N=12
N=11
B
Number of DRB loci
8
7
6
5
4
3
2
1
0
N=13
N=3
N=11
N=9
N=4
C
Substitution rate at ABS
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
dN at ABS
dS at ABS
N=14
N=5
N=31
N=12
N=11
Carnivora
Chiroptera
Primate
Rodentia
Ungulate

Figure 4.2.

CHAPTER 6

CONCLUSION

Most evolutionary forces, such as drift and directional selection, tend to reduce genetic diversity in natural populations so a key question is what counteracts these forces to preserve genetic variation? Haldane suggested over 60 years ago that parasites may select for host genetic diversity (Haldane 1949). This dissertation focused on the role of parasites as agents of selection because parasites have intimate evolutionary relationships with their hosts, the potential to drive host population dynamics, and they have consequences for individual fitness. All of these levels of connection can be observed at the gene level. Whereas a wealth of evidence from human and laboratory studies have provided the foundation for this claim, this dissertation makes a novel contribution to the field by focusing on a non-model system in the wild. First, Chapter 2 demonstrated that parasites can track montane vole populations and were related to host traits and higher condition. Chapter 4 found that parasite load was greater in voles with greater genetic diversity and was associated with specific MHC alleles. Overall, these results suggest that parasites likely had little effect on the fitness of individual montane voles or on their overall population dynamics, although cumulative, sub-lethal effects could influence lifetime reproduction. Chapter 3 investigated the signals of historic balancing selection at the molecular level in the montane vole and across 16 rodent species and found that MHC diversity and purifying selection relate to rodent population dynamics and gene duplication.

With the growing availability of cost-effective Next Generation sequencing methods (Babik *et al.* 2009), non-model species with varying life histories and population dynamics will

increasingly provide evidence for the role of natural selection on MHC variation in wild populations (Bernatchez & Landry 2003; Ekblom *et al.* 2007; Eizaguirre *et al.* 2012). While a growing body of evidence supports that parasites can drive variation in MHC diversity in natural populations, this might not be the only or even main selective force. Chapter 5 investigated the contribution of parasitism and sexual selection as mechanisms explaining among-species variation in MHC diversity across diverse orders. It showed that both parasites and sexual selection are important for MHC variation across mammals, but sexual selection may be more broadly importantly than was previously considered.

This research has major conservation implications. As populations of wildlife around the globe continue to shrink, and the pull of genetic drift grows stronger, it becomes ever more important to understand the forces maintaining functional genetic variation in the wild.

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APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER 2

Recapture Analysis on Body Condition and Infection Status

Recaptured voles were assigned to 1 of 4 infection transition groups: 0-0 (uninfected at both first and second capture); 0-1 (uninfected at first capture and infected at second capture); 1-1 (infected at both captures); and 1-0 (infected at first capture and uninfected at second capture). Infection groups were assigned separately for cestode and *Eimeria* infections, and the final model included transition group, age, sex, site, and the duration of days between capture events as independent variables. We also examined cestode intensity as a predictor of body condition change between captures. We used infection categories of low and high, (low = 0 to 200 eggs/g and high = > 200eggs/g) and assigned recaptured animals to one of four transition groups (low to low, low to high, high to high, high to low). Our sample size was inadequate to repeat this analysis for *Eimeria* intensity.

Results

Thirty-five animals were recaptured between 5 and 20 days after the original capture date and were used to test whether infection was associated with temporal changes in body condition. Changes in *Eimeria* or cestode presence or intensity did not predict changes in any measure of host body condition.

Spatial Effects on Relationship between Density and Prevalence

Ripley's *K* function tests for departure from the null hypothesis of spatial randomness. We compared separately the distribution of *Eimeria* and cestode infected voles against the distribution of the underlying vole population (under natural environmental heterogeneity) at each grid for years of relatively low population density (2008) and high density (2009) to identify differences in spatial clustering of prevalence in low versus high density years. The K function was calculated at set distances of the radius between 0m and 20m to identify departures from the natural background variation in the population at various spatial scales (Diggle & Chetwynd 1991). We ran the simulation 1000 times to create a 95% confidence envelope, outside of which would indicate statistically significant patterns.

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APPENDIX B

SUPPORTING INFORMATION FOR CHAPTER 3

We followed procedures for genotyping non-model species from previously published papers to filter out artifacts (Galan *et al.* 2010; Kloch *et al.* 2010; Zagalska-Neubauer *et al.* 2010). We based our filtering procedure on a series of thresholds under the assumption that true alleles will be more common than artifacts across all individuals and within individuals (Babik *et al.* 2009). First, we called sequence variants putative alleles if they were present in at least 3 reads and made up at least 3% of reads of all variants within an individual (Babik *et al.* 2009). Second, we looked across individuals and retained putative alleles that were present in at least two individuals, representing the gold-standard two-PCR criterion of MHC studies (i.e., where an allele must be obtained from two independent PCRs to guard against PCR artifacts (Babik 2010). Only 1 putative allele that qualified based on length, read number per individual, and > 3 base pair differences from higher frequency alleles was found in a single individual and was retained in the final dataset. Third, we checked all putative alleles for indels and removed those that had insertions or deletions less than 3bp in length, which would result in a frameshift mutation. Fourth, to check for low frequency true alleles, we examined each individual with sequence variants between 2-3% frequency and retained variants that differed by at least three substitutions from the most similar higher frequency allele (Kloch *et al.* 2010). All those that differed by 3-bp or less were at low frequency (< 3%) and low read number (< 3 reads), and always co-occurred with the same more frequent alleles. These represented 59 putative alleles

out of 82 and were discarded from the final dataset. Two alleles were greater than 3-bp differences from the most frequent alleles, yet were only found in a single individual each, had less than 3 reads, and were at 1% frequency within their respective amplicons. These were removed as well. Fifth, we checked for chimeras by visually inspecting alleles per individual and determined whether putative alleles always co-occurred with putative parental alleles and using the program Chimaera (Posada & Crandall 2001) in the RDP3 program (Martin *et al.* 2010). This represented one putative allele, which was removed from the full set. Finally, we tested the coverage threshold of 46 reads per individual using linear regression between sequence number and putative alleles, and found a slight increase in allele numbers associated with increasing read counts ($P = 0.02$; adj.$R^2 = 0.032$). This relationship ceased to be significant at a threshold of 54 reads per individual ($P = 0.06$; adj.$R^2 = 0.023$).

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TABLES

Table S2.1. The comparative dataset of 16 rodent species used in the analysis.

	Dependent variables			Predictor variables									
Species	Tajima's D	dN/dS at ABS	dS-dN at nonABS	No. DRB loci	Log population size	Log Ne[A]	Log body mass(g)	Population dynamics[B]	Log sample size	SqrtArcsin dS at ABS	SqrtArcsin dS at nonABS	π	Log no. alleles	MHC References
Apodemus flavicollis	2.1	3.77	-0.05	1	10.03		1.50	Cyclic	2.56	0.32	0.14	0.095	1.41	Musolf et al. 2004; Meyer-Lucht and Sommer 2005; Meyer-Lucht and Sommer 2009
Apodemus sylvaticus	1.35	3.28	-0.06	1	9.59		1.34	Bottleneck	2.21	0.37	0.13	0.114	1.58	Musolf et al. 2004
Arvicola terrestris	1.5	4.48	-0.04	2	10.87		2.43	Cyclic	3.37	0.32	0.18	0.114	1.36	Oliver and Piertney 2006; Bryja et al. 2007; Oliver et al. 2009; Tollenaere et al. 2011
Castor fiber	0.72	5.25	-0.02	2	10.51	1.42	4.28	Bottleneck	1.88	0.21	0.13	0.062	1	Babik et al. 2005
Ctenomys talarum	0.3	0	0.06	1	11.95	2.42	1.32	Stable	2.59	0.37	0.28	0.031	1.41	Cutrera and Lacey 2006; Cutrera et al. 2011; Cutrerea et al. 2012
Dipodomys spectabilis	-0.23	3.09	-0.01	4	9.4	2.02	2.15	Bottleneck	0	0.28	0.27	0.155	0.7	Busch et al. 2008
Gerbillurus paeba	0.2	3.53	-0.01	2	9.94		2.10	Stable	1.6	0.32	0.23	0.095	1.52	Harf and Sommer 2005
Hypogeomys antimena	1.59	2.88	0	1	6.6		1.41	Bottleneck	2.43	0.26	0.3	0.100	0.6	Sommer et al. 2002; Sommer 2003;
Microtus montanus	0.89	0.76	0.13	2	10.87	2.3	3.07	Cyclic	2.1	0.63	0.46	0.133	1.32	This study
Microtus oeconomus	2.16	0.73	0.26	3	9.78	1.88	1.63	Cyclic	1.72	0.74	0.59	0.155	1.26	Klotch et al. unpublished; Radwan pers. comm
Myodes glareolus	1.2	1.31	0.29	4	10.11		1.52	Cyclic	2.52	0.64	0.64	0.194	2.03	Axtner and Sommer 2007; Babik and Radwan 2007; Kloch et al. 2010; Guivier et al. 2010
Peromyscus maniculatus	0.77	2.53	-0.02	1	8.43	4.95	1.30	Stable	1.83	0.43	0.3	0.129	1.45	Richman et al. 2001; Richman et al. 2003
Rattus rattus	0.18	2.67	0.02	1	8.32		2.30	Bottleneck	1.76	0.41	0.31	0.134	0.7	Sommer 2008
Rhabdomys pumilio	-0.18	2.57	0	1	9.28		1.71	Stable	2.72	0.34	0.24	0.086	2.41	Froeschke and Sommer 2005; Froeschke and Sommer 2012
Spermophilus citellus	0.63	6.94	0	1	4.32		2.60	Bottleneck	2.58	0.23	0.29	0.115	0.6	Říčanová et al. 2011
Spermophilus suslicus	0.61	5.4	0.01	1	9.66		2.40	Bottleneck	2.56	0.26	0.29	0.111	1.26	Biedrzycka and Radwan 2008; Biedrzycka et al. 2011

A. Ne references: Milishnikov 2004, Busch et al. 2007, Sommer et al. 2002b, Winternitz unpublished data, Galbreath and Cook 2004, Zheng et al. 2003.

B. Population dynamic references:

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Hypogeomys antimena: Sommer et al. 2002, Sommer and Hommen 2000, Durbin, J. & Goodman, S. 2008. Hypogeomys antimena. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1.

Microtus montanus: Pinter 1986; Smith unpublished data; Linzey, A.V. & NatureServe (Hammerson, G.) 2008. Microtus montanus. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1.

Microtus oeconomus: Zub et al. 2012; Linzey, A.V., Shar, S., Lkhagvasuren, D., Juškaitis, R., Sheftel, B., Meinig, H., Amori, G. & Henttonen, H. 2008. Microtus oeconomus. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1.

Myodes glareolus: Hörnfeldt 1994; Amori, G., Hutterer, R., Kryštufek, B., Yigit, N., Mitsain, G., Palomo, L.J., Henttonen, H., Vohralík, V., Zagorodnyuk, I., Juškaitis, R., Meinig, H. & Bertolino, S. 2008. Myodes glareolus. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1.

Peromyscus maniculatus: Gilbert and Krebs 1991; Linzey, A.V. 2008. Peromyscus maniculatus. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1.

Rattus rattus: Innes et al. 2001; Sommer 2008; Amori, G., Hutterer, R., Kryštufek, B., Yigit, N., Mitsain, G. & Palomo, L.J. 2008. Rattus rattus. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1.

Rhabdomys pumilio: Baxter and Hansson 2001; Coetzee, N. & van der Straeten, E. 2008. Rhabdomys pumilio. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1.

Spermophilus citellus: Millesi et al. 1999; Říčanová et al. 2011; Coroiu, C., Kryštufek, B., Vohralík, V. & Zagorodnyuk, I. 2008. Spermophilus citellus. In: IUCN 2012. IUCN Red List of Threatened Species. Version 2012.1.

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Table S2.2. Results from codon-based Z tests for a) departures from neutrality (dN/dS ≠ 1) at the antigen binding sites (ABS) (Brown et al. 1993) and b) negative selection (dS-dN > 1) at non-ABS across 16 rodent species and *Tupaia*. The species are categorized by presence of duplicated DRB loci (0,1) and presence of cyclic population dynamics (0,1). Significant P-values are in bold.

		dN/dS		dS-dN			
Species	**No. Sequences**	**ABS**	**P-value**	**nonABS**	**P-value**	**Duplicated**	**Cyclic**
Apodemus flavicollis	26	3.770	**0.001**	-2.331	1.000	0	0
Apodemus sylvaticus	38	3.281	**0.000**	-3.040	1.000	0	0
Arvicola terrestris	23	4.481	**0.000**	-1.697	1.000	1	1
Castor fiber	10	5.252	**0.014**	-0.917	1.000	1	0
Ctenomys talarum	26	0.000	0.459	1.332	0.093	0	0
Dipodomys spectabilis	5	3.086	0.111	-0.255	1.000	1	0
Gerbillurus paeba	33	3.526	**0.001**	-0.396	1.000	1	0
Hypogeomys antimena	4	2.883	0.275	-0.053	1.000	0	0
Microtus montanus	21	0.761	0.585	1.895	**0.030**	1	1
Microtus oeconomus	18	0.730	0.468	2.027	**0.022**	1	1
Myodes glareolus	106	1.308	0.396	2.434	**0.008**	1	1
Peromyscus maniculatus	28	2.528	**0.004**	-0.691	1.000	0	0
Rattus rattus	5	2.665	**0.023**	0.462	0.322	0	0
Rhabdomys pumilio	257	2.571	**0.020**	0.065	0.474	0	0
Spermophilus citellus	4	6.937	**0.000**	0.030	0.488	0	0
Spermophilus suslicus	18	5.402	**0.001**	0.467	0.321	0	0
Tupaia belangeri	28	2.628	**0.020**	1.470	**0.046**	1	0

Table S2.3. The subset of highly supported generalized linear models within four AICc units from the top model explaining the following dependent variables: Tajima's D, Log number of alleles, nucleotide diversity (π), dN/dS at ABS, and dN – dS at nonABS. All models included a significant intercept term and had full model *P* values and *Q* values (corrected *P* values using the False Discovery Rate) < 0.05. Abbreviated terms are described as follows: dS: synonymous substitutions; dN: non-synonymous substitutions; ABS: antigen binding site. AICc weights (*w*) describe the normalized relative likelihood of the model, or the probability the model is the best, given the data and set of candidate models.

Models for Tajima's D	AICc	ΔAICc	*w*	adj r^2	*P*	*Q*
Population dynamics	35.078	0.000	0.286	0.43	0.001	0.005
Population dynamics + number of DRB loci	35.459	0.381	0.237	0.52	0.000	0.002
Population dynamics + Log body mass(g)	35.863	0.785	0.193	0.50	0.000	0.002
Population dynamics + number of DRB loci + Log body mass(g)	36.934	1.856	0.113	0.58	0.000	0.001
Population dynamics + Log sample size	37.561	2.483	0.083	0.45	0.002	0.005
Population dynamics + Log population size	38.618	3.540	0.049	0.41	0.004	0.007
Population dynamics + Log body mass(g) + Log sample size	39.060	3.982	0.039	0.53	0.000	0.002

Models for Log number of alleles	AICc	ΔAICc	*w*	adj r^2	*P*	*Q*
Population dynamics	23.267	0.000	0.463	0.40	0.002	0.006
Log population size	25.679	2.412	0.139	0.19	0.034	0.036
Population dynamics + Log sample size	26.256	2.988	0.104	0.40	0.004	0.007
Log population size + Log body mass(g)	26.295	3.028	0.102	0.28	0.021	0.026
Log population size + Log sample size	26.382	3.115	0.097	0.27	0.022	0.026
Population dynamics + Log population size	26.421	3.154	0.096	0.40	0.005	0.007

Models for π (nucleotide diversity)	AICc	ΔAICc	*w*	adj r^2	*P*	*Q*
Number of DRB loci + Log population size	-61.305	0.000	0.463	0.49	0.000	0.002
Number of DRB loci	-60.524	0.781	0.313	0.37	0.002	0.005
Number of DRB loci + Log population size + Log body mass(g)	-58.629	2.676	0.121	0.50	0.000	0.002
Number of DRB loci + Log body mass(g)	-58.300	3.006	0.103	0.38	0.004	0.007

Models for dN/dS at ABS	AICc	ΔAICc	*w*	adj r^2	*P*	*Q*
Log population size + Log body mass(g)	64.888	0.000	0.591	0.42	0.002	0.005
Log population size	67.499	2.611	0.160	0.20	0.028	0.030
Log population size + Log body mass(g) + Log sample size	67.923	3.035	0.130	0.42	0.003	0.007
Number of DRB loci + Log population size + Log body mass(g)	68.088	3.200	0.119	0.42	0.003	0.007

Models for dS-dN at nonABS	AICc	ΔAICc	*w*	adj r^2	*P*	*Q*
Number of DRB loci	-27.327	0.000	0.477	0.32	0.005	0.007
Number of DRB loci + Log sample size	-26.547	0.780	0.323	0.39	0.003	0.007
Number of DRB loci + Log body mass(g)	-24.589	2.739	0.121	0.31	0.014	0.018
Number of DRB loci + Log population size	-23.709	3.618	0.078	0.27	0.024	0.028

Table S2.4. Support for the subset of generalized linear models with the highest support (< 4 delta AICc units from the top model) after controlling for phylogeny using phylogenetic generalized least squares regression (PGLS). The subset of models from the GLM analysis (Table S1) is shown; see Table S1 for explanation of abbreviated terms. Pagel's λ was estimated using the rodent phylogeny (see Figure 3) and observed trait data and significance determined by likelihood ratio tests comparing models assuming the maximum likelihood estimate of λ to models assuming no phylogenetic signal (λ=0).

Models for Tajima's D	**AICc**	**ΔAICc**	***w***	**λ**	**λ *P***
Population dynamics	31.442	0.000	0.197	0	1.000
Population dynamics + number of DRB loci	31.095	-0.346	0.235	0	1.000
Population dynamics + Log body mass(g)	31.499	0.057	0.192	0	1.000
Population dynamics + number of DRB loci + Log body mass(g)	31.601	0.159	0.182	0	1.000
Population dynamics + Log sample size	33.197	1.756	0.082	0	1.000
Population dynamics + Log population size	34.254	2.813	0.048	0	1.000
Population dynamics + Log body mass(g) + Log sample size	33.726	2.285	0.063	0	1.000

Models for Log number of alleles	**AICc**	**ΔAICc**	***w***	**λ**	**λ *P***
Population dynamics	19.631	0.000	0.439	0	1.000
Log population size	22.602	2.971	0.099	0	1.000
Population dynamics + Log sample size	21.892	2.261	0.142	0	1.000
Log population size + Log body mass(g)	22.659	3.028	0.097	0	1.000
Log population size + Log sample size	22.746	3.115	0.093	0	1.000
Population dynamics + Log population size	22.058	2.426	0.131	0	1.000

Models for π (nucleotide diversity)	**AICc**	**ΔAICc**	***w***	**λ**	**λ *P***
Number of DRB loci + Log population size	-65.434	0.000	0.481	0.36	0.483
Number of DRB loci	-64.259	1.175	0.267	0.5	0.418
Number of DRB loci + Log population size + Log body mass(g)	-62.992	2.441	0.142	0	1.000
Number of DRB loci + Log body mass(g)	-62.474	2.960	0.110	0.53	0.463

Models for dN/dS at ABS	**AICc**	**ΔAICc**	***w***	**λ**	**λ *P***
Log population size + Log body mass(g)	61.251	0.000	0.653	0	1.000
Log population size	64.422	3.171	0.134	0	1.000
Log population size + Log body mass(g) + Log sample size	67.923	6.671	0.023	NA	NA
Number of DRB loci + Log population size + Log body mass(g)	63.724	2.472	0.190	0	1.000

Models for dS-dN at nonABS	**AICc**	**ΔAICc**	***w***	**λ**	**λ *P***
Number of DRB loci	-30.404	0.000	0.408	0	1.000
Number of DRB loci + Log sample size	-30.184	0.221	0.366	0	1.000
Number of DRB loci + Log body mass(g)	-28.225	2.179	0.137	0	1.000
Number of DRB loci + Log population size	-27.346	3.059	0.088	0	1.000

Table S2.5. Phylogenetic signal in rodent traits measured by Blomberg's *K* and Pagel's *λ*. Values of Blomberg's K were estimated from continuous traits only using the *Picante* package of R, and significance was determined based on variance of phylogenetically independent contrasts relative to 1000 tip shuffling randomizations of trait values on the rodent phylogenetic tree extracted from the mammalian supertree (Bininda-Emonds et al. 2008). Pagel's λ was estimated using the *Caper* package of R with significance determined by likelihood ratio tests comparing models assuming the maximum likelihood estimate of λ to models assuming no phylogenetic signal (λ=0). Significant *P*-values (α<0.05) are in bold.

	Blomberg's *K*		**Pagel's *λ***	
Trait	***K***	***P***	***λ***	***P***
Number of DRB loci	0.35	0.06	0.60	0.14
Log population size	0.21	0.31	0.00	1.00
Log body mass(g)	0.19	0.42	0.00	1.00
Population dynamics	NA	NA	0.92	0.23
Bottleneck	NA	NA	0.57	0.07
Cyclic	NA	NA	0.78	0.08
Duplicated	NA	NA	**0.81**	**0.00**
Log sample size	0.22	0.32	0.00	1.00
dS at ABS	**0.50**	**0.02**	0.64	0.09
dS at nonABS	0.34	0.07	0.21	0.68

Table S2.6. Model-averaged estimates of the different parameters in the subset of models with high confidence (Δ AICc < 4 from the top model), as well as the unconditional variance, 95% confidence intervals, and importance. Model-averaged coefficients for each parameter were calculated in the *glmulti* package of R. Unconditional variance was estimated using the 'Buckland method' (Buckland *et al.* 1997). Importance describes the sum of the parameter weights in the subset of models in which the parameter is present. Parameter estimates with 95% CIs that did not cross zero are in bold.

Dependent: Tajima's D		95% CI			
Predictor trait	**β**	**Lower**	**Upper**	**Uncond. var.**	**Importance**
Log population size	-0.004	-0.024	0.016	8.55358E-05	0.049
Log sample size	0.030	-0.104	0.164	0.004	0.122
Log body mass(g)	-0.106	-0.459	0.247	0.026	0.346
Number of DRB loci	-0.087	-0.372	0.197	0.017	0.350
(Intercept)	1.054	-0.133	2.240	0.297	1.000
Population dynamics: cyclic	**0.906**	**0.148**	**1.665**	**0.122**	**1.000**
Population dynamics: stable	-0.530	-1.320	0.260	0.132	1.000

Dependent: Log number of alleles		95% CI			
Predictor trait	**β**	**Lower**	**Upper**	**Uncond. var.**	**Importance**
Log body mass(g)	-0.022	-0.114	0.071	0.002	0.095
Log sample size	0.036	-0.115	0.187	0.005	0.188
Log population size	0.049	-0.100	0.198	0.005	0.405
Population dynamics: cyclic	0.325	-0.357	1.008	0.100	0.619
Population dynamics: stable	0.469	-0.425	1.362	0.171	0.619
(Intercept)	0.580	-0.655	1.815	0.327	1.000

Dependent: π (nucleotide diversity)		95% CI			
Predictor trait	**β**	**Lower**	**Upper**	**Uncond. var.**	**Importance**
Log body mass(g)	-0.002	-0.012	0.008	2.10628E-05	0.224
Log population size	-0.005	-0.016	0.006	2.61544E-05	0.584
(Intercept)	0.119	0.019	0.220	0.002	1.000
Number of DRB loci	**0.026**	**0.010**	**0.041**	**5.42244E-05**	**1.000**

Dependent: dN/dS at ABS		95% CI			
Predictor trait	**β**	**Lower**	**Upper**	**Uncond. var.**	**Importance**
Number of DRB loci	-0.037	-0.220	0.145	0.007	0.109
Log sample size	0.060	-0.224	0.344	0.017	0.118
Log body mass(g)	0.983	-0.251	2.217	0.326	0.854
Log population size	-0.491	-1.011	0.028	0.058	0.910
(Intercept)	5.577	0.000	11.154	6.665	1.000

Dependent: dS-dN at nonABS		95% CI			
Predictor trait	**β**	**Lower**	**Upper**	**Uncond. var.**	**Importance**
Log population size	0.000	-0.002	0.003	1.33E-06	0.078
Log body mass(g)	-0.003	-0.018	0.012	4.77E-05	0.121
Log sample size	0.017	-0.041	0.075	0.001	0.323
(Intercept)	-0.110	-0.320	0.101	0.010	1.000
Number of DRB loci	**0.065**	**0.015**	**0.115**	**0.001**	**1.000**

APPENDIX C

SUPPORTING INFORMATION FOR CHAPTER 4

Analysis of contemporary selection- comparison with neutral markers

The genetic structure of neutral genes (microsatellites) was compared with that of the DRB gene thought to be under selection. Because we could not unambiguously delineate Mimo alleles to specific DRB loci, we used the Average Percent Difference (APD) of MHC and microsatellite loci between pairs of individuals within populations (Miller & Lambert 2004; Miller *et al.* 2010), calculated in excel after the method of Yuhki, O'Brien (1990). Owing to limitations in sample size, our power to detect significant correlations and differences between neutral and adaptive markers is low, so we focus on highlighting trends. We tested the correlation between APD and allelic richness in MHC and microsatellites using Spearman rank correlations in SPSS v.20. A positive correlation would support genetic drift as the main driver of diversification for both types of loci.

Strength of genetic drift

Signals of genetic drift based on positive correlations between microsatellite and MHC APD were stronger for 2008 ($R^2 = 0.79$; $P < 0.001$) than 2009 ($R^2 = 0.24$; $P = 0.67$; Table S7). This indicates that genetic drift affected both MHC and microsatellite markers more during the lower density year (2008). We could not perform more formal tests using outlier analysis because we could not assign MHC alleles to specific loci and generate exact genotypes. Year was not a significant predictor of differences in microsatellite heterozygosity and d^2 (heterozygosity: T =

0.01, df =118, P = 0.99; d^2: T = -0.39, df =118, P = 0.70), implying that these measures of neutral diversity were similar during high and low density years.

TABLES

Table S3.1. Properties of the six microsatellite loci used to gentotype M. montanus. Panel refers to the multiplexing group, dye indicates the fluorescent dye attached to the 5' end of the F primer, Ho indicates observed heterozygosity.

Panel	Dye	Locus	Primer sequence (5'-3') F, forward; R, reverse	Repeat motif	Size range (bp)	No. of alleles	Ho	Reference for initial characterization	GenBank Accession no.
A	HEX	Mar076	F: TCACCAGGACCTACTGAGCA	(AC)16	110-136	14	0.870	Walser and Heckel 2008	EF666987
	FAM	Ma68	F: GACTTTTTCATAGAATGAGGTTTTAG	(AC)10GC(AC)12	116-142	13	0.899	Gauffre et al. 2007	EF177201
	NED	Ma88	F: AAGGACGATGAGGACCAACC	(GA)27	137-161	11	0.858	Gauffre et al. 2007	EF177199
B	NED	Msmoe02	F: CATCTGATGAGTCCCTGAGG	(GT)17	135-185	17	0.884	Van de Zande et al. 2000	AF268903
	HEX	AV13	F: CTGGCTCTATCTATCTGTCTATC	(GATA)14	195-271	19	0.923	Stewart et al. 1998	Y16552
	FAM	Ma54	F: CGAATATGCTGTCTCACTTCC	(TG)3(CG(TG)8CG(TG)9	209-233	13	0.868	Gauffre et al. 2007	EF177197

Table S3.2. Quantification of genotyping error rate across the 6 microsatellite loci. Number of samples re-genotyped ranged from 10-18 across loci.

Locus	Correct	Number of duplicates	Number of mistyped duplicates	Number of mistyped alleles	Error rate per reaction	Error rate per allele
Mar076	18	18	0	0	0	0
Ma68	17	17	0	0	0	0
Ma88	10	10	0	0	0	0
AV13	17	17	0	0	0	0
Ma54	17	18	1	1	0.056	0.028
Msmoe02	16	16	0	0	0	0
Overall	**95**	**96**	**1**	**1**	**0.000579**	**0.000145**

Table S3.3. Partitioning of neutral molecular variance within and among years 2008 and 2009 by sites and across individuals using the analysis of molecular variance (AMOVA) method.

Source of variation	**d.f.**	**SS**	**VC**	**FST**	**%**	***P*-value**
Among sites	2	7.427	0.010	0.005	0.460	0.190
Among years within sites	3	7.033	0.012	0.006	0.590	0.180
Among individuals within sites by year	132	268.207	0.000	0.000	-0.008	0.730
Within individuals	138	285.000	2.065	0.003	99.750	0.540

SS, sums of squares; VC, variance component; FST, fixation index; %, percentage of genetic variation; *P*-value, level of significance based on 10100 permutations.

Table S3.4. Fst estimates for all 6 microsatellite loci standardized according to the method of Hedrick (2005). Variance was estimated using bootstrapping methods with 10,000 randomizations.

Locus	Diversity Parameter	Bootstrapped Est.	95% CI Min	95% CI Max
Mar076	G'_{ST_est}	0.084	0.015	0.170
Ma68	G'_{ST_est}	0.091	0.011	0.186
Ma88	G'_{ST_est}	0.193	0.107	0.294
AV13	G'_{ST_est}	0.223	0.117	0.341
Ma54	G'_{ST_est}	0.156	0.054	0.268
Msmoe02	G'_{ST_est}	0.178	0.083	0.284

G'_{ST_est} = standardized measure of genetic differentiation (Hedrick 2005). Calculated in SMOGD: Software for the Measurement of Genetic Diversity (version 1.2.5)

Table S3.5. Allele frequencies of MHC class II DRB alleles for *M. montanus* in the total population, in subpopulations, and years.

Level	**All**	**Subpopulations**			**Years**	
Category		KP1	KP2	Rmed	2008	2009
N	274	67	98	109	33	241
Mimo-DRB*01	6.8	4.5	9.2	5.5	3.0	7.1
Mimo-DRB*02	40.0	40.3	29.6	30.3	36.4	32.0
Mimo-DRB*03	7.8	9.0	2.0	11.0	3.0	7.9
Mimo-DRB*04	16.4	6.0	7.1	8.3	12.1	6.6
Mimo-DRB*05	11.8	11.9	5.1	3.7	6.1	6.2
Mimo-DRB*06	7.8	3.0	2.0	4.6	0.0	3.7
Mimo-DRB*07	16.7	11.9	14.3	8.3	15.2	10.8
Mimo-DRB*08	0.7	0.0	0.0	1.8	0.0	0.8
Mimo-DRB*09	2.8	1.5	1.0	5.5	0.0	3.3
Mimo-DRB*10	5.2	0.0	2.0	0.0	3.0	0.4
Mimo-DRB*11	1.6	1.5	3.1	0.0	0.0	1.7
Mimo-DRB*12	0.3	0.0	0.0	0.9	0.0	0.4
Mimo-DRB*13	11.0	3.0	8.2	7.3	12.1	5.8
Mimo-DRB*14	0.9	1.5	0.0	0.9	0.0	0.8
Mimo-DRB*15	0.5	1.5	0.0	0.0	0.0	0.4
Mimo-DRB*16	0.9	1.5	1.0	0.0	0.0	0.8
Mimo-DRB*17	0.7	0.0	2.0	0.0	0.0	0.8
Mimo-DRB*18	8.0	3.0	11.2	8.3	9.1	7.9
Mimo-DRB*19	1.3	0.0	1.0	2.8	0.0	1.7
Mimo-DRB*20	0.3	0.0	0.0	0.9	0.0	0.4
Mimo-DRB*21	0.4	0.0	1.0	0.0	0.0	0.4

Abbreviations: KP1, Kettle Ponds 1; KP2, Kettle Ponds 2; Rmed, Research Meadow; N, sample size.

Table S3.6. Partitioning of MHC allele frequency variance within and among years 2008 and 2009 by sites and across individuals using the analysis of molecular variance (AMOVA) method.

Source of variation	d.f.	SS	VC	FST	%	*P*-value
Among sites	2	1.018	0.006	0.014	1.410	0.201
Among years within sites	3	0.927	-0.006	-0.015	-1.490	0.822
Within years by site	255	108.798	0.427	-0.001	100.080	0.632

SS, sums of squares; VC, variance component; FST, fixation indice; %, percentage of genetic variation; *P*-value, level of significance based on 10100 permutations.

Table S3.7. Estimates of microsatellite and MHC Average Percent Difference (APD) by site per year. N, sample size; A, average number of alleles observed; SE, standard error.

Sample year	Sample site	N	A	APD ± SE
Microsatellite loci				
2008				
	KP1	6	6.17	30.56 ± 2.05
	KP2	6	6.33	38.8 ± 5.02
	Rmed	11	8.67	45.35 ± 2.51
	Metapopulation	23	7.06	41.58 ± 4.28
2009				
	KP1	32	11.00	37.78 ± 0.54
	KP2	38	11.67	35.74 ± 0.61
	Rmed	45	13.17	40.88 ± 0.54
	Metapopulation	115	11.94	38.53 ± 1.49
MHC putative loci				
2008				
	KP1	2	2.00	33.3 ± 0
	KP2	4	6.00	34.17 ± 4.76
	Rmed	11	8.00	40.55 ± 2.83
	Metapopulation	17	9.00	39.81 ± 2.28
2009				
	KP1	30	14.00	32.6 ± 2.42
	KP2	38	15.00	34.41 ± 1.65
	Rmed	38	15.00	35.56 ± 1.59
	Metapopulation	106	21.00	35.52 ± 0.82

Table S3.8. Results of generalized linear models showing the effect of genetic variables, host variables, population variables, and design variables on (i) the probability of *Eimeria* infection, (ii) the probability of cestode infection, (iii) *Eimeria* morphotype A intensity, (iv) cestode morphotype A load (including uninfected animals, (v) cestode morphotype A intensity (excluding uninfected animals), and (vi) endoparasite species richness. MHC predictor variables were run in separate models.

	Dependent	Eimeria presence N=96		Cestode presence N=96		Eimeria intensity N=90		Cestode intensity N=25 infected		Endoparasite species richness N=96	
Independent		df	χ^2	df	χ^2	df	χ^2	df	χ^2	df	χ^2
Genetic variables	Microsatellite heterozygosity									1	5.374*
	Microsatellite d^2							1	5.228*		
	MHC heterozygosity					1	2.28			1	3.316
Host variables	Age			2	4.613						
	Sex										
	Condition							1	13.815***		
Population variables	Site	2	6.836*			2	10.045**				
	Density										
Design variables	Trap period							3	8.656*		
	Year	1	3.024					1	5.849*		

	Dependent	Eimeria presence N=88		Cestode presence N=88		Eimeria intensity N=88		Cestode intensity N=22 infected		Endoparasite species richness N=88	
Independent		df	χ^2	df	χ^2	df	χ^2	df	χ^2	df	χ^2
Genetic variables	Microsatellite heterozygosity			1	3.004					1	5.470*
	Microsatellite d^2										
	MHC divergence										
Host variables	Age			2	4.006						
	Sex										
	Condition							1	6.303**		
Population variables	Site	2	6.557*			2	10.303**				
	Density			1	2.997						
Design variables	Trap period										
	Year	1	2.211								

	Dependent	Eimeria presence N=96		Cestode presence N=96		Eimeria intensity N=90		Cestode intensity N=25 infected		Endoparasite species richness N=96	
Independent		df	χ^2	df	χ^2	df	χ^2	df	χ^2	df	χ^2
Genetic variables	Microsatellite heterozygosity									1	5.464*
	Microsatellite d^2							1	5.228*		
	MHC number of supertypes					1	3.275			1	3.323
Host variables	Age			2	4.613						
	Sex										
	Condition							1	13.815***		
Population variables	Site	2	6.836*			2	11.654**				
	Density										
Design variables	Trap period							3	8.656*		
	Year	1	3.024					1	5.849*		

Note: Only the results for final simplified models are given (full model: dependent variable = microsatellite heterozygosity + microsatellite d^2 + MHC variable + specific MHC alleles + age + sex + condition + site + host denisty + trap period + year). Test statistics are Wald χ^2 for binary (0 or 1) response variables (probability of Eimeria and cestode infection) and likelihood ratio χ^2 tests for other dependent variables. *, P<0.05; **, P<0.01; ***, P<0.001.

FIGURE LEGENDS

Figure S3.1. Relationships between microsatellite and MHC Average Percent Difference (APD) by year. Lines indicate best linear fit.

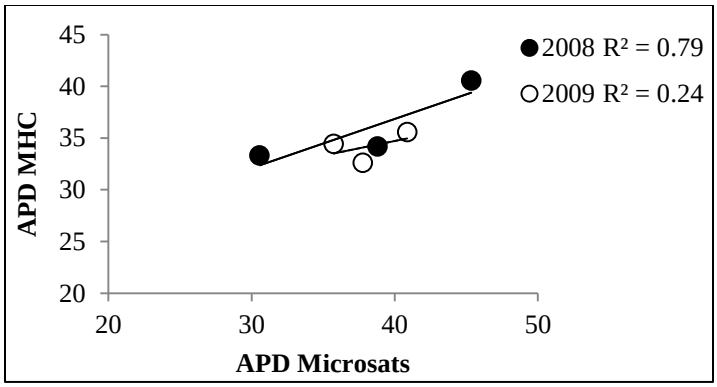
45
40
35
30
25
20
20
30
40
50
APD MHC
APD Microsats
2008 R² = 0.79
2009 R² = 0.24

Figure S3.1.

APPENDIX D

SUPPORTING INFORMATION FOR CHAPTER 5

APPENDIX FOR CHAPTER 5

Data provided as CSV file from author by request.

TABLES

Table S4.1. Full dataset for comparative analysis. PSR, parasite species richness. (Provided as CSV file from author by request).

Table S4.2. Phylogenetic signal in estimates of MHC genetic diversity and mammal life history traits measured by Blomberg's K and Pagel's λ. Values of Blomberg's K were estimated from continuous traits only using the Picante package of R, and significance was determined based on variance of phylogenetically independent contrasts relative to 1000 tip shuffling randomizations of trait values on the rodent phylogenetic tree extracted from the mammalian supertree (Bininda-Emonds et al. 2008). Pagel's λ was estimated using the Caper package of R with significance determined by likelihood ratio tests comparing models assuming the maximum likelihood estimate of λ to models assuming no phylogenetic signal (λ=0). Significant P-values (α<0.05) are in bold.

	Blomberg's *K*		**Pagel's *λ***	
Trait	***K***	***P***	***λ***	***P***
dN at ABS	0.35	**0.00**	0.37	**0.00**
Realtive allelic richness	0.23	0.17	0.00	1.00
Relative testes size	0.41	**0.00**	0.70	**0.00**
Mating system	NA	NA	0.18	**0.05**
Corrected PSR	0.18	0.65	0.01	0.93
Relative body mass	0.37	**0.00**	0.32	**0.00**
Transformed dS at ABS	0.26	0.08	0.47	**0.00**
Log population size	0.73	**0.00**	0.78	**0.00**

Table S4.3. Summary of phylogentic generalized least squares (PGLS) models with the top support (lowest AICc values). The three top models from the GLM analysis (Table 1 and 2) are shown; see Table 1 for explanation of abbreviated terms. Pagel's λ was estimated using a subset of the mammal supertree phylogeny (Bininda-Emonds et al. 2008) and observed life history trait data, and model significance was determined by likelihood ratio tests comparing models assuming the maximum likelihood estimate of λ to models assuming no phylogenetic signal (λ=0). All ML values for λ were zero.

Model	Effect statistics			
dN at ABS	**r**	**Lower CI**	**Upper CI**	***p-value***
Full model	$\lambda = 0.000, F_{12,48} = 5.249$, n = 61			0.000
Taxon Carnivora	0	.	.	.
Taxon Chiroptera	0.173	**0.088**	**0.257**	**0.046**
Taxon Primate	0.117	**0.084**	**0.150**	**0.001**
Taxon Rodentia	0.000	-0.045	0.044	0.996
Taxon Ungulate	0.034	-0.009	0.078	0.436
Relative testes size	0.053	**0.038**	**0.069**	**0.001**
Corrected PSR	-0.014	-0.034	0.007	0.511
Transformed dS at ABS	0.191	**0.097**	**0.285**	**0.047**
Taxon Carnivora*Corrected PSR	0	.	.	.
Taxon Chiroptera*Corrected PSR	-0.036	-0.122	0.051	0.683
Taxon Primate*Corrected PSR	0.025	-0.002	0.053	0.366
Taxon Rodentia*Corrected PSR	0.114	**0.065**	**0.162**	**0.022**
Taxon Ungulate*Corrected PSR	-0.009	-0.054	0.036	0.841
dN at ABS	**r**	**Lower CI**	**Upper CI**	***p-value***
Full model	$\lambda = 0.000, F_{15,48} = 3.577$, n = 71			0.000
Taxon Carnivora	0	.	.	.
Taxon Chiroptera	0.118	**0.005**	**0.231**	0.301
Taxon Primate	0.097	**0.017**	**0.177**	0.231
Taxon Rodentia	-0.002	-0.093	0.089	0.983
Taxon Ungulate	-0.179	**-0.290**	**-0.069**	0.111
Corrected PSR	-0.026	**-0.047**	**-0.006**	0.212
Transformed dS at ABS	0.012	-0.149	0.173	0.939
Taxon Carnivora*Corrected PSR	0	.	.	.
Taxon Chiroptera*Corrected PSR	-0.008	-0.072	0.057	0.907
Taxon Primate*Corrected PSR	0.022	-0.006	0.050	0.430
Taxon Rodentia*Corrected PSR	0.152	**0.103**	**0.200**	**0.003**
Taxon Ungulate*Corrected PSR	0.063	**0.019**	**0.108**	0.162
Taxon Chiroptera*Transformed dS at ABS	0.070	-0.267	0.406	0.837
Taxon Primate*Transformed dS at ABS	0.099	-0.122	0.321	0.656
Taxon Rodentia*Transformed dS at ABS	0.097	-0.128	0.322	0.667
Taxon Ungulate*Transformed dS at ABS	1.009	**0.604**	**1.415**	**0.016**
Relative allelic richness	**r**	**Lower CI**	**Upper CI**	***p-value***
Full model	$\lambda = 0.000, F_{5,67} = 3.455$, n = 72			0.008
Taxon Carnivora	0	.	.	.
Taxon Chiroptera	0.353	-0.160	0.866	0.494
Taxon Primate	0.557	**0.238**	**0.876**	0.085
Taxon Rodentia	0.307	-0.080	0.695	0.431
Taxon Ungulate	-0.693	**-1.090**	**-0.296**	0.086

Table S4.4. Summary of phylogentic generalized least squares (PGLS) models predicting relative testes size with the top support (lowest AICc values). See Table 1 for explanation of abbreviated terms. (Full model: Relative testes size = Taxon + Mating system + Corrected PSR + Log male mass).

Model	**Effect statistics**			
dN at ABS	**r**	**Lower CI**	**Upper CI**	***p-value***
Full model	$\lambda = 0.707$, $F_{9,53} = 3.723$, n = 63			0.001
Taxon Carnivora	0	.	.	.
Taxon Chiroptera	-1.947	**-2.838**	**-1.056**	**0.033**
Taxon Primate	0.099	-0.621	0.819	0.891
Taxon Rodentia	0.071	-0.696	0.838	0.926
Taxon Ungulate	1.256	**0.473**	**2.038**	0.115
Mating system (monogamous)	0	.	.	.
Mating system (polygamous)	0.333	**0.060**	**0.605**	0.228
Mating system (promiscuous)	0.797	**0.549**	**1.045**	**0.002**
Corrected PSR	-0.143	**-0.233**	**-0.053**	0.117
Log male body mass	-0.424	**-0.562**	**-0.286**	**0.003**

Table S4.5. Predictor variables from confidence set of models within 4 ΔAICc of model with highest support. Model averaged effect size, 95% confidence intervals, unconditional variance, and number of models including the variable. Variables with estimates that do not cross zero are in bold.

Dependent variable: Relative number of alleles						
Selection variables: Relative testes size and corrected PSR						
Predictor variable	**β**	**Lower CI**	**Upper CI**	**Uncond. var.**	**Importance**	**N**
Log population size	-0.001	-0.041	0.039	0.000	0.131	2
Corrected PSR	-0.003	-0.052	0.046	0.001	0.158	3
Relative testes size	0.022	-0.078	0.122	0.002	0.171	2
Relative body mass	0.048	-0.156	0.252	0.010	0.313	5
Taxon Chiroptera	0.550	-0.635	1.734	0.350	0.821	8
Taxon Primate	0.445	-0.257	1.147	0.123	0.821	8
Taxon Rodentia	-0.078	-0.809	0.653	0.133	0.821	8
Taxon Ungulate	-0.461	-1.350	0.427	0.197	0.821	8
Selection variables: Mate choice and corrected PSR						
Log population size	-0.001	-0.049	0.047	0.001	0.158	2
Corrected PSR	-0.004	-0.063	0.056	0.001	0.191	3
Relative body mass	0.047	-0.159	0.253	0.011	0.311	4
Taxon Chiroptera	0.513	-0.665	1.691	0.346	0.784	6
Taxon Primate	0.440	-0.277	1.156	0.128	0.784	6
Taxon Rodentia	-0.054	-0.745	0.637	0.119	0.784	6
Taxon Ungulate	-0.426	-1.300	0.449	0.191	0.784	6
Dependent variable: dN at ABS						
Selection variables: Relative testes size and corrected PSR						
Predictor variable	**β**	**Lower CI**	**Upper CI**	**Uncond. var.**	**Importance**	**N**
Relative body mass	0.000	-0.005	0.004	0.000	0.139	1
Corrected PSR	0.001	-0.005	0.006	0.000	0.152	1
Log population size	-0.001	-0.005	0.004	0.000	0.156	1
Taxon Chiroptera	**0.229**	**0.112**	**0.346**	**0.003**	**1.000**	**4**
Taxon Primate	**0.131**	**0.066**	**0.196**	**0.001**	**1.000**	**4**
Taxon Rodentia	0.059	-0.028	0.146	0.002	1.000	4
Taxon Ungulate	0.058	-0.028	0.145	0.002	1.000	4
Relative testes size	**0.058**	**0.027**	**0.089**	**0.000**	**1.000**	**4**
dS at ABS	**0.289**	**0.114**	**0.463**	**0.008**	**1.000**	**4**
Selection variables: Mate choice and corrected PSR						
Log population size	0.000	-0.003	0.003	0.000	0.123	2
Corrected PSR	0.000	-0.005	0.004	0.000	0.129	2
Relative body mass	0.000	-0.006	0.005	0.000	0.129	2
Mate choice: Polygamous	-0.007	-0.062	0.049	0.001	0.581	5
Mate choice: Promiscuous	0.034	-0.047	0.115	0.002	0.581	5
dS at ABS	0.224	-0.012	0.460	0.014	0.870	8
Taxon Chiroptera	**0.231**	**0.082**	**0.381**	**0.006**	**1.000**	**10**
Taxon Primate	**0.185**	**0.110**	**0.260**	**0.001**	**1.000**	**10**
Taxon Rodentia	**0.134**	**0.042**	**0.226**	**0.002**	**1.000**	**10**
Taxon Ungulate	**0.124**	**0.016**	**0.231**	**0.003**	**1.000**	**10**
Dependent variable: Relative testes size						
Predictor variable	**β**	**Lower CI**	**Upper CI**	**Uncond. var.**	**Importance**	**N**
Log population size	0.005	-0.037	0.047	0.000	0.149	1
Corrected PSR	-0.182	-0.430	0.066	0.015	0.814	2
Mate choice: Polygamous	**0.662**	**0.038**	**1.286**	**0.097**	**1.000**	**3**
Mate choice: Promiscuous	**0.891**	**0.329**	**1.453**	**0.078**	**1.000**	**3**
Taxon Chiroptera	**-1.775**	**-3.126**	**-0.424**	**0.453**	**1.000**	**3**
Taxon Primate	0.312	-0.359	0.983	0.112	1.000	3
Taxon Rodentia	0.326	-0.536	1.188	0.184	1.000	3
Taxon Ungulate	**0.962**	**0.220**	**1.705**	**0.137**	**1.000**	**3**
Log male body mass	**-0.352**	**-0.604**	**-0.101**	**0.016**	**1.000**	**3**
dS at ABS	-1.234	-2.713	0.246	0.543	1.000	3