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MHC genetic diversity and avian malaria prevalence in Mokoia Island saddlebacks

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Abstract: Studies of wildlife populations have identified associations between disease resistance and diversity at genes of the major histocompatibility complex (MHC), which are involved with adaptive immunity. We compare MHC class II B (MHCIIIB) and microsatellite genetic diversity in a population of New Zealand passerine birds, North Island saddlebacks on Mokoia Island, that was also tested for avian malaria. Prevalence of infection was low, and we found no conclusive evidence that infection status was linked to particular MHCIIIB alleles. Individual-level MHCIIIB nucleotide diversity, and the number of alleles per bird were both lower in infected compared with uninfected samples; however, neither trend was statistically significant. Genetic structure was observed in the MHCIIIB dataset, suggesting that selective forces are shaping MHCIIIB diversity in this population. Future research would benefit from long-term monitoring of allele frequencies and larger sample sizes where possible. Additionally, including disease intensity data (i.e. the degree of infection in an individual) alongside prevalence data (i.e. presence/absence of infection in an individual) may enhance the ability to detect phenotype-genotype associations in future research.

Keywords: bottleneck; infectious disease; mosquito-vectored; next generation sequencing; (*Philesturnus rufusater*; *Plasmodium*)

Introduction

Small populations with reduced genetic diversity may be highly susceptible to infection by novel pathogens. Although still equivocal, there is growing evidence that wild populations with low diversity at genes of the major histocompatibility complex (MHC) may be especially compromised (e.g. Siddle et al. 2007, reviewed in Radwan et al. 2010). MHC genes initiate adaptive immune responses by encoding cell-surface proteins that are responsible for recognising and binding to foreign peptides (Klein & O'Uigin 1993). Class I MHC molecules are primarily associated with intracellular pathogens, such as viruses, while class II molecules target peptides derived mainly from extracellular pathogens (Hughes & Yeager 1998; Pierny & Oliver 2006). Evidence from wild populations indicates that disease associations can occur for specific class I and class II alleles, as well as for groups of alleles and/or overall MHC diversity (e.g. Hill et al. 1991; de Campos-Lima et al. 1993; Thursz et al. 1995; Paterson et al. 1998; Trachtenberg et al. 2003; Wegner et al. 2003; Westerdahl et al. 2005; Bonneaud et al. 2006; Siddle et al. 2007; Oliver et al. 2009; Jarvi et al. 2013; Sepil et al. 2013). Additionally, while negative associations with disease may be interpreted as certain MHC genotypes providing qualitative protection against a pathogen, positive associations may result from quantitatively resistant genotypes that limit pathogen load and/or the deleterious effects of the disease, without completely eradicating it (Sepil et al. 2013). The latter phenomenon is also referred to as

'tolerance'. Characterising MHC-disease associations may be particularly useful for species of conservation concern, as such information may help guide management actions. For example, eastern and northwestern populations of Tasmanian devils (*Sarcophilus harrisii*) have different MHC profiles, and maintaining isolation between these populations has been proposed for managing outbreaks of an infectious cancer (Siddle et al. 2010). In New Zealand, genetic information has increasingly been incorporated into wildlife management strategies, particularly for avian species, and there is mounting effort to understand and incorporate patterns of immune gene diversity (e.g. Jamieson 2015).

Many endemic New Zealand bird species are threatened due to population bottlenecks over the last century that have also led to losses of genetic diversity, including reduced MHC diversity (Holdaway 1989; Miller & Lambert 2004; Jamieson et al. 2008; Sutton et al. 2013; Jamieson 2015; Sutton et al. 2015). It has been speculated that this reduced genetic diversity may increase the vulnerability of these species to disease (Hale 2007; Hale & Briskie 2007). One concern is the potential susceptibility of New Zealand birds to introduced strains of avian malaria. Avian malaria is a mosquito-transmitted disease caused by protozoan parasites of the genus *Plasmodium* (Order Haemosporida). These parasites asexually multiply in bird red blood cells (Valkiūnas et al. 2005; Braga et al. 2011), and undergo sexual reproduction in mosquitoes (Diptera: Culicidae). In infected birds, pathological effects can include altered pigmentation of the blood, anaemia,

morbidity, and mortality (Braga et al. 2011). The first exposure to avian malaria usually results in an acute infection phase with the most severe fitness consequences (Atkinson & van Riper 1991), after which the infection may persist for years with little or no quantifiable effects on fitness (i.e. chronic infection; Atkinson & van Riper 1991). Naïve species with no prior exposure to malaria can be particularly susceptible to the disease, leading to dramatic losses of avifauna following spread of the parasites and their mosquito vectors (van Riper III et al. 1986; Atkinson & van Riper 1991). In Hawai'i, for example, introduced avian malaria and its introduced mosquito vector, *Culex quinquefasciatus*, play a key role in the ongoing declines and extinctions of many endemic forest bird species (van Riper III et al. 1986; Atkinson et al. 2014).

Unlike Hawaiian species, New Zealand native birds may have had an association with *Plasmodium* spp. that pre-dates human colonisation (i.e. before 1000 years ago; Baillie & Brunton 2011; Ewen et al. 2012; Howe et al. 2012; Schoener et al. 2014). There are at least two endemic *Plasmodium* lineages (Ewen et al. 2012), and of the twelve native mosquito species, at least one, *C. pervigilans*, is a confirmed vector (Derraik 2004; Massey et al. 2007). Additionally, non-native *Plasmodium* have been co-introduced with exotic birds, likely since the 1800s (Duncan et al. 2006; Ewen et al. 2012), and one introduced species, *P. relictum*, can be highly virulent in some avian hosts (Atkinson et al. 1995; Palinauskas et al. 2008). Range expansion over the past 35 years by the exotic mosquito *C. quinquefasciatus*, which is a competent vector of *P. relictum*, has prompted concern that negative impacts on native avifauna could mirror those seen elsewhere in the world (Tompkins & Gleeson 2006). To date, avian malaria has been reported in several native New Zealand species (reviewed in Derraik et al. 2008, Howe et al. 2012, and Schoener et al. 2014), and has been implicated in the deaths of mōhua (*Mohoua ochrocephala*), New Zealand dotterel (*Charadrius obscurus*; Alley 2002; Derraik et al. 2008), South Island saddleback (*Philesturnus carunculatus*; Alley et al. 2010), great spotted kiwi (*Apteryx haastii*; Howe et al. 2012), and hihi (*Notiomystis cincta*; Howe et al. 2012).

Recently, New Zealand North Island saddlebacks (*Ph. rufusater*: Calledidae) on Mokoia Island were discovered to be infected with multiple lineages of *Plasmodium* at low prevalence (6.3% of 319 sampled birds), and it is believed that this population harbours chronic infections (Thorne 2007; Castro et al. 2011). Previous research has shown that North Island saddlebacks have moderately variable MHC class II diversity (Sutton et al. 2013, 2015), but to the best of our knowledge no published studies have aimed to link avian malaria susceptibility to MHC variation in any native New Zealand species. Elsewhere, studies have identified associations between malaria and diversity at MHC class I and class II genes (e.g. Westerdahl et al. (2005) for associations with class I genes; Sepil et al. (2013) for associations with class II genes; Hill et al. (1991) for associations with both classes to human malaria). Here, we investigate whether avian malaria infection is associated with MHC class II B (hereafter, MHCII B) genetic diversity in the Mokoia Island North Island saddleback population. We predict that if specific MHCII B alleles convey resistance/susceptibility to avian malaria in North Island saddlebacks, then infected and uninfected birds will have different MHCII B profiles (i.e. some alleles will occur at high frequencies in one group, but will be absent or at low frequency in the other). Additionally, as individuals with greater MHCII B diversity may be able to respond to a

wider array of pathogens, possibly including a wider range of *Plasmodium* lineages, uninfected birds may have greater MHCII B diversity. For comparison with MHCII B results, we use microsatellite markers to test whether patterns at MHC genes deviate from those expected under neutrality.

Methods

Sample collection, disease testing & genotyping

Blood samples from 319 Mokoia Island saddlebacks were collected between January 2007 and March 2008 (i.e. spring/summer/autumn; concurrent with the saddleback breeding season), and screened for *Plasmodium* spp. infection as described in Castro et al. (2011). Twenty (6.3%) birds were positive from a PCR-based test (Castro et al. 2011). To acquire MHCII B and microsatellite profiles for subsets of uninfected and infected birds, we first used a modified chelex method (Walsh et al. 1991; Casquet et al. 2012) to extract genomic DNA from blood stored in lysis buffer (Seutin et al. 1991). For our MHCII B analysis, we performed sample preparation, sequencing, and bioinformatics based on the protocol outlined in Sutton et al. (2013, 2015) for 14 uninfected and 16 infected birds. Note that because samples for this project were sequenced alongside those for concurrent studies, we had to limit our sample sizes so that we could maximise sequencing coverage per individual. To ensure very good coverage, we chose to sequence a total of 30 saddlebacks from Mokoia Island, 16 of which came from the infected pool of samples, and 14 from the uninfected pool. Briefly, our protocol involved amplifying each sample in triplicate with barcoded primers PhcaMHCII.F.1 and PhcaMHCII.Ex2.R.1 (Sutton et al. 2013) to target 240 bp (~90%) of exon 2, followed by pooling and purifying (Agencourt® AMPure XP, Beckman Coulter) the amplified products from each individual. We then quantified each purified product (Qubit™, Invitrogen™), and pooled equimolar amounts of all samples for next generation sequencing (Ion Torrent™, Life Technologies (Rothberg et al. 2011)). We filtered sequences by length and quality, and removed low frequency sequencing reads (<0.001 of variants). We retained sequences that were 314–320 bp, which represented the expected amplicon size (with barcoded primers) ±3 bp to allow for ±1 codon differences. Our quality restrictions retained sequences for which ≥90% of nucleotides had Phred scores of at least 15. The per-base quality (Phred) scores obtained from the sequencing files are defined by the expression $q = -10 \times \log(p)$, where p is the probability of error (Ewing & Green 1998; Ewing et al. 1998). Thus, a score of 15 indicates 96.8% accuracy. Finally, we assigned sequences to individual birds based on unique barcode combinations (see Sutton et al. 2013, 2015 for complete bioinformatics protocol). Although individual sequences could not be assigned to specific loci due to the nature in which passerine MHC loci are typically co-amplified (e.g. Babik et al. 2009; Zagalska-Neubauer et al. 2010; Sepil et al. 2012), we hereafter refer to individual sequences as 'alleles' for simplicity. We estimated the repeatability of our methods for validating and assigning alleles to individuals by comparing the alleles assigned to replicate samples from 23 saddlebacks included in this and concurrent studies. We calculated our repeatability scores as 1–APD (average percent difference; Yuhki & O'Brien 1990).

For our microsatellite analysis, we used multiplex PCR to amplify 23 loci (Table S1 in online Supplementary Material) in all 20 infected and a subset of 20 uninfected birds. We

dried 10–15 ng of DNA into wells of 384-well plates before PCR. Each 2 μ l reaction contained 1 μ l Type-it Master Mix (Qiagen), and 1 μ l primer mix containing forward (0.02–0.44 μ M) and reverse (0.08–1.76 μ M) locus-specific primers in addition to M13-tagged (Schuelke 2000) fluorescent ‘dyes’ (6-FAM, VIC, NED, PET; 0.16 μ M \times number of loci). We were consistent with our assignment of dye to locus in order to avoid genotyping error due to dye-associated allele size shifts (Sutton et al. 2011). Our cycling conditions were 95°C for 5 min, followed by a touchdown sequence comprising 94°C for 30 s, annealing for 90 s and extension at 72°C for 45 s. During the touchdown cycles, the annealing temperature started at 60°C and reduced 1 degree per cycle for 8 cycles. This was followed by 25 cycles at 94°C for 30 s, 52°C for 90 s and 72°C for 45 s with a final 30 min hold at 60°C. Following amplification, we resolved fragment sizes on an ABI 3730 Genetic Analyser (Applied Biosystems; Genetic Analysis Services, University of Otago) with GeneScan 500 (LIZ)TM size standard, and used Genemapper v4.0 (Applied Biosystems) to score alleles. To check data for null alleles and scoring errors, we used Micro-checker v2.2.3 (van Oosterhout et al. 2004). Deviations from Hardy-Weinberg equilibrium (HWE) were calculated in Genepop v4.0.10 (Raymond & Rousset 1995; Rousset 2008), using the exact probability test (Guo & Thompson 1992) with Markov chain parameters employing 10 000 dememorisations, 1000 batches and 10 000 iterations per batch. To test for linkage disequilibrium, we used Genpop probability tests (Fisher’s exact tests) with the same Markov chain parameters used for testing HWE. We applied sequential Bonferroni correction (Holm 1979; Rice 1989) to the resulting p-values.

MHC comparisons

When considering multi-locus MHCIIIB profiles (i.e. all the MHCIIIB alleles identified as present in an individual), only four genotypes occurred more than once (one occurred twice, two occurred three times, and one occurred eight times; the genotype that occurred twice, and the genotype that occurred eight times were found in uninfected and infected individuals). Therefore, to investigate differences in MHCIIIB profiles between uninfected and infected birds, we focused on testing for associations between infection status and the presence or absence of each allele independently. We used Fisher’s exact tests implemented in R v3.1.2 (R Core Team 2014) to assess whether the presence of each allele was significantly different between uninfected and infected birds. As sample sizes for MHC genotypes were small and slightly different for uninfected and infected birds (14 vs 16), we also used R to randomly assign individuals to each infection status without replacement 10 000 times (i.e. permutations). We used these permutations to generate a predicted mean and 95% confidence interval of the presence of each allele given a null hypothesis that presence was not associated with infection status.

We used in-house R code to generate an individual fasta file of the alleles present in each bird so that we could calculate individual-level statistics for the total number of alleles (A_i), the total number of polymorphic sites (S_i ; using the R function ‘seg.sites’ in package ape; (Paradis et al. 2004)), and the nucleotide diversity (π_i ; using the R function ‘nuc.div’ in package pegas (Paradis 2010)). We used generalised linear models (GLMs, implemented in R) to compare estimates between infected and uninfected birds. Finally, we tested for genetic structure (i.e. patterns of genetic relationships) by using Genalex v6.41 (Peakall & Smouse 2006) to first calculate genetic distance

between uninfected and infected samples, and then to perform Principal Components Analysis (PCA) for visualising the patterns of genetic structure contained in the distance matrix. Distance matrices can be calculated for binary as well as codominant genetic markers, so to estimate genetic distance for our MHCIIIB data we treated each allele as either present (1) or absent (0) in each sample, and we selected the distance calculation option for binary (haploid) data.

In addition to treating alleles as being distinct in terms of their effect on phenotype, we also tested for the effects of MHC ‘functional supertypes’ on infection status. Functional supertypes consist of groups of alleles predicted to have similar antigen-binding properties based on their amino acid sequences (e.g. Trachtenberg et al. 2003; Sepil et al. 2013). We considered five MHCIIIB functional supertypes that were previously identified for North Island saddlebacks (Sutton 2013), based on the amino acid properties of each individual allele (z -descriptors; Sandberg et al. 1998; Doytchinova & Flower 2005; Schwensow et al. 2007), and by k -means clustering using the adegenet package in R (Jombart 2008). To test whether the number of supertypes per individual was different between uninfected and infected birds, we used GLMs with binomial distributions implemented in R.

Microsatellite comparisons

To investigate differences in microsatellite profiles between uninfected and infected birds, we used Genalex to calculate expected and observed heterozygosities (respectively, H_E and H_O (Nei 1978)) for each group. We used Fstat v2.9.3.2 (Goudet 1995, 2002) to calculate allelic richness (number of alleles per population corrected for sample size (Mousadik & Petit 1996; Petit et al. 1998)), and we assessed genetic differentiation in Arlequin v3.5 (Excoffier et al. 2005) by using Slatkin’s linearised F_{ST} (Slatkin 1995). Significance of F_{ST} values was tested using 30 000 permutations. As with our MHCIIIB data, we used Fisher’s exact tests to assess whether the presence of each allele was significantly different between uninfected and infected birds. To visualise genetic structure, we implemented PCA and assignment tests in Genalex.

Results

MHC

Sequencing results

A total of 69 498 reads were assigned to this study based on exact matches of barcodes on both the forward and reverse primers. The average number of reads per individual was 2316 (± 628 SD, range 975–3685), with >0.95 estimated repeatability of our genotyping methods. Our data yielded a total of 15 putatively functional MHCIIIB alleles (Genbank accessions: KF225751-KF225754; KF225756-KF225763; KF225765-KF225766; KP125357) that did not contain stop codons, and which displayed additional characteristics of classical MHC alleles (e.g. they did not cluster with a group of non-classical MHCIIIB sequences that were previously identified; Sutton et al. 2013). All alleles were 240 bp, and differed from each other by at least 5 nucleotides (2.1%). Each allele translated a unique amino acid sequence.

MHC genetic diversity

All 15 MHCIIIB alleles were present in the sample of infected birds, while 14 were observed in the uninfected birds (Fig. 1).

Most alleles were observed in relatively equal proportions between the two groups of samples, and the one allele that was missing from the uninfected birds (PhruMHCII001) was only present in 19% of infected birds (Fig. 1). The allele that had the greatest disparity between groups was PhruMHC027, which occurred in 36% of uninfected birds and 75% of infected birds (Fig. 1, Table 1). However, the presence of individual MHCII alleles was not significantly different between the two groups (Fisher's exact tests, Table 1). The number of copies of individual alleles fell within the 95% confidence interval from 10 000 permutations for each infection status, further demonstrating that allele presence was not significantly associated with infection status.

When we compared individual-level variation in terms of the number of alleles per bird (A_i), the number polymorphic sites (S_i), and nucleotide diversity (π_i), we observed non-significant trends of lower diversity in infected birds compared to uninfected birds (Table 2 & Fig. 2). Of the five MHC supertypes identified previously (Sutton 2013), all five were

found in both uninfected and infected birds. Although infected birds had, on average, fewer supertypes per individual (mean \pm standard deviation: uninfected = 4.86 ± 0.36 ; infected = 4.13 ± 1.15), this difference was not significant (GLM: z-value = 1.778 p-value = 0.075).

MHC genetic structure

Plotting the first two axes of the PCA, which was based on pairwise genetic differences, showed that individuals could be divided into two groups (Fig. 3a). These first two axes explained 73% of the MHCII variation in the 30 Mokoia Island saddleback samples. Nineteen birds clustered into a 'low diversity' group to the left along axis 1, while eleven birds clustered into a 'high diversity' group on the right (Fig. 3a). Of the 15 total MHCII alleles, 40% were missing from the low diversity group, while none were absent from the high diversity group. Clustering did not appear to be associated with infection status (Fig. 3a).

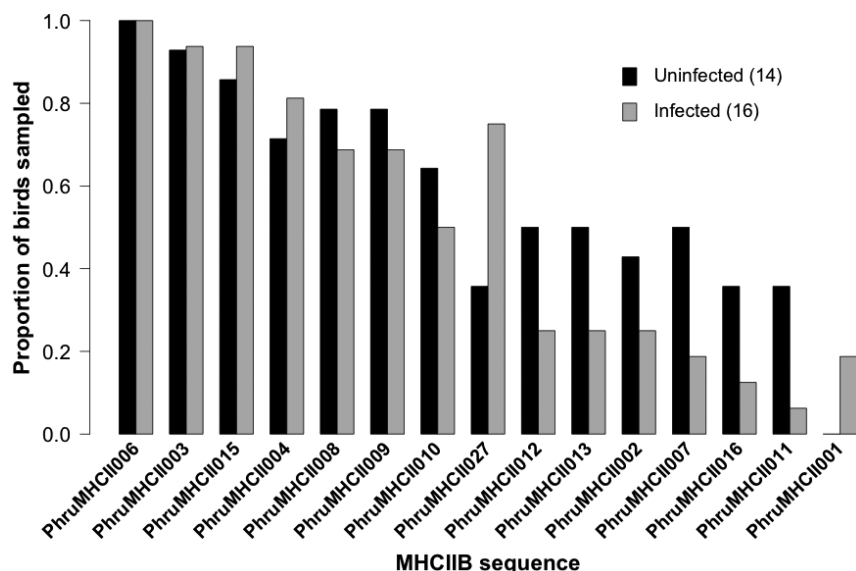


Figure 1: Identities of MHCII sequences and proportions at which each was present in Mokoia Island saddlebacks infected and uninfected with avian malaria. Legend numbers indicate sample sizes.

Table 1: Results of Fisher's exact tests comparing the presence of each MHCII allele in Mokoia Island saddlebacks infected and uninfected with avian malaria.

Allele	N	Number of uninfected birds (max N=14)	Number of infected birds (max N=16)	Fisher's odds ratio	Fisher's p-value	Permutation mean in uninfected birds (95% CI)	Permutation mean in infected birds (95% CI)
PhruMHC001	30	0	3	0.000	0.228	1.4 (0–3)	1.6 (0–3)
PhruMHC002	30	6	4	2.188	0.442	4.7 (2–7)	5.3 (3–8)
PhruMHC003	30	13	15	0.871	1.000	13.1 (12–14)	14.9 (14–16)
PhruMHC004	30	10	13	0.588	0.675	10.7 (8–13)	12.3 (10–15)
PhruMHC006	30	14	16	na	na	16 (16–16)	14 (14–14)
PhruMHC007	30	7	3	4.111	0.122	4.7 (2–7)	5.3 (3–8)
PhruMHC008	30	11	11	1.639	0.689	10.3 (8–13)	11.7 (9–14)
PhruMHC009	30	11	11	1.639	0.689	10.3 (8–13)	11.7 (9–14)
PhruMHC010	30	9	8	1.765	0.484	7.9 (5–11)	9.1 (6–12)
PhruMHC011	30	5	1	7.771	0.072	2.8 (1–5)	3.2 (1–5)
PhruMHC012	30	7	4	2.887	0.257	5.1 (3–8)	5.9 (3–8)
PhruMHC013	30	7	4	2.887	0.257	5.1 (3–8)	5.9 (3–8)
PhruMHC015	30	12	15	0.412	0.586	12.6 (11–14)	14.4 (13–16)
PhruMHC016	30	5	2	3.712	0.204	3.3 (1–5)	3.7 (2–6)
PhruMHC027	30	5	12	0.197	0.063	7.9 (5–11)	9.1 (6–12)

Table 2: Individual-level MHCIIB exon 2 sequence polymorphism in Mokoia Island saddlebacks infected and uninfected with avian malaria based on the average number of sequences per bird (A_i), the average number polymorphic sites (segregating, S_i) among sequences, and nucleotide diversity (π_i). Numbers in brackets indicate standard deviations. Z- and p-values come from GLMs with binomial distributions, for which degrees of freedom were 29 (total) and 28 (residual) for all models.

MHC diversity	Uninfected	Infected	z-value	p-value
A_i	8.71 (2.43)	7.63 (2.19)	1.259	0.208
S_i	78.43 (6.41)	76.06 (7.07)	0.962	0.336
π_i	0.133 (0.004)	0.131 (0.002)	1.576	0.115

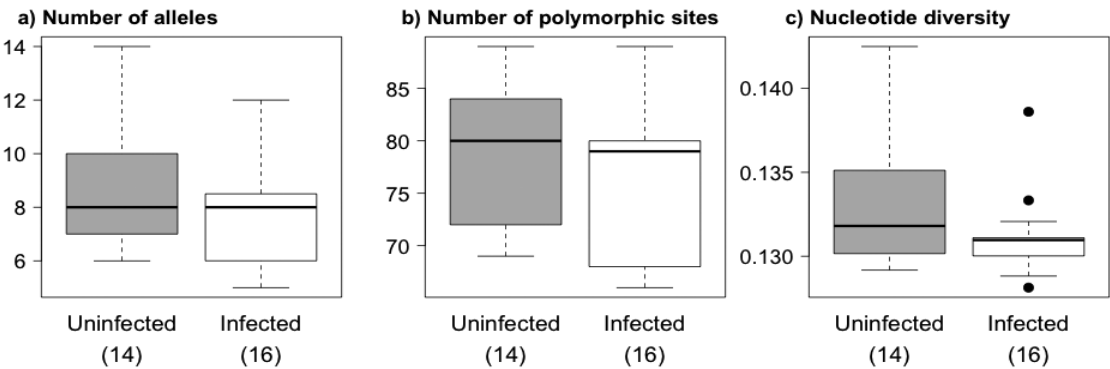


Figure 2: Individual-level MHCIIB exon 2 polymorphism in Mokoia Island saddlebacks infected and uninfected with avian malaria. Horizontal bars represent median values. Boxes above and below median values indicate the interquartile range (the 25th to 75th percentile). Where there are no outliers, the whiskers represent the maximum and minimum values. Where there are outliers (filled circles), the whiskers represent 1.5 times the interquartile range (approximately two standard deviations). Values in brackets along the x-axis indicate sample sizes.

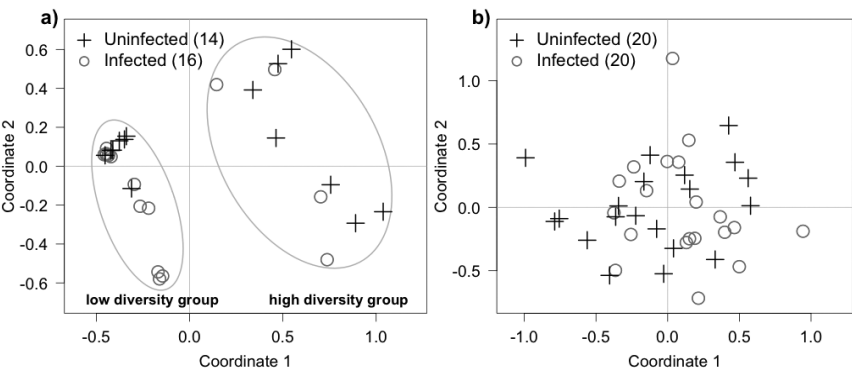


Figure 3: Plot of the first two axes of a PCA based on presence/absence of a) 15 MHCIIB sequences, and b) microsatellite genotype (n loci = 19) in Mokoia Island saddlebacks infected and uninfected with avian malaria. Data-points are jittered to reduce overlap. The two axes in panel (a) explain 73% of the total variation. The two axes in panel (b) explain 44% of the total variation.

Microsatellites

Tests for Hardy-Weinberg departures and genotyping errors
Of the 23 microsatellite loci that we amplified, 19 were polymorphic in Mokoia Island saddlebacks (loci CK5A4B, K3/K4, PCC02 and POCC6 (Table S1) were monomorphic). Among the 19 polymorphic loci, there were no deviations from Hardy-Weinberg proportions, and no evidence of null alleles or linkage disequilibria. All samples amplified at least 17 loci, and 93% of samples amplified all 19 polymorphic loci. We estimated genotyping error rate using data from a concurrent study involving 486 samples, which included 40 Mokoia Island samples. Across the 19 loci, the proportion of

samples that were re-genotyped ranged from 15.5% to 42.3% per locus (mean = 23.8%), and resulted in an overall error rate of 0.27%.

Microsatellite genetic diversity

In total, 47 alleles were recorded across 19 microsatellite loci in 40 Mokoia Island saddleback samples. Allelic richness standardised to sample size ($n = 19$) was 2.36 for uninfected and infected birds. There was no difference between mean H_O and mean H_E within each group, and mean H_O was similar between uninfected and infected birds (mean $H_O \pm$ standard error: uninfected = 0.392 ± 0.048 ; infected = 0.381 ± 0.050).

Microsatellite genetic structure

There were two private microsatellite alleles (i.e. alleles that only occur in one group) in the uninfected birds (one each at loci 2F9 and 2H8/Pca05) and two private alleles in the infected birds (one each at loci 4H2/Pca14 and Pcc42). Each private allele was rare, occurring at a frequency of 0.025. For comparison with MHC data, this frequency corresponded to 5% of either infected or uninfected individuals. Fisher's exact tests indicated that the presence of each allele did not differ significantly between uninfected and infected birds. The pairwise F_{ST} was zero, indicating no differentiation between infected and uninfected birds based on their microsatellite genotypes (p-value = 0.614). A two-dimensional PCA plot (Fig. 3b) showed no clustering, and the variation explained by the first two axes was 44%. Population assignment tests correctly assigned only 48% of samples according to their infection status.

Discussion

We examined MHC and microsatellite genetic diversity in a population of New Zealand North Island saddlebacks that was exposed to avian malaria on Mokoia Island. Genes of the MHC are associated with immunity, while diversity at microsatellite loci is assumed to be neutrally evolving. Thus, comparing patterns of MHC and microsatellite diversity is a useful strategy for testing for selection, and assessing phenotype-genotype associations. We found no significant difference in the presence of individual MHCII alleles between infected and uninfected birds. However, we note that MHC allele PhruMHC001 was only found in infected birds (19% of infected individuals), and MHC allele PhruMHC027 occurred in a substantially larger proportion of infected than uninfected birds (75% vs 36%). By comparison, differences between microsatellite alleles were much smaller. As our sample sizes were necessarily low due to low prevalence of avian malaria at the time of this study, it would be worthwhile monitoring the frequencies of MHC alleles over time in North Island saddlebacks, particularly PhruMHC001 and PhruMHC027, to further assess the possibility of a phenotype-genotype association. For example, if there is a weak phenotype-genotype association (e.g. if an allele has a small effect on tolerance/susceptibility to avian malaria), then the frequency of that allele in the population should change slowly rather than quickly over time. This could explain the moderately large, but non-significant, disparity between the presence of PhruMHC027 in uninfected and infected birds at the time of sampling. Alternatively, if birds with a particular allele succumb quickly to avian malaria during the acute infection stage, then that allele would be difficult to detect in wild populations where sick or dead individuals may be not sampled (Westerdahl et al. 2005).

In terms of overall genetic diversity and structure, we observed a non-significant trend toward higher individual-level MHCII polymorphism in uninfected birds. Further, there was evidence of genetic structuring in MHCII genotypes, which did not occur for neutrally evolving microsatellite loci. Although a difference in genetic structuring between MHC and microsatellite diversity is suggestive of balancing selection acting on the MHC (Piertney & Oliver 2006; Alcaide 2010), we did not find conclusive evidence that MHC genetic structuring was associated with avian malaria infection status in the Mokoia Island saddleback population. Thus, we cannot identify specific factors that may be responsible for MHC

genetic structuring. We also cannot discount the possibility that there may be an association between MHC diversity and intensity of infection, which we were unable to test at this time.

Assuming that phenotype-genotype associations exist, then a failure to detect associations could result from a number of factors aside from small sample sizes. As described above, highly susceptible individuals might be omitted during sampling if they are less active than healthy individuals, or if they die from their infections (Westerdahl et al. 2005). Second, as native New Zealand birds are probably not naïve to some avian malaria parasites or their mosquito vectors, it is possible that this co-evolutionary history imparts a measure of resilience against introduced lineages. In this scenario, historical selection pressures could have led to contemporary birds having similar, resistant genotypes. At least two *Plasmodium* lineages (BELL01 and KOKAK001) are thought to be endemic to New Zealand (Baillie & Brunton 2011; Ewen et al. 2012; Howe et al. 2012), and a native New Zealand mosquito, *C. pervigilans*, is a confirmed *Plasmodium* vector (Massey et al. 2007). Thus, birds that become infected with new strains of *Plasmodium* may not necessarily suffer severe infection. Third, most non-native *Plasmodium* species were likely co-introduced to New Zealand during colonial times (Ewen et al. 2012). Thus, even if a history of co-evolution with one *Plasmodium* lineage did not bestow resistance to a novel strain, enough time may have passed for some New Zealand species/populations to have experienced evolution in response to non-native *Plasmodium*, similar to what appears to be occurring in low elevation populations of the Hawaiian amakihi (*Hemignathus virens*) after introductions of *P. relictum* to the Hawaiian Islands in the early 1800s (Foster et al. 2007).

Contrary to the idea that a co-evolutionary history with native *Plasmodium* imparts a degree of resilience to introduced lineages, or that enough time has passed for resistance to have evolved, infection by new or multiple strains, or in combination with other parasites, may raise parasitaemia levels beyond the capability of the host's immune system. At least two of the *Plasmodium* lineages identified in North Island saddlebacks from Mokoia Island phylogenetically resemble *P. relictum*, but other lineages, including the native KOKAK001, were also found (Castro et al. 2011; Howe et al. 2012). One lineage that was commonly identified in saddlebacks on Mokoia Island was *P. (Huffia) elongatum* (Castro et al. 2011), which has also been identified in other species, including the carcasses of South Island saddlebacks co-infected with avian pox (Alley et al. 2010; Howe et al. 2012). Although it may be difficult to determine the primary causative agent following disease-related mortality, future research may benefit by comparing MHC profiles in birds that succumb vs birds that survive disease outbreaks. For example, comparisons of MHC profiles following experimental infections of avian malaria have identified specific groups of alleles associated with either survival or susceptibility in Hawaiian honeycreepers (Jarvi et al. 2013).

Spread of non-native avian malaria parasites and their mosquito vectors can have catastrophic consequences when naïve hosts become infected. In Hawai'i, introductions of *P. relictum* and the mosquito vector *C. quinquefasciatus* have decimated several endemic bird populations, and continue to impede conservation efforts (Atkinson et al. 1995, 2000, 2014). On a global scale, associations between avian malaria resistance/susceptibility and the MHC have been identified in several species (e.g. Westerdahl et al. 2005, 2012; Bonneaud et al. 2006; Loiseau et al. 2008; Radwan et al. 2012; Dunn et

al. 2013; Jarvi et al. 2013; Sepil et al. 2013). Excluding studies in which birds were experimentally infected, one factor that these cited studies have in common is that malaria prevalence was high ($\geq 30\%$) in nearly all populations studied, and ranged from 33.3% to 81.6% (mean = 52.2%; standard deviation = 14.2) in populations for which associations between MHC variation and infection probability were identified. Thus, higher prevalence than that observed in the Mokoia Island saddleback population may increase power to detect correlations between MHC variation and infection status. Additionally, more extensive datasets that include parasite intensity could help elucidate complex host-disease dynamics, which are likely to be context specific, and correlated with such factors as host age and reproductive status, food availability, and environmental variation (Knowles et al. 2011). Saddleback populations that have higher prevalence (e.g. Hen Island, E.R. Schoener, New Zealand, pers. comm.), as well as other New Zealand species with higher prevalence (e.g. bellbird (*Anthornis melanura*) populations in the Hauraki Gulf (Baillie et al. 2012)) are candidates for such research, and would make excellent comparisons for low prevalence populations like Mokoia Island saddlebacks.

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Supplementary Material

Additional supporting information may be found in the online version of this article:

Table S1: Microsatellite primers and fluorophore labels used to quantify neutral genetic variation in North Island saddlebacks (*Philesturnus rufusater*) from Mokoia Island.

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