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Fluctuating Parasite Prevalence Is Not Linked to Patterns of MHC Class II-ß Diversity in an Island Endemic Reptile

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Fluctuating Parasite Prevalence is Not Linked to Patterns of MHC Class II-ß Diversity in an Island Endemic Reptile

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Abstract

Few studies have explored the evolutionary mechanisms that maintain adaptive immunogenetic diversity in nature. We took advantage of museum samples to test for evidence of parasite-mediated fluctuating selection at MHC Class II-ß loci in an endemic island reptile. The Saban anole *Anolis sabanus* is commonly infected with three species of malaria (*Plasmodium*). Proportions of each parasite species detected in anole blood samples fluctuated over space and time, suggesting competitive interactions between parasites or differences in vector ecology. Our analyses of parasite prevalence and MHC Class II-ß allelic variation found that malaria infection was not associated with patterns of host immunogenetic diversity. We found that infection was contingent on sex, with males being more likely to test positive for malaria. These results indicate that malaria parasites do not impose significant selective pressures on *A. sabanus* or that genetic drift in this island population overwhelms the effects of parasite-mediated selection.

Introduction

The mechanisms underpinning the patterns and maintenance of genetic variation in nature are of significant interest to evolutionary and conservation biologists. Eco-evolutionary relationships between parasites and their hosts play a key role in maintaining genetic variability in wild populations, notably by selecting for immunogenetic polymorphism in the host and promoting reciprocal evolution in parasites (Edwards & Hedrick, 1998; Eizaguirre et al., 2009; Meyer & Thomson, 2001; Spurgin & Richardson, 2010). This evolutionary "arms race" between parasites and their hosts is a classic example of the Red Queen dynamic (Van Valen, 2015), in which evolutionary advantages gained by one species cause negative impacts on opposing species and thus select for reciprocal adaptation (Schulte et al., 2010).

Greater immunogenetic variability is generally considered to be advantageous to the host because it provides a broader range of potential immune defenses (Jackson & Tinsley, 2005). In vertebrates, responses to parasitic infection are moderated by a suite of both innate and adaptive immunity genes (Boehm, 2012; Hirano et al., 2011; Litman et al., 2010). Allelic variants of genes involved in conferring immunity may vary widely between populations and even among individuals within the same population (Evans et al., 2009; Miller et al., 2010; Spurgin & Richardson, 2010). While the function and evolution of key vertebrate immunity genes in the context of host-parasite systems have been the subject of extensive laboratory-based research (Chen et al., 2021; Eizaguirre et al., 2012; Phillips et al., 2018; Savage & Zamudio, 2011), the specific mechanisms by which parasite-mediated selection maintains observed patterns of immunogenetic diversity in natural populations remain relatively understudied.

The major histocompatibility complex (MHC) is a highly variable multigene family that mediates the adaptive immune response in jawed vertebrates (Piertney & Oliver, 2006; Westerdahl, 2005). Although the MHC was originally studied because of its importance in

determining organ transplant compatibility in humans, the remarkable degree of variability observed at MHC loci has led to a more recent focus on the gene family as a paradigm for studying adaptive genetic variation in wildlife populations (Hedrick, 1994). Genes within this family encode molecules that bind peptides from inside the cell membrane and transport them to specialized receptor cells, initiating an immune response (Herdegen-Radwan et al., 2021; Piertney & Oliver, 2006). MHC loci involved in adaptive immunity are classified into two major subgroups: MHC Class I molecules are located on the surfaces of all nucleated cells and present both self and non-self antigens to effector T lymphocytes, while MHC Class II molecules are limited to a range of specialized antigen-presenting cells and bind only to peptides of foreign origin (Neefjes et al., 2011).

The high levels of variation observed at MHC loci are thought to be due to one or more forms of parasite-mediated balancing selection (Herdegen-Radwan et al., 2021; Westerdahl, 2005). The fluctuating selection hypothesis proposes that temporal or spatial differences in parasite pressure select for greater host immunogenetic variation than would be expected in a system where the degree of parasite pressure remained consistent (Hedrick, 2002). Previous field studies have supported a key prediction of this hypothesis by showing that parasite prevalence and other selective pressures on immunity genes vary across time and space, but the effects of these fluctuations on MHC allelic diversity in natural populations are still poorly understood (Fraser et al., 2010; Larson et al., 2014). The role of fluctuating selection in shaping observed patterns of MHC diversity has been relatively understudied due to the need for long-term datasets with sufficient spatial replication and knowledge of past parasite population dynamics (Bakuza & Nkwengulila, 2009; Schall & Marghoob, 1995). Moreover, studies that examine “snapshots” of the relationship between MHC allelic diversity and parasite pressure in one population at a single

point in time fail to account for evolutionary responses to parasite pressure, underscoring the importance of long-term field studies that repeatedly sample the same populations (Herdegen-Radwan et al., 2021).

The present study takes advantage of a longitudinal dataset obtained from three populations of the endemic Saban anole *Anolis sabanus*. Blood samples were collected from these populations repeatedly over two decades, allowing for the effects of parasite-mediated selection to be measured across both space and time. Saba is a small (5 km^2) but environmentally heterogeneous volcanic island in the Lesser Antilles with a variety of microclimates shaped by variation in precipitation, insolation, and prevailing wind exposure on different sides of its central peak. Saban anoles are common across all habitat types on the island and are known to be infected with three species of malaria parasites: *Plasmodium floridense, P. leucocytica,* and *P. azurophilum* (Perkins, 2001; Schall & Staats, 2002). Only seven other terrestrial reptile species exist on Saba, reducing the likelihood that complex species interactions might impact malaria transmission (Eustatius & Powell, 2006). Levels of malarial infection are not uniform across the island, possibly due to differences in transmission dynamics of the vector species or variation in selective pressures across different habitat types (Hudson et al., 2002; Wood et al., 2007). Saban anoles in wetter habitats have been found to be infected with malaria at twice or even three times the rate of those in cooler and drier habitats, providing strong evidence that different habitats on the island impose different selective pressures (Schall & Staats, 2002; Staats & Schall, 1996). The observed variability in selective pressures across habitats, existence of long-term parasite infection data, and the insular nature of this anole species makes *A. sabanus* and its malaria parasites an ideal system in which to test for parasite-mediated fluctuating selection.

In this study we use a next-generation sequencing approach to compare levels of variation at MHC Class II-ß loci between spatially and temporally distinct populations of *A. sabanus* in order to test the hypothesis that fluctuations in malaria prevalence drive host immunogenetic variability in this system via positive selection for specific immune alleles in different populations. We target the peptide-binding region of *A. sabanus* MHC Class II molecules at a group of loci known as *mhc2b* (Santonastaso, 2020). Furthermore, we used neutral microsatellite markers as a backdrop against which to examine the effects of selection at *mhc2b* loci, allowing us to compare the degree of neutral genetic structuring between populations to that observed at adaptive MHC loci (Alcaide, 2010; Gong et al., 2021; Knafler et al., 2017). We predict that if population sub-structuring is greater at *mhc2b* than at neutral microsatellite loci, localized selection may be leading to adaptive differentiation in *A. sabanus*. We also compare the incidence of specific MHC alleles and overall allelic richness in both infected and uninfected *A. sabanus* individuals across spatiotemporally distinct populations to test our prediction that infection by *Plasmodium spp*. promotes immunogenetic variation between host populations via positive selection. If the most common alleles detected at *mhc2b* vary significantly across years, sampling sites, or both, we can infer that variation in parasite prevalence maintains host immunogenetic variability in accordance with the fluctuating selection hypothesis. Conversely, if no significant differences in MHC variability between populations are detected across space and time, genetic drift or unknown factors may be the most influential drivers of *mhc2b* variability in this system. Finally, we aim to determine whether overall prevalence and community composition of *Plasmodium spp*. remains stable or varies on spatial and temporal scales, a key component of the fluctuating selection hypothesis that has been documented in other anole-malaria associations (Otero et al., 2019).

Materials and Methods

Sampling

The blood samples used in this study were obtained from a long-term longitudinal dataset archived at the American Museum of Natural History that spans nearly two decades of field collection across the island of Saba. Three field sites were chosen based on differing habitat profiles: the Crispeen site is in a tropical rainforest, Troy Hill is in a town, and Ladder is a coastal site with significant elevation changes throughout (**Figure 1**). Blood dots from a total of 189 *A. sabanus* individuals were selected from sample collections made from these three sites in the years 2004, 2009, and 2014. Anoles were captured at each site using catch poles and processed at a field station overnight. One distal toe was removed from the hind leg of each lizard to collect blood smears for DNA extraction. Clipped toes were sterilized with ethanol after blood sampling, after which the wound was sealed with a drop of sterile glue to reduce pain and limit the possibility of infection. All lizards were released at their original capture sites within 24 hours (Santonastaso, 2020). All animal handling and sampling involving University of New Orleans (UNO) personnel was completed in accordance with the UNO IACUC protocol 14-001.

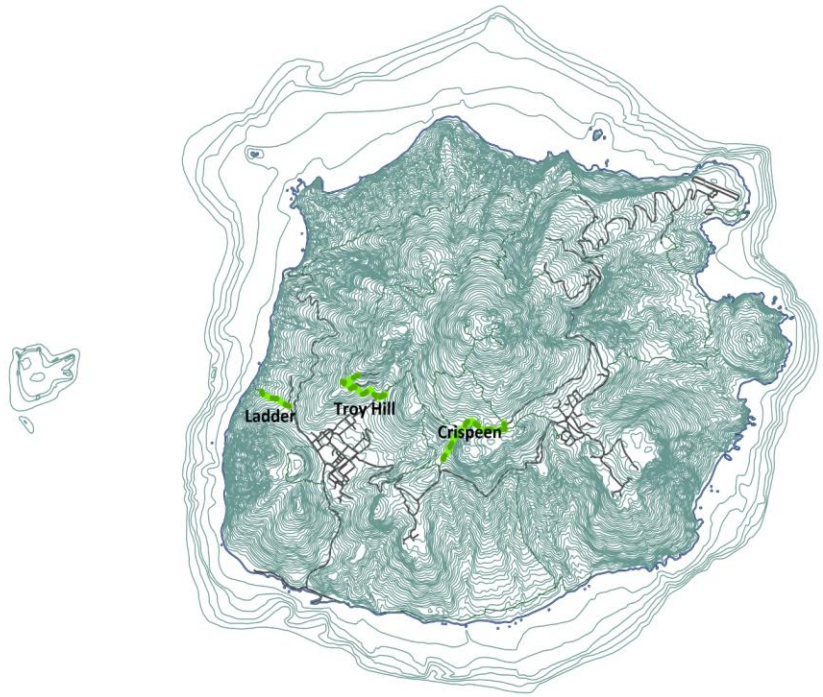

Figure 1: Topographic map of Saba Island and location of sites where *A. sabanus* was collected. All sampling sites used in this study are highlighted in green.

Plasmodium screening

Each *A. sabanus* blood sample was screened for the presence of *Plasmodium spp.* using a nested polymerase chain reaction (PCR) diagnostic based on the mitochondrial *cytochrome b* gene (Perkins & Schall, 2002). Genomic DNA was extracted from blood dot samples using the DNeasy Blood and Tissue kit and associated protocol (QIAGEN, MD). The concentration of extracted DNA was assessed using a NanoDrop 1000 (ThermoScientific, DE) and diluted to 10-100 ng/ul prior to PCR amplification. The first-round PCR reactions used the primers DW2 5' TAA TGC CTA GAC GTA TTC CTG ATR ATC CAG 3' and 3932R 5’ GAC CCC AAG GTA ATA CAT AAC CC 3’ and were carried out using the following conditions: an initial denaturation step at 95°C for 12 minutes followed by 34 cycles of 95°C for 30 seconds, annealment at 52°C for 30 seconds, and an extension at 72°C for 60 seconds with a final extension period at 72°C. The second

round of the nested PCR used the primers DW1 5' TCA ACA ATG ACT TTA TTT GG 3’ and 3932R with the following protocol: an initial denaturation step at 95°C for 12 minutes followed by 34 cycles of 95°C for 30 seconds, annealment at 45°C for 30 seconds, and an extension at 72°C for 60 seconds with a final extension period at 72°C before being lowered to a holding temperature of 12°C. The presence or absence of PCR products was then assessed using agarose gel electrophoresis and considered to be positive for *Plasmodium spp.* infection if a band appeared at approximately 600 bp in the second-round PCR.

Microsatellite analyses

A panel of nine polymorphic microsatellite loci was assembled from existing resources (Stenson et al., 2000; Wordley et al., 2011) and organized into three multiplexes for PCR amplification (**Table 1**). The 5’ ends of forward primers were modified with one of three different fluorophores (TET, 6-FAM and HEX; Integrated DNA Technologies, Coralville, IA). PCR reactions for Multiplex 1 were completed using 0.2 µM of each primer, 5 uL 2X Multiplex PCR Kit, and 4-7 ng of DNA for a total reaction volume of 10 µL. PCR reactions were carried out using the following conditions: an initial denaturation step at 95°C for 15 minutes followed by 34 cycles of 95°C for 30 seconds, annealment at 56°C for 90 seconds, and an extension at 72°C for 60 seconds with a final extension period at 60°C for 30 minutes. Multiplexes 2 and 3 were completed with the same PCR reagents but using a touchdown thermocycling protocol with an initial denaturation step at 95°C for 15 minutes followed by 10 cycles of denaturation at 95°C for 30 seconds, annealment at 63°C for 90 seconds, and extension at 72 °C for 60 seconds, and then 30 additional cycles of denaturation at 94°C for 30 seconds, annealment at 53°C for 90 seconds, extension at 72°C for 60 seconds, and a final extension step at 60°C for 30 minutes.

Microsatellite genotyping was carried out using Geneious Prime version 2020.1.2 for trace analysis and peak calling. The program ARLEQUIN v. 3.5.2.2 (Excoffier & Lischer, 2010) was used to test for linkage disequilibrium between loci and for deviations from Hardy-Weinberg equilibrium. We also used ARLEQUIN to quantify average expected heterozygosity (He) and allelic richness (ARmsat) and to perform two analyses of molecular variance (AMOVA), grouping individuals by either year or site to test if there was significant neutral genetic structure between populations and/or time intervals. The program STRUCTURE was used to implement a Bayesian clustering approach to assess spatial population structure based on microsatellite data where the number of populations (K) varied from 1 to 9. Each analysis was repeated three times with a burn-in period of 1000 steps followed by 100,000 MCMC chain repetitions. The online resource Structure Harvester (Earl & Vonholdt, 2012) was used to determine the optimal number of subpopulations based on average rates of change in the log likelihood values between consecutive changes in K (Evanno et al., 2005).

Bioinformatic analyses of MHC allelic variation

The forward primer MHC_Anolis_F1 5' GTGAATTTCTTGTACCAAGTGA 3' and reverse primer MHC_Anolis_R2 5' GAGGGAGAAGGGATCGAAGA 3' were used to amplify a 250bp fragment of the MHC Class II ß subunit's peptide-binding region (*mhc2b*) using two rounds of PCR amplification. First-round PCR amplifications were completed using 0.2 µM of forward and reverse primers, 0.125 uM of MgCl2, 6.5 uL of BSA (20 mg/mL), 12.5 uL of 2X GoTAQ HotStart Colorless MasterMix (Promega Corporation, Madison, WI) and 4-7 ng of DNA for a total volume of 25 µL in volume with an initial denaturation step at 95°C for 5 minutes and 10 cycles of the following protocol: 95°C for 30 seconds, an annealment step that started at 55°C and decreased successively by 0.5°C in each cycle, an extension period at 72°C for 90 seconds, and 32 additional

cycles of denaturation at 95°C for 30 seconds, annealment at 50°C for 30 seconds, and extension at 72°C for 90 seconds with a final extension at 72°C for 12 minutes. In the second round PCR, linker sequences for Illumina adaptors were added to the original primers and used to re-amplify the PCR product using 1 µL of first-round PCR product as a DNA template under the same thermocycling protocol. One individual from each candidate subpopulation (n=9) was chosen as a replicate and amplified twice during separate reactions.

PCR products were sent to the Georgia Genomics and Bioinformatics Core at the University of Georgia for 150 cycles of paired-end sequencing using the Illumina MiSeq platform. Bioinformatic analysis of the dataset was carried out as follows: The cutPrimers tool (Kechin et al., 2017) was used first to remove primer sequences and Illumina adapters used in the amplification process from the raw Illumina MiSeq data. TRIMMOMATIC (Bolger et al., 2014) was then used to remove Illumina adapters and sequences of improper length, after which the AmpliSAT software pipeline (Sebastian et al., 2016) was used to demultiplex, cluster, and filter sequence variants in order to identify the number of unique alleles in each amplicon. Minimum amplicon depth was set to 200 bp and all other settings left at the recommended platform defaults for Illumina MiSeq data. Artefact sequences and chimeras were discarded along with length variants that caused a frameshift and alleles that amplified to a significantly lower extent than other alleles in the same individual, as determined by a combination of manual genotyping and the Degree of Change method (Lighten et al., 2014). Replicates from each population were used to validate allele calling based on repeatability between replicates.

Statistical analyses

The program DNASp was used to create a haplotype list from which we calculated the MHC nucleotide diversity values *k*, Θ-*s*, and π at the population level. ARLEQUIN was then used to

perform an AMOVA, quantifying the degree of microsatellite and MHC structuring due to time or sampling site to test the prediction that the degree of structuring differs between neutral and adaptive loci. The program Molecular Evolutionary Genetics Analysis (MEGA-X; Kumar et al., 2018) was used to create a phylogeny of allelic variants retrieved from *A. sabanus* in our study as well as a phylogeny comparing *mhc2b* sequences from *A. sabanus* to other squamates. A two-way contingency table analysis with *Plasmodium* infection status as a response variable was used to determine whether sampling site or year of sample collection influenced infection status in *A. sabanus*. A second contingency analysis was also performed to test whether *Plasmodium* infection was contingent on sex.

A generalized linear model with Poisson errors was used to test whether *mhc2b* allelic variability differed between year and site in accordance with our prediction that allele frequencies would fluctuate on spatial and temporal scales. A logistic regression with a Poisson error distribution was used to assess whether specific MHC alleles were correlated with higher or lower infection prevalence across populations, testing our prediction that certain alleles provide a selective advantage against *Plasmodium* infection. A binomial logistic regression was used to determine whether MHC nucleotide diversity values *k*, Θ-*s*, and π significantly impacted the likelihood of infection, testing the prediction that overall immunogenetic variability protects against malaria infection. A second binomial logistic regression was also used to test the effects of sampling year, site, sex, and interactions between these factors on observed MHC variability. Except where otherwise noted, all statistical analyses were conducted in R v.4.1.1 (R Core Development Team, 2021).

Results

Plasmodium infection

The nested PCR diagnostic determined that 65 anoles were infected with *Plasmodium spp*. across all sampled populations. Sanger sequencing and Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990) surveys of the NCBI genetic database revealed that all three species of *Plasmodium* parasites were present at each site (**Figure 2**).

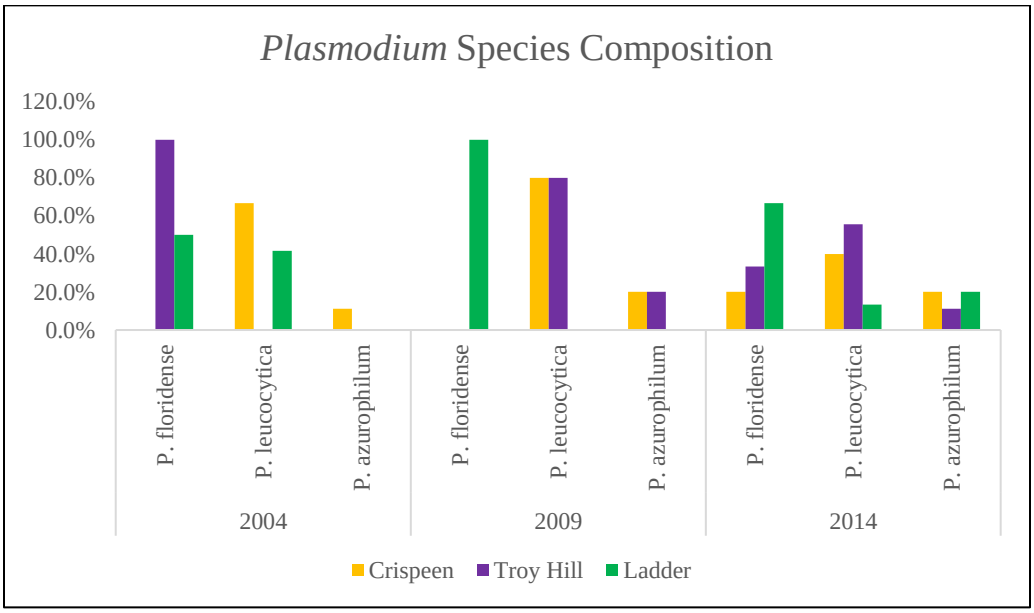

Figure 2: Percentage of infections from 2004, 2009, and 2014 where each of the three *Plasmodium* species were identified using a nested PCR diagnostic.

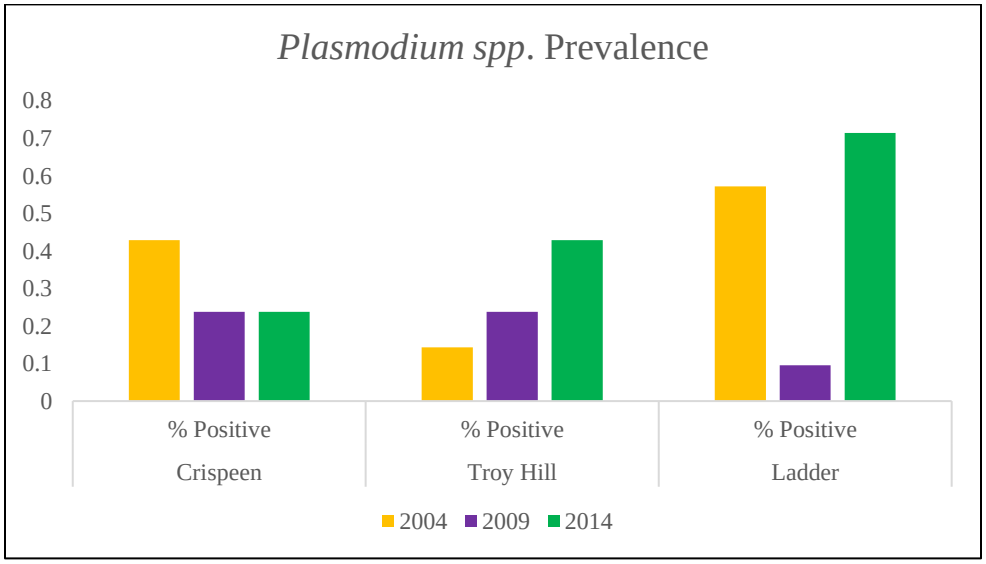

Figure 3: *Plasmodium spp*. infection rates in 2004, 2009, and 2014 at each sampling site.

Changes in *Plasmodium spp*. prevalence across years varied by site (**Figure 3**). Crispeen had a 43% infection rate in 2004, which had declined by 2009 and remained lower in 2014. Troy Hill's infection prevalence was 14% in 2004 and steadily increased, reaching 43% by 2014. Ladder had the highest initial prevalence at 57% in 2004, declining steeply by 2009 and then rebounding to 71% in 2014 (**Figure 3**). Malaria species composition also varied between years and sites (**Figure 2**), with *P. leucocytica* being the most common species identified (n=28) and *P. azurophilum* the least common (n=8). The two-way contingency table analysis that tested whether malaria infection status was contingent on year or sampling site did not yield significant results. However, the two-way contingency analysis that included sex and infection status as variables indicated that malaria infection was contingent on sex, with males being significantly more likely than females to be infected with *Plasmodium spp*. ($p=0.0063$; $p=0.6918$).

Microsatellite analysis

There were no consistent deviations from Hardy-Weinberg equilibrium or linkage equilibrium between the nine microsatellite loci used in this study (**Table 1**). Observed microsatellite heterozygosity (He) was not significantly different from expected values ($p > 0.99$). AMOVA analyses using the program ARLEQUIN indicated that 99.47% of observed microsatellite variation was within populations regardless of whether populations were grouped by year or site, indicating a lack of population structure (**Table 3**).

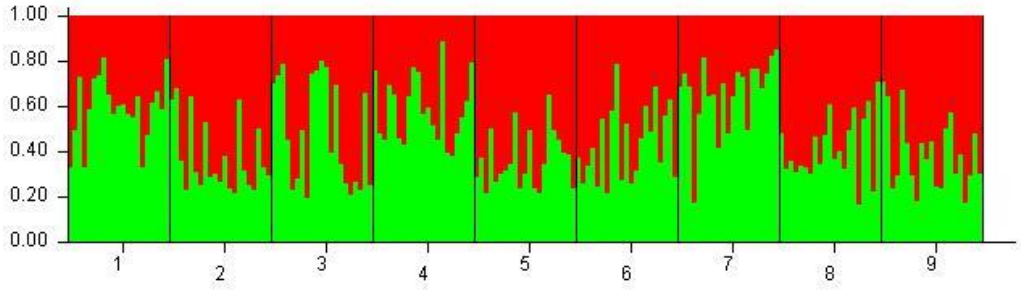

Figure 4: STRUCTURE bar plot (K=2) showing no significant sub-structuring between populations at the genotyped microsatellite loci.

Structure Harvester determined that the optimal number of sub-populations (K) based on microsatellite genotypes was two. Results from STRUCTURE itself based on a model with 2 sub-populations did not indicate significant neutral population structuring (**Figure 4**). Since STRUCTURE cannot identify K=1 as the best estimate using the Evanno method, it is possible that there is only one functional population of *A. sabanus* and that gene flow occurs readily across the island.

Table 1. Microsatellite multiplex primer series for *Anolis sabanus*. Primer names were retained from the original publications (Stenson et al., 2000; Wordley et al., 2011). The number of samples successfully genotyped (n), repeat motif, number of alleles, and expected heterozygosity (H_e) from the current study and reference studies (Ref) are listed. 189 individuals were genotyped at all multiplexes.

						No. alleles		H_e	
	Primer	n	Forward Primer 5' - 3'	Reverse Primer 5' - 3'	Repeat Motif	Asaba	Ref	Asaba	Ref
MultiPlex 1	AoSA18	183	AGCTCGAATTGCCACCAATA	TCCGACTCTTTGCACAGTTG	$(CA)_{10}$	10	5	0.39	0.71
	AoGT2	184	GAAGTAACTTTTTGATTCTAGGTT	TTACTCAGCACACCTTCCTG	$(GT)_{10}$	13	3	0.46	0.84
	AoBA36	184	TGTTATGCCATGCCTGATGT	GCAACAGGACATACCACCGT	$(GT)_{10}$	9	3	0.40	0.81
	Acar9	188	AAAGGCAATGGCAGAGAAAA	TAATGGGAAAGGAGGCAGTG	$(AAGG)_{52}$	8	10	0.24	0.86
Multiplex 2	Acar1	167	CCAAAAACCAAAAAGGCTGA	TGGACACACATACACCCACA	$(AC)_{38}$	38	6	0.84	0.74
	Acar8	181	CCCAATAGAGGAAAGGGACC	AGAATCACGCCTTCTGCTTT	$(AAAG)_{76}$	13	9	0.57	0.89
	Acar10	141	GGATGTGTGTGTTTGTGTTGG	GGCTGTTGAGGGATTCTTGA	$(ACAT)_{28}$	11	6	0.56	0.66
Multiplex 3	Ao10;13	179	AAGTCAAATCATGCTAAATA	ACCCTAAAGTAAAAATAATA	$(CT)_{10}(AT)(AC)_7$	32	10	0.82	0.81
	AoGT9	187	CATCTGTGGCTCATGGCTTT	CTTTCTCCCACCTGGACATT	$(TG)_5(TA)_4(TG)_{12}$	35	9	0.83	0.76

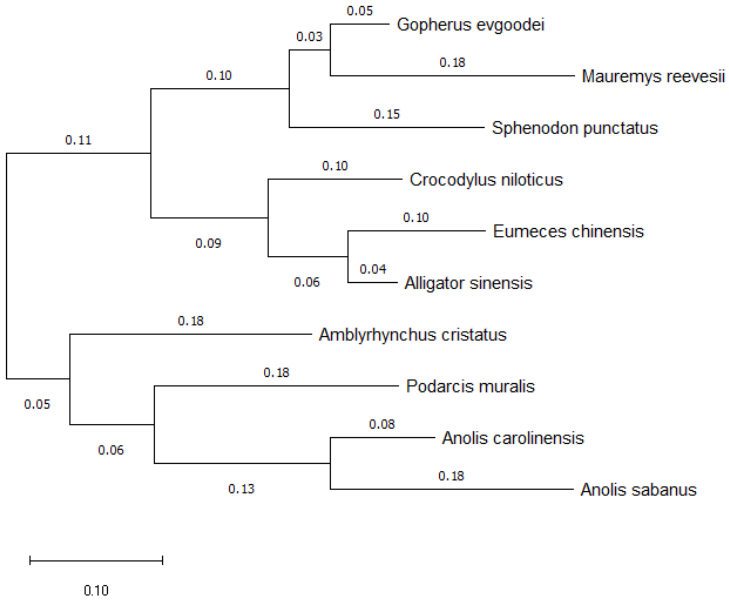

Figure 5: Maximum likelihood tree illustrating phylogenetic relationships between *mhc2b* sequences from *A. sabanus* and homologs created with MEGA-X. Sequences were aligned using the program's Clustal-W function and the Tamura-Nei nucleotide substitution model.

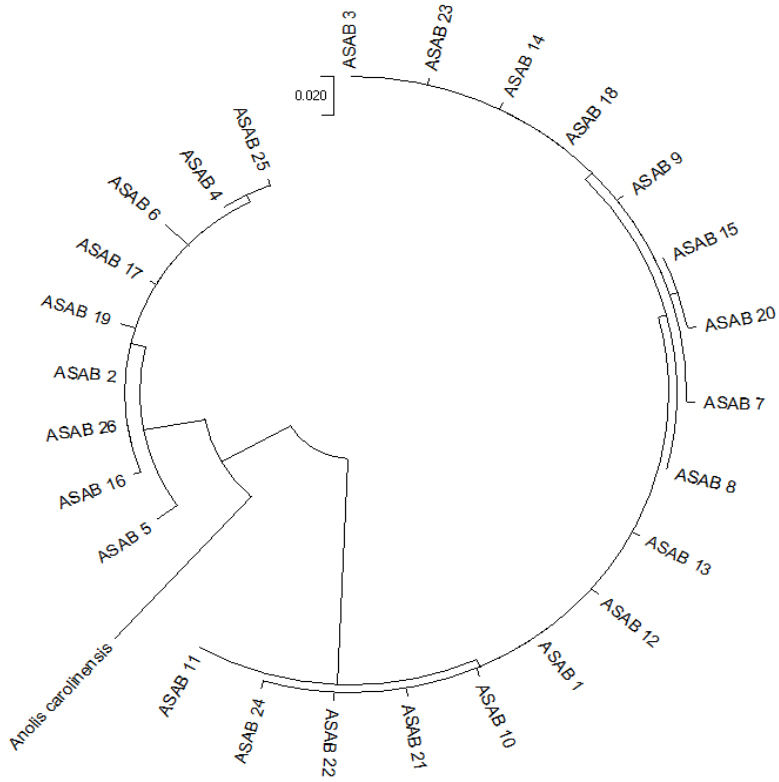

Figure 6: Circular maximum likelihood phylogeny showing the grouping of 26 different *mhc2b* sequences from *A. sabanus* and one outgroup sequence from *Anolis carolinensis* into two clusters based on the presence of a 12bp deletion.

Patterns of MHC variability

Fifty-seven distinct allele variants of 239-328 bp in length were amplified from 189 *A. sabanus* at the *mhc2b* locus using the custom primers MHC_Anolis_F1 and MHC_Anolis_R2. All sequences were BLASTed against the NCBI database of genetic data to confirm identity as *Anolis* MHC class II – β sequences. A total of 26 allele variants remained after the removal of unidentifiable sequences and those that the BLAST tool identified as *Anolis* in origin but were either chimeras, not derived from the MHC, or had amplification levels below the DOC statistical threshold (Lighten et al., 2014). Alleles that appeared in only one or two individuals and did not occur in replicated samples were included in the sequence phylogeny but excluded from further analysis, leaving five dominant variants we henceforth refer to as A, B, C, D, and E. Analysis with the program MEGA-X revealed that the sequences fell into two main groups, one with a deletion spanning 12 bp and one without. Phylogenetic analysis indicated that the most common *mhc2b* sequence obtained from *A. sabanus* grouped with related species *Anolis carolinensis* when compared to a variety of homologues from other reptiles (**Figure 4**). Maximum likelihood analysis of the phylogeny including all 26 *A. sabanus* alleles in MEGA-X grouped the *A. carolinensis* outgroup sequence with the *A. sabanus* sequences in which the 12 bp deletion was absent (**Figure 5**). Of the 189 individuals sequenced at *mhc2b*, 20 were excluded from further analysis due to Illumina sequencing failure of an unknown origin. Out of 9 replicates, two were excluded due to this sequencing failure. Replicability of allelic identity in the remaining samples was nevertheless high (85.71%).

Table 2. MHC allelic diversity values across sub-populations of *A. sabanus*.

	Site	n	He[a]	Ai[b]	Θk[c]	A[d]	>2[e]
2004	Crispeen	183	0.55	2.45	1.97	7	10
	Troy Hill	184	0.49	2.90	1.85	7	14
	Ladder	184	0.47	2.67	2.44	8	11
2009	Crispeen	167	0.63	2.63	1.55	6	12
	Troy Hill	181	0.50	2.42	0.85	4	8
	Ladder	141	0.54	2.61	4.02	11	11
2014	Crispeen	179	0.61	2.17	2.17	7	6
	Troy Hill	187	0.53	2.72	0.84	4	9
	Ladder		0.49	2.68	3.28	10	11

Average alleles per individual (Ai) across populations ranged from 2.17-2.90 (**Table 2**). Of the 169 individuals for whom *mhc2b* allelic composition could be determined, 14 (8%) possessed only one allele, 63 (37%) possessed two alleles, 71 (42%) possessed three alleles, and 21 (12%) possessed four alleles. All populations contained at least one individual with more than two *mhc2b* alleles.

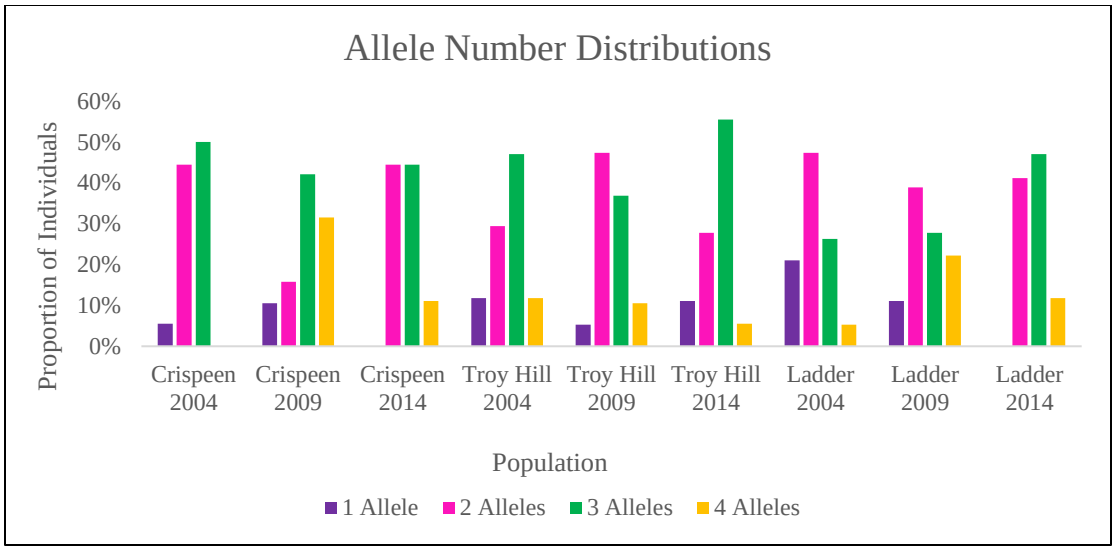

Figure 7: Distribution of MHC alleles per individual (Ai) in each sub-population of *A. sabanus*.

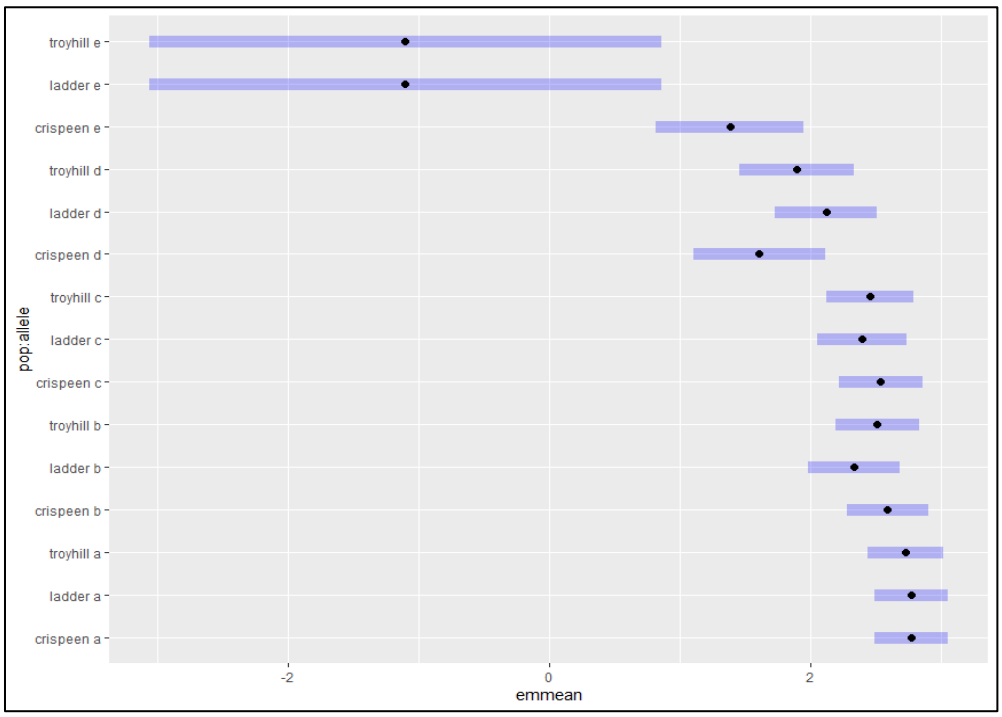

Figure 8: Estimated marginal means plot showing variation in allele prevalence at each sampling site. Allele "e" occurs at a lower frequency than all other alleles at the Troy Hill and Ladder sites.

The generalized linear model with Poisson errors determined that sampling year had no effect on MHC allelic richness, but the model did retain a significant interaction between "allele" and "population". An estimated marginal means plot generated from the generalized linear model

indicated that the prevalence of the *mhc2b* allele coded as "E" was lower in Troy Hill and Ladder populations than all other alleles in other populations. The logistic regression with Poisson errors determined that both year and site significantly impacted the frequency of *Plasmodium spp*. infection within a population but that presence of specific *mhc2b* alleles did not. The binomial logistic regression that tested MHC nucleotide diversity against likelihood of Plasmodium infection did not yield significant results, nor did the binomial logistic regression that tested sampling year, site, and sex against MHC variability.

Comparison of microsatellite and MHC variability

AMOVA analyses indicated that nearly all observed covariance at microsatellite and MHC loci was within populations (99.47% and 100.57%, respectively), with negligible levels of differentiation among populations at either neutral or adaptive markers (**Table 3**). This indicates a lack of significant population structuring at both neutral and adaptive loci.

Table 3. Comparison of AMOVA results at microsatellite and MHC loci.

		df	SS	Percentage Covariance	P-Value
Microsatellite	Among Groups	183	0.55	2.45	1.97
	Among Pops.	184	0.49	2.90	1.85
	Within Pops.	184	0.47	2.67	2.44
	Total	167	0.63	2.63	1.55
MHC	Among Groups	181	0.50	2.42	0.85
	Among Pops.	141	0.54	2.61	4.02
	Within Pops.	179	0.61	2.17	2.17
	Total	187	0.53	2.72	0.84

Supplementary Table 1. Significance matrix for linkage disequilibrium across microsatellite loci.

	Significant Linkage Diseq.								
	SA18	*GT2*	*Ba36*	*Acar9*	*Acar1*	*Acar8*	*Acar10*	*10;13*	*AoGT9*
SA18	*	-	-	-	-	-	-	-	-
GT2	-	*	+	-	+	-	-	-	+
Ba36	-	+	*	+	-	-	-	-	-
Acar9	-	-	+	*	-	-	-	-	-
Acar1	-	+	-	-	*	-	-	-	-
Acar8	-	-	-	-	-	*	+	-	-
Acar10	-	-	-	-	-	+	*	-	-
10;13	-	-	-	-	-	-	-	*	-
AoGT9	-	+	-	-	-	-	-	-	*

Supplementary Table 2. Significance matrix for FST across microsatellite loci.

	FST Significance Matrix								
	SA18	GT2	Ba36	Acar9	Acar1	Acar8	Acar10	10;13	AoGT9
SA18		-	-	-	-	-	-	-	-
GT2	-		-	+	+	-	+	-	+
Ba36	-	-		+	+	-	+	-	+
Acar9	-	+	+		-	+	-	-	+
Acar1	-	+	+	-		-	+	-	+
Acar8	-	-	-	+	-		+	-	-
Acar10	-	+	+	-	+	+		-	+
10;13	-	-	-	-	-	-	-		+
AoGT9	-	+	+	+	+	-	+	+	

Discussion

We used a long-term dataset to test the hypothesis that fluctuations in prevalence of malaria infection are a significant driver of immunogenetic diversity in the island endemic *A. sabanus*. PCR-based malaria diagnostics and Illumina MiSeq analysis of *A. sabanus* MHC amplicons confirmed the prediction that both parasite prevalence and allele frequencies at immune loci fluctuate over space and time in this system (**Figure 2**). However, our results do not provide compelling evidence that these fluctuations in the prevalence of *Plasmodium spp*. drive adaptive immunogenetic variability in the host.

Although we found that 34.4% of all sampled anoles were positive for malaria, we were unable to link patterns of infection to fluctuation in MHC allele frequencies across populations. Parasite-mediated selection is generally accepted as a main driver of MHC variability in host species (Aguilar et al., 2004; Spurgin & Richardson, 2010), but several studies have demonstrated that MHC variation declines in small populations when the degree of selective pressure exerted by parasites can be overwhelmed by the effects of drift (Eimes et al., 2011; Höglund et al., 2015; Santonastaso et al., 2017; Zhang et al., 2016). We did not detect population sub-structuring at neutral loci (**Figure 4**) or find evidence of localized adaptation at *mhc2b*, suggesting that *A. sabanus* functions as a single population and that gene flow occurs between our sampling sites on Saba despite differences in habitat type (Firman et al., 2019; Han et al., 2010; Valenzuela-Quiñonez et al., 2016).

MHC Class II sequences from *A. sabanus* were similar to those from related species *A. carolinensis*, but the presence of a unique 12bp deletion in many of the high-frequency MHC alleles retrieved in our study warrants further investigation (**Figure 5**). Deletions of this size remove four amino acids and have the potential to significantly impact phenotypes, but whether this allelic variant is adaptive or deleterious has yet to be determined. A 12 bp deletion in an MHC Class II allele retrieved from a population of brown bears (*Ursus arctos*) was determined to be a pseudogene through cDNA analysis, so applying this methodology to the *A. sabanus* alleles we detected in this study may shed light on the nature of the alleles where the deletion is present (Goda et al., 2009). The significantly lower prevalence of MHC allele "E" in two of our study populations (**Figure 8**) may suggest unknown differences in the adaptive value of various MHC alleles across space and time or simply be caused by drift.

Variation in overall infection prevalence across sites and time periods indicates that differences in habitat type and other environmental factors influence parasite ecology (Beckley et al., 2016; Escobar et al., 2016). Our prediction that the dominant species of *Plasmodium* found in anoles that tested positive would also shift over space and time was supported (**Figure 3**), but no clear pattern could be detected and our diagnostic methods did not account for the possibility of co-infection with multiple parasite species. The dynamics between malaria species infecting the same host are still poorly understood, but changes in relative proportions of parasite species over time and space may suggest competitive interactions between *Plasmodium spp*. or ecological differences among vectors (Otero et al., 2019).

Malaria infection was contingent on sex but not on year or site, indicating that differences between sexes are an important factor in this host-parasite system. Sex differences in parasite prevalence and virulence are common across many vertebrate taxa, particularly those in which sexes are dimorphic and intersexual selection is strong (Briggs et al., 2020; Freeman-Gallant et al., 2001; Klein, 2004; Zuk & McKean, 1996). In accordance with this trend, male anoles tend to be larger than females and spend much of their time performing sexual displays and defending territory (Butler et al., 2007; Stehle et al., 2017). These differences likely make male anoles more conspicuous to the insect vectors that transmit *Plasmodium spp*. and drive observed differences in malaria prevalence between sexes (Otero et al., 2019; Schall et al., 2000). Differences in the incidence of malaria infection between sexes may also be attributed to physiological reasons: testosterone is a known inhibitor of immune responses against malaria in other vertebrates (Benten et al., 1991; Krücken et al., 2005; vom Steeg et al., 2019). The observed differences in malaria incidence between male and female *A. sabanus* likely reflect sex-based tradeoffs between

immunity and reproductive success, which is a common phenomenon across animal taxa (Lindsey & Altizer, 2009).

Fitness consequences for infected anoles may vary depending on which parasites are present since the type of blood cells impacted differs between malaria species, with *P. floridense* and *P. azurophilum* infecting erythrocytes while *P. leucocytica* targets several classes of white blood cells (Perkins, 2001). However, overall virulence of all three malaria parasites found on Saba is strikingly low (Schall & Staats, 2002). Parasitemia and subsequent reduction of hemoglobin in infected *A. sabanus* is much less severe than in other lizard-malaria associations where malaria infection has significant hormonal and behavioral consequences (Dunlap & Schall, 2015). Although parasite pressure is known to inhibit the development of male secondary sexual ornaments in many systems, infection status also had no effect on the strongly dimorphic dorsal coloration of male *A. sabanus* (Schall & Staats, 2002; Vergara et al., 2012). The remarkably low virulence of malaria in this system suggests that *Plasmodium* parasites on Saba have limited access to hosts due to strong vector seasonality or that transmission intensity is low regardless of seasonal effects, favoring parasites that maintain low virulence and prolong host lifespan due to reduced availability of new hosts (Ebert & Mangin, 1997; Ewald, 1994). It is also possible that *Plasmodium* infection removes more susceptible anoles from the population before they can be sampled, underscoring the need for laboratory studies to understand the true prevalence and fitness costs of malaria in this system (Bonneaud et al., 2017). Although our results indicate that malaria infection does not impose strong selective pressure on *A. sabanus*, sampling anoles repeatedly throughout the year and incorporating environmental variables to account for the effects of vector seasonality may reveal signatures of host-parasite coevolution that our study design was unable to detect (Doan et al., 2019; Otero et al., 2019; Schall & Staats, 2002).

Conclusions

Our findings provide evidence that MHC variation in the island endemic anole *A. sabanus* is not linked to fluctuations in malaria prevalence and that rates of malaria infection in this species differ significantly between sexes. Future work on this system should account for the effects of environmental variables on parasite populations and investigate the possibility of strong vector seasonality. The present study provides the first sequences of MHC Class II genes for *A. sabanus* and adds substantially to the existing body of knowledge regarding reptilian MHC and the evolutionary forces that maintain its variability in nature. Our use of molecular tools to determine malaria infection status and parasite identity represents a methodological advance from visual inspection of blood smears alone, providing greater diagnostic accuracy and allowing for reliable identification of parasite species.

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Vita

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