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Wild cyclic voles maintain high neutral and MHC diversity without strong evidence for parasite-mediated selection

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Abstract The major histocompatibility complex (MHC) is an important component of vertebrate immune defense involved with self/nonself recognition and disease susceptibility. The high variability of genes of the MHC is thought to arise from both parasite-mediated and sexual selection. An outstanding question involves the degree to which balancing selection can oppose genetic drift to maintain high MHC diversity in the face of population bottlenecks. To address this question we examined genetic diversity and population structure at neutral (microsatellite) and MHC genes in montane voles [*Microtus montanus* (Peale, 1848)] subject to high amplitude population fluctuations, and compared these to measures of infection by common gastrointestinal parasites. We found high neutral and MHC allelic variability, indicating low impacts of genetic drift despite large fluctuations in population size. Greater MHC diversity did not predict lower parasite richness or infection by the two most common endoparasites (cestodes and coccidian protozoa), as

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might be expected if genotypic composition confers resistance to infection. One specific MHC allele predicted lower cestode intensity, but we found no other associations between MHC and infection measures. Neutral heterozygosity was positively associated with total parasite richness, possibly owing to greater parasite tolerance among heterozygous relative to more inbred hosts. Overall, these results suggest that factors beyond the parasites examined here, such as high inter-patch migration, mate choice, gene conversion or other infectious agents, are likely maintaining the high levels of MHC diversity observed in wild montane voles.

Keywords Major histocompatibility complex · Host-parasite relationship · Balancing selection · *Microtus montanus* · Cestodes · *Eimeria* · Microsatellites

Introduction

Genetic diversity can determine individual fitness and the adaptive potential of populations in response to environmental change (Thompson 1998; Keller and Waller 2002; Spielman et al. 2004b). Understanding how population dynamics impact genetic diversity is a fundamental goal of wildlife genetics. In particular, fluctuating populations that undergo periodic bottlenecks can experience short-term losses of genetic diversity through drift that can recover following natural gene flow or artificial translocations (Johnson et al. 2010). Long-term maintenance of neutral genetic diversity depends on interacting factors including the relative strength of genetic drift versus population interconnectedness (Saccheri et al. 1998; Frankham et al. 2002; Hess et al. 2002), with selection further governing the maintenance of adaptive genetic variation (Reed and Frankham 2001).

The major histocompatibility complex (MHC) is well-suited for studies of the adaptive maintenance of genetic variation because of its known immunogenetic function (Klein 1986) and the large body of empirical evidence pointing towards balancing selection favoring MHC diversity in wild populations (Bernatchez and Landry 2003; Sommer 2005; Piertney and Oliver 2006). This region contains the most diverse set of coding genes in vertebrates, with most species examined to date showing extraordinary levels of allelic diversity and heterozygosity (Edwards and Hedrick 1998; Knapp 2005). How this variation persists in populations that experience strong declines or population bottlenecks is a crucial 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 and Richardson 2010; Oliver and Piertney 2012).

A number of studies have documented reduced MHC diversity in populations subject to extreme or frequent fluctuations in size (reviewed in Radwan et al. 2010; Sutton et al. 2011). In rarer cases, populations can maintain high MHC diversity despite evidence from neutral diversity suggesting severe population bottlenecks (Aguilar et al. 2004; but see Hedrick 2004; van Oosterhout et al. 2006; Oliver and Piertney 2012). 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 test 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. In particular, three modes of parasite mediated selection that could operate on MHC are: (1) the heterozygote advantage, whereby individuals with more alleles will recognize and combat a greater diversity of parasites (Doherty and Zinkernagel 1975; Penn et al. 2002); (2) the rare-allele advantage, whereby parasites adapt to overcome more common alleles, such that rare alleles confer resistance (Takahata and Nei 1990; Spurgin and Richardson 2010); and (3) the fluctuating selection hypothesis, whereby temporal and/or geographic variation in parasite risk selects for different sets of MHC alleles at different times and locations (Hill et al. 1991). This final mode of selection further predicts that MHC alleles will show greater population structure than neutral markers (Spurgin and Richardson 2010).

Here, we examined patterns of both presumed neutral and MHC genetic diversity and tested evidence for their associations with parasitism in montane voles (*Microtus montanus*), a species that inhabits alpine grassy meadows of North America ranging from Colorado to Utah (Sera and Early 2003). Voles from the Arvicolinae subfamily (to which montane voles belong) are a useful system to investigate MHC variation because they undergo frequent multi-annual population cycles (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; Winternitz et al. 2012). The factors responsible for these population cycles are currently unknown, and could include resources, predators, parasites, social effects, or a combination of these (reviewed in Krebs 2013). Montane voles also harbor multiple well-characterized parasites (Winternitz et al. 2012) and have 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 2013). This previous study found evidence for historic balancing selection through trans-species persistence of alleles (Garrigan and Hedrick 2003), but weak evidence of positive selection at the functionally important antigen binding sites (ABS).

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 and contemporary balancing selection on the MHC Class II DRB locus. We also tested whether several species of gastrointestinal parasites could serve as potential agents of selection on MHC variation, and if so, identify the mode of selection. Associations between MHC diversity and gastrointestinal helminthes have been observed in wild populations (reviewed in Sommer 2005) and MHC recognition of gastrointestinal parasites is initiated when helminth antigens activate intestinal epithelial cells to produce inflamatory cytokines that induce the T helper 2 type immune response, upregulating MHC class II expression and recruiting antigen presenting cells (dendritic cells and basophils) to the site of infection (Paul and Zhu 2010). We expected to find negative associations between measures of MHC diversity and parasitism at the individual level, consistent with the heterozygote-advantage hypothesis (e.g. Wegner et al. 2003). We also tested for associations between parasite infection and specific MHC alleles and functional supertypes, as previous work showed that particular alleles might confer protection against specific parasites (e.g. Westerdahl et al. 2005; Tollenaere et al. 2008; Froeschke and Sommer 2012) and to test the rare-allele advantage and fluctuating selection hypotheses. 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; Townsend and Jamieson 2013).

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, USA (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 0.5 km at approximately 2,900 m elevation. A total of 262 voles were captured using Longworth live traps 4–5 consecutive days per site every 2 weeks throughout the breeding season (Jun 15–Aug 15). No marked animals were captured from >1 site. Vole density changed by a factor of 30 between trapping intervals within each year (2008, 2009, 2010) and by a factor of 20 between years (Winternitz et al. 2012). Mean low year density was approximately 15 voles/ha, and high year density was 322 voles/ha. Mean vole density also differed among sites, with a full analysis of vole density provided in Winternitz et al. (2012). Only data from 2008 to 2009 are included in this study owing to low density and insufficient samples in 2010 where only 5, 2, and 12 animals were trapped during the entire breeding season in KP1, KP2, and Rmed, respectively. Animals received numbered ear tags (National Brand Tag Company) and sex, age, body mass (g) and length (mm) were recorded. Tissue was obtained from adults via a 2 mm tail tip stored in 95 % ethanol at 5 °C after anesthetizing the animals with isoflurane gas for 1 min. Fecal samples were stored in 10 % formalin. Permission for this study was granted by the Colorado Department of Natural Resources Division of Wildlife (Scientific Collections Licenses #08-10TR2006); animals were handled as per guidelines of the American Society of Mammalogists (Sikes and 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).

Parasitism measures

Methods for parasite identification and quantification are detailed in Winternitz et al. (2012). Briefly, parasite oocytes and eggs were examined using salt flotation to determine the presence of infection and to estimate parasite intensity per gram of feces. Fecal egg counts are commonly used in longitudinal studies to non-invasively determine infection status (Keymer and Hiorns 1986; Scott 1988; Ferrari et al. 2004; Froeschke and Sommer 2005). Morphological measurements were used to assign parasites to one of three taxonomic groups: coccidia (*Eimeria,* (Levine and Ivens 1965)), cestodes and nematodes. Although the fitness consequences of parasites for montane voles are unknown, these parasites can reduce host fitness in other vertebrate species through reducing body condition, survival and reproductive success (Wiger 1977; Scott and Lewis 1987; Scott 1988; Fuller and Blaustein 1996; Vorisek et al. 1998; Lello et al. 2005; Hakkarainen et al. 2006; Turner et al. 2012), thus, creating the potential for them to be agents of selection for montane voles. We identified five *Eimeria* morphospecies at a combined 53 % prevalence, two cestode species at a combined 24 % prevalence (Andrya spp. and Paranoplocephala infrequens; Bakke and Wiger 1975) and one nematode (*Syphacia spp.)* at 2 % prevalence

(Winternitz et al. 2012). Five measures of infection status were examined for individual voles: parasite species richness (which could vary from 0 to 8), and for cestodes and *Eimeria* separately, the presence/absence and intensity of infection (combining data for all morphospecies). Cestode intensity was estimated as the number of oocytes/eggs per gram of feces, and *Eimeria* intensity followed a scale of 0–3 based on oocysts per sample (1 = 1–10 oocysts, 2 = 11–100 oocysts, and 3 = >100 oocysts).

Microsatellite genotyping

We extracted genomic DNA using the PureGene DNA isolation kit (Gentra Systems), following the manufacturer's protocol. Six microsatellite loci were used to estimate the amount of neutral genetic variation in populations (Table S1): *Mar076* (HEX), *Ma68* (FAM), *Ma88* (NED), *Msmoe02* (NED), *AV13* (HEX), and *Ma54* (FAM) (Stewart et al. 1998; Van de Zande et al. 2000; Gauffre et al. 2007; Walser and Heckel 2008). 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), 4 μL of 5× reaction buffer, 2 μL of 2.5 mM MgCl2, 2 μL of a mix of 10 mM deoxyribonucleotide triphosphates, and 0.2 μL of (5u/ μL) GoTaq®Flexi DNA Polymerase (Promega M8295). Loci *Mar076*, *Ma68*, and Ma88 were amplified in multiplex panel 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 settings for both panels: an initial denaturation step at 95 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 90 s, elongation at 72 °C for 60 s, and a final extension at 60 °C for 30 min. 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

We focused on the MHC class II DRB gene exon 2 because it has previously been shown to contain most of the functionally important ABS and is, therefore, the most likely candidate for detecting balancing selection acting on MHC class II genes (Hughes and Yeager 1998). For genotyping, we used oligonucleotide forward primer JS1 and reverse primer JS2 described by Schad et al. (2004) to amplify a 171 bp fragment of the second exon of the DRB gene that includes part of the functional ABS. This primer system has amplified sequences from multiple loci in rodents when the DRB locus has been duplicated (Galan et al. 2010). To assign 454 sequencing reads to 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 and Glenn 2011). PCR conditions and further 454 sequencing and genotyping procedures are described in Winternitz and Wares (2013).

Initial analysis (Winternitz and Wares 2013) 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 (2013).

Statistical analysis

Estimates of neutral genetic variation

We tested whether 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 Fisher exact tests using Markov chain methods in GENEPOP version 4.0.10 (Raymond and Rousset 1995b; Rousset 2008) and corrections for multiple tests were performed using the false discovery rate (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 (F_{is}), and was calculated using the program GenAlEx (Peakall and 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 per 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 Markov chain method with exact tests of Guo and Thompson (1992) in GENEPOP. Significant P values were determined by correcting for multiple hypotheses testing using the FDR approach.

Interpopulation differences in genotype frequencies between years and sites were assessed with Markov chain Monte Carlo approximations of Fisher exact tests (Raymond and Rousset 1995a) calculated in Arlequin ver. 3.5 (Excoffier et al. 2005; Excoffier and Lischer 2010). The measure of genetic differentiation estimated for small sample size G_{ST_est} (Nei and Chesser 1983) and standardized by the greatest observed diversity G_{ST_est}' (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 and 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 and 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 by site and 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 and Rozas 2009). We did not include heterozygosity estimates for MHC because we were not able to assign alleles to specific loci and therefore, estimates may not be accurate. 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 and Flower 2005; Schwensow et al. 2007). The average number of supertypes observed was calculated per site and year.

The genetic structure of populations and years at the MHC was investigated using the AMOVA approach in Arlequin, with significance ($P < 0.05$) determined using 10,000 permutations. 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 and Rousset 1995a) in Arlequin 3.5.

Analysis of contemporary selection and genetic predictors of infection

To test whether MHC genes show lower among-population divergence measures relative to neutral markers (as expected under balancing selection), we used the average percent difference (APD) between pairs of individuals within populations (Miller and Lambert 2004; Miller et al. 2010), described further in the Appendix. We next tested whether MHC genotypes and microsatellite diversity were associated with three measures of parasitism: (1) parasite presence/absence and (2) intensity were calculated for both *Eimeria* and cestodes separately, and (3) total parasite species richness. Individual genetic variability at the MHC was calculated in several ways. For each individual, we calculated the presence-absence of each of the 21 Mimo-DRB alleles and the presence-absence of five MHC supertypes. We also recorded four measures of diversity: the total number of alleles per individual, the number of alleles based on unique amino acid sequences, the total number of supertypes and the pairwise divergence between alleles calculated in Mega 5 (Tamura et al. 2011). Count variables were log-transformed when necessary to meet normality assumptions. To estimate neutral 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).

Prior to analyses, we tested for independence of the genetic predictor variables.. Mean microsatellite heterozygosity was not significantly associated with d^2 (Spearman's $R = 0.142$, $P = 0.12$). Neutral variables (individual heterozygosity and d^2) and continuous MHC variables (listed above) were not significantly correlated based on Spearman's Rank tests (R ranges from -0.02 to -0.04, all $P > 0.05$). Because all MHC variables (aside from presence/absence of specific alleles) were significantly positively correlated (R ranges from 0.35 to 0.89, $P < 0.001$), we focused on the log number of alleles as the principal measure of MHC diversity. To test whether specific MHC alleles or supertypes were associated with parasitism, we first narrowed the set of predictors using an exhaustive search based on AICc in the *glmulti* package (Calcagno and de Mazancourt 2010) in R Team (2012). Variables from models with strong support (Δ AICc values within 4 units of the lowest AICc value; Burnham and Anderson 2002) and 95 % confidence intervals that did not overlap with zero were retained in the subsequent analyses. Parasite response variables (presence-absence of *Eimeria* and cestodes, *Eimeria* load, cestode intensity, and

endoparasite species richness) were tested against the binomial predictors 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). Generalized linear models (GLMs) involving presence-absence parasite measures used binomial error structures with logit link functions, tests for *Eimeria* load and endoparasite species richness used Poisson error structures with loglinear link functions, and tests for log cestode intensity (considering only positive individuals) used linear error structures and identity link functions.

As we had multiple predictors (and competing hypotheses), we chose to use the more robust method of multi-model inference to identify biologically reasonable explanatory variables averaged across strongly supported models (Grueber et al. 2011; Symonds and Moussalli 2011). After specific allele and supertype selection (1–4 specific alleles and 1 supertype retained per dependent variable), we ran full GLMs using *glmulti* for associations between parasitism and MHC and neutral diversity measures. We included the following ecological variables and host characteristics in all models, as these were previously shown to predict infection status in the field (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. Finally, we tested whether neutral genetic diversity and variation in MHC were associated with host body condition, using the above mentioned predictor variables and condition as the response variable with a linear identity link.

Results

Neutral genetic variation

A total of 139 voles 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 S2). No null alleles were detected and there was no evidence of linkage disequilibrium (Table 1) or departure from Hardy–Weinberg Equilibrium after FDR corrections. Microsatellite allelic richness and heterozygosity (observed and expected) were high across all sites and years, and the fixation index F_{is} did not indicate substantial inbreeding (Table 1). One site (Rmed) was out of Hardy–Weinberg equilibrium (lower than expected heterozygosity) when pooling samples by year, as was the year 2009 and the metapopulation overall (after FDR corrections).

There was no evidence of population structure between years or sites based on AMOVA tests ($P > 0.05$, Table S3). Estimates of migration between sites using private alleles corrected for sample size were high (15.0 migrants per year, 17.7 pooling years). Exact tests of sample differentiation based on genotype frequencies also showed no evidence of population structure when performed for each site by year ($P = 0.12 \pm 0.04$; 100,000 Markov steps) or across years combined ($P = 0.43 \pm 0.05$; 100,000 Markov steps). Estimated G_{ST} ranged from 0.01 to 0.03 and corrected G_{ST}' ranged from 0.08 to 0.23 across the six loci (Table S4).

MHC diversity

A total of 21 DRB alleles were detected from 123 individuals that were reliably genotyped based on a sufficient minimum coverage of 54 reads (mean 140.7, SD = 93.3, range

Table 1 Estimates of microsatellite genetic diversity for each sampling site by year, including sample sizes (N), average number of alleles observed (A), allelic richness (AR; for sample sizes >5 individuals per site/year, 20 individuals per metapopulation, and 35 individuals per site), 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)

Year	Site	N	A	AR	Ho	He	Fis	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
	Total	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
	Total	115	11.94	10.83	0.850	0.866	0.018	**0.008**
2008 + 2009	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**
	Total	138	12.61	12.20	0.855	0.878	0.014	**0.015**

Abbreviations for sampling locations are given in methods text

54–526). The frequencies of alleles and supertypes were similar across sites and over time (Table 2; Fig. 1, Table S5) even though total peak density including all sites was 132 voles/ha for 2008 and 510 voles/ha for 2009. 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. Counter to the expectation of fluctuating selection (Spurgin and Richardson 2010), we found no evidence for spatial or temporal differences in allele or type frequencies using AMOVA ($P > 0.05$, Table S3) or exact tests (year: $P = 0.46 \pm 0.04$; population: $P = 0.92 \pm 0.01$). There was some evidence for genetic drift based on APD correlations between MHC and neutral markers during the low density year, but this correlation disappeared during the high density year, indicating that genetic drift was not a significant factor affecting montane vole functional or neutral diversity in the years studied (see Appendix and Table S6 for more detail).

Associations between parasitism and neutral genetic diversity

Mean microsatellite heterozygosity was positively associated with endoparasite species richness (Table S7; Fig. 2a). 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. Individual characteristics (age, condition) and ecological variables (site) included as covariates demonstrated associations with parasitism in montane voles similar to previous work (Winternitz et al. 2012). Site was a significant predictor of *Eimeria* intensity (Table S7), sub-adults had lower cestode prevalence than adults (Table S7) and host body condition was positively associated with cestode intensity (Table S7).

Table 2 MHC class II DRB exon 2 variability in *M. montanus* for each sampling site and year

Level	N	A	A_{super}	A_{priv}	π	K	$N_{EST2008}$	$N_{EST2009}$	$N_{EST2010}$
All	123	21	5	0	0.133	22.700	88 (71–106)	322 (248–396)	16 (14–17)
Location									
KP1	32	14	5	1	0.089	15.133	122 (115–130)	175 (164–188)	18 (15–23)
KP2	42	15	5	3	0.079	13.540	61 (56–68)	410 (387–436)	12 (11–14)
Rmed	49	15	5	3	0.096	16.424	82 (75–91)	382 (365–400)	16 (15–17)
Year									
2008	17	9	4	0	0.076	12.991			
2009	106	21	5	11	0.090	15.329			

Average number of alleles observed (A), average number of allele supertypes (A_{super}), number of private alleles, (A_{priv}), nucleotide diversity (π), and number of pairwise basepair changes (K). Estimated population density (per ha) over the population cycle with 95 % confidence intervals: $N_{EST2008}$ (increasing phase), $N_{EST2009}$ (peak phase), $N_{EST2010}$ (low phase)

Associations between MHC diversity, specific alleles and parasitism

MHC diversity measures were not associated with total parasite richness, the presence/absence of *Eimeria* and cestodes, or measures of parasite intensity (Table S7). Tests of associations between measures of parasitism and the presence or absence of specific MHC alleles showed that only the presence of Mimo-DRB*07 (at 17 % frequency within the population, Fig. 1) was negatively related to cestode intensity (Table S7, Fig. 2b). Specific MHC alleles were not significantly associated with the presence/absence of *Eimeria* and cestodes, as would be expected if certain alleles conferred complete resistance to parasites ('qualitative resistance', sensu Westerdahl et al. 2013).

Body condition and genetic diversity

Vole body condition, as a proxy for the ability to procure resources and overall fitness, was higher for adults than for sub-adults, and was not associated with microsatellite or MHC diversity (Table S7). The presence of Mimo-DRB*14 was associated with poorer body condition (Table S7), but this allele was found in only 2 of 94 adults with condition scores available.

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). In fact, montane voles had slightly more MHC alleles recovered (controlling for sampling effort) when compared to 16 other rodent species (21 vs. 15 alleles; Winternitz and Wares 2013), possibly due to their duplicated loci. We found no evidence for genetic sub-structuring among sampling locations or years based on neutral markers or MHC data. Fixation indices for both marker types were low or zero, and genetic variability did not differ significantly by site or year, indicating that genetic drift and population sub-structuring were weak or absent in this population. Although our study

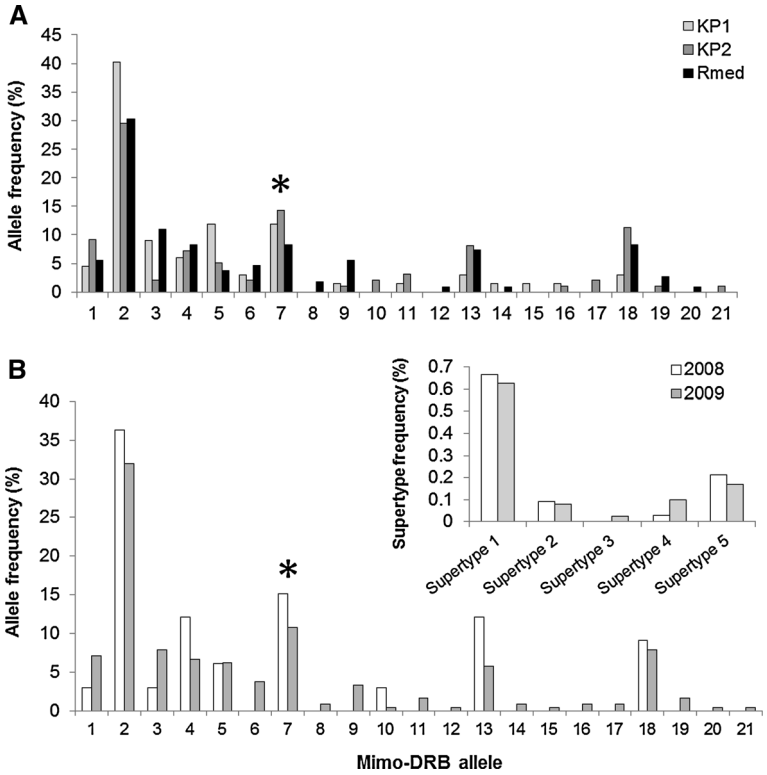

Fig. 1 Allele frequencies of MHC class II DRB alleles for *M. montanus* (Mimo-DRB) were generally consistent across sampling locations (**a**) and years (**b**). MHC supertype frequencies by year were also consistent and are shown in the inset of Fig. 1b. *Asterisks* indicate the allele negatively associated with cestode intensity. *Abbreviations for locations*: *KP1* Kettle Ponds 1, *KP2* Kettle Ponds 2; *Rmed* Research Meadow

found evidence for private DRB alleles among sites, differences in private alleles were eroded during the high density year (2009), most likely due to unobserved movement between sites. Thus, migration across populations and between years could produce a "rescue effect" that replenishes allelic diversity during peak density years (Berthier et al. 2006). Indeed, migration among sites within a meta-population organization is probably the most important factor influencing both neutral and adaptive diversity for this population.

The frequency distribution of specific MHC alleles was similar across sites and over the short timescale examined, counter to what we might have expected if fluctuating selection was responsible for the high MHC diversity observed here. This timeframe relates to approximately six generations of voles; and we acknowledge that a longer time series would be useful in determining whether allele frequencies vary over time. For example, a study on nine wild populations of guppies (*Poecilia reticulate)* over 2 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). However, a study on island water voles (*Arvicola amphibious*) showed that strong balancing selection can equalize MHC allele frequencies

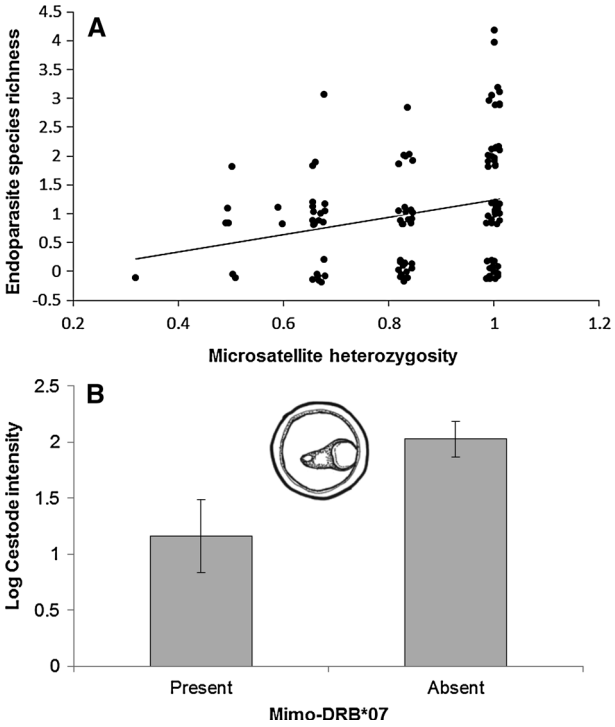

Fig. 2 Significant relationships were observed between (**a**) endoparasite species richness and average microsatellite heterozygosity, and (**b**) log cestode intensity and the presence of Mimo-DRB*07 in *M. montanus*. In (**a**), data points are jittered to show the true number of samples and the line is the linear best-fit for visualization. Tests of other associations between neutral and MHC diversity and parasitism were non-significant, and no other specific alleles or supertypes were associated with measures of parasite infection examined here. The illustration in (**b**) depicts a cestode egg

and increase heterozygosity after a population bottleneck in a single generation (Oliver and Piertney 2012).

We expected to find negative associations between MHC allelic diversity and parasitism, especially if heterozygous individuals can combat a broader spectrum of parasites than homozygous animals (as in Penn et al. 2002). Instead, we found that high MHC diversity did not predict lower infection by gastrointestinal helminths and protozoa. Our analyses of specific alleles showed that Mimo-DRB*07 predicted low cestode intensity, suggesting it might confer quantitative resistance (Westerdahl et al. 2013). MHC alleles have previously been found to associate with cestode infection in wild rodents (Tollenaere et al. 2008; Axtner and Sommer 2012), and with *Eimeria* infection in laboratory mice (Schito et al. 1996) and chickens (Lillehoj et al. 1989). We also note that allele Mimo-DRB*14, negatively associated with vole condition, could be related to an unmeasured parasite of montane voles.

Our findings of similar MHC allele frequencies across populations and a lack of association between MHC diversity and infection levels, point away from mechanisms of fluctuating selection, rare-allele advantage, and heterozygote advantage as key drivers of MHC diversity. Instead, our analyses were most consistent with high inter-patch migration

supporting maintenance of both neutral and adaptive variation. By comparison, work on experimental populations of three-spined sticklebacks (*Gasterosteus aculeatus*) showed that parasite resistance alleles increased in frequency in the offspring generation, providing strong support for both frequency dependent selection and fluctuating selection (Eizaguirre et al. 2012). Importantly, we acknowledge that analyses of mechanisms of selection operating on specific MHC alleles are more powerful when performed over longer timescales, to observe temporal changes in allele-parasite frequencies, and to test whether these associations are consistent or change over time (Spurgin and Richardson 2010).

Parasites have also been shown to correlate negatively with neutral genetic diversity, possibly through inbreeding depression lowering parasite resistance (Coltman et al. 1999; Acevedo-Whitehouse et al. 2005; Rijks et al. 2008). Here, we asked whether neutral diversity might predict lower infection risk, as observed in studies of Soay sheep (*Ovis aries*) and sea lions (*Zalophus californianus*) (Coltman et al. 1999; Acevedo-Whitehouse et al. 2003). Instead, we found that greater neutral diversity predicted higher endoparasite species richness. One explanation for the trend found in our study could be that animals with greater genome-wide diversity might better tolerate heavy infections (e.g., through hybrid vigor; Baird et al. 2012) 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 (*Melospiza melodia*), New Zealand robins (*Petroica australis*), Soay sheep and other species showed 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 and Waller 2002; Jamieson et al. 2007). As our study found no evidence for inbreeding based on genetic markers, there is little support for this hypothesis. An alternative hypothesis is that parasites have little impact on host fitness, and that 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 hypothesis, we showed that animals with greater cestode loads were older and also had higher measures of age-specific body condition, possibly because heavier animals ingested more food and, as a result, incidentally consumed more parasite infectious stages (see also Winternitz et al., 2012). A lack of negative associations between indices of fitness and parasitism, combined with previous evidence of lifelong susceptibility to infection, points away from these parasites having strong fitness effects (Winternitz et al. 2012). Support for the second hypothesis is equivocal as post hoc analyses showed no evidence of higher microsatellite heterozygosity in animals recaptured compared to those captured only once (one-way ANOVA $F_{1,116} = 0.003$, $P = 0.96$, $N = 36$ recaptured animals), or between adults and sub-adults ($F_{1,116} = 0.670$, $P = 0.415$, $N = 38$ sub-adults and 80 adults). However, it would be useful to re-examine the importance of neutral genetic diversity for parasite infection using more markers during ecologically stressful intervals or for younger animals for which infection status and survival could be tracked over time (Coltman et al. 1999; Rijks et al. 2008).

Overall, our results showed that montane voles maintain high neutral and adaptive genetic diversity despite repeated high amplitude fluctuations in abundance. One underlying mechanism for this variation could be that alleles are exchanged between vole subpopulations during periods of high density when migration supports gene flow. In terms of neutral diversity, our analyses showed that endoparasite richness was positively associated with diversity based on microsatellite markers. We suggested two exclusive hypotheses for this association: (1) parasites kill off the hosts with low genetic diversity but

not genetically diverse hosts that can tolerate parasitism, or (2) parasites have little or no impact on hosts, but genetically diverse hosts survive longer and hence accumulate more parasites over time. Our results favor the second hypothesis, as there was little evidence for gastrointestinal parasites having strong fitness effects on this population. Surprisingly, most measures of infection reported here did not covary with measures of MHC diversity. However, it is important to note that other parasites (beyond those measured here) could represent selective forces affecting MHC diversity. For example, blood borne protozoa are known to negatively affect fitness in other vole populations (Watkins et al. 1991; Smith et al. 2005; Kloch et al. 2013), and ectoparasitic arthropods (fleas, ticks and lice) were negatively associated with MHC variability in some rodent species (Oliver et al. 2009b; Schad et al. 2012). Yet no signals of historic positive selection on the DRB codons most likely to be involved with antigen recognition were found (Winternitz and Wares 2013), and evidence of contemporary parasite-mediated selection on specific alleles and supertypes was absent in the present study. We note that factors other than parasitism – especially sexual selection and gene conversion – have been suggested as maintaining high MHC diversity, either independently of or in conjunction with parasitism (reviewed in Penn and Potts 1999; Milinski 2006; Havlicek and Roberts 2009; Spurgin et al. 2011; Winternitz et al. 2013). As a final point, other ecological pressures could limit parasite transmission and lower their impacts as agents of selection. Such pressures might come from predators, which are known to have high impacts on vole population sizes (Korpimäki et al. 2002), and could pre-empt parasites from acting as a significant selective force (Hass 1989). 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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