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ORIGINAL ARTICLE

Functional variation at an expressed MHC class IIβ locus associates with *Ranavirus* infection intensity in larval anuran populations

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Abstract

Infectious diseases are causing catastrophic losses to global biodiversity. Iridoviruses in the genus *Ranavirus* are among the leading causes of amphibian disease-related mortality. Polymorphisms in major histocompatibility complex (MHC) genes are significantly associated with variation in amphibian pathogen susceptibility. MHC genes encode two classes of polymorphic cell-surface molecules that can recognize and bind to diverse pathogen peptides. While MHC class I genes are the classic mediators of viral-acquired immunity, larval amphibians do not express them. Consequently, MHC class II gene diversity may be an important predictor of *Ranavirus* susceptibility in larval amphibians, the life stage most susceptible to *Ranavirus*. We surveyed natural populations of larval wood frogs (*Rana sylvatica*), which are highly susceptible to *Ranavirus*, across 17 ponds and 2 years in Maryland, USA. We sequenced the peptide-binding region of an expressed MHC class IIβ locus and assessed allelic and genetic diversity. We converted alleles to functional supertypes and determined if supertypes or alleles influenced host responses to *Ranavirus*. Among 381 sampled individuals, 26% were infected with *Ranavirus*. We recovered 20 unique MHC class IIβ alleles that fell into two deeply diverged clades and seven supertypes. MHC genotypes were associated with *Ranavirus* infection intensity, but not prevalence. Specifically, MHC heterozygotes and supertype ST1/ST7 had significantly lower *Ranavirus* infection intensity compared to homozygotes and other supertypes. We conclude that MHC class IIβ functional genetic variation is an important component of *Ranavirus* susceptibility. Identifying immunogenetic signatures linked to variation in disease susceptibility can inform mitigation strategies for combatting global amphibian declines.

Keywords Major histocompatibility complex · Ranidae · Amplicon primers · Roche · 454 · *Rana sylvatica* · *Lithobates sylvaticus*

Introduction

Infectious disease results from the interaction among pathogens, hosts, and their shared environment (Scholthof 2007). Understanding the relative roles of these factors in driving disease outbreaks and severity has required multiple investigations into a variety of variables in natural and controlled settings. Common goals in conservation genetics are to assess whether hosts can persist with pathogens given their current genetic diversity and to determine the genetic potential for host disease resistance, a research focus with a host-centric perspective (Spielman et al. 2004; Allendorf et al. 2010). The major gap, and one of the management relevance, is the elucidation of how host genetics responds across environmental and pathogen pressure gradients (Smith et al. 2009).

Within a host, receptor molecules in the acquired immune system of vertebrate animals interact directly with pathogens. In particular, genes of the major histocompatibility complex (MHC) encode diverse cell surface molecules that can

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recognize and bind to a wide array of pathogen-derived peptides (Cresswell 1994). Class I MHC genes play a large role in self-nonself recognition and defense against intracellular pathogens such as viruses. Class II MHC gene code for the primary molecules can initiate acquired immunity against foreign organisms, particularly bacterial, protozoal, and fungal pathogens (Cooke and Hill 2001). However, recent advances in immunology have revealed significant cross-talk among all MHC classes (Dengjel et al. 2005), suggesting that either class I or class II molecules can respond to microbial pathogens. While class I genes also show high allelic diversity and play critical roles in adaptive immunity, class II MHC genes are of particular interest in wildlife disease studies because they have the highest levels of allelic polymorphism across all vertebrate genes (Flajnik and Kasahara 2001; Sommer 2005), show extensive copy number variation (Schaschl et al. 2009; Siddle et al. 2010), are often under strong positive selection based on nonsynonymous to synonymous substitution rate ratios (Hughes and Nei 1989; Aguilar et al. 2004), and can persist across closely related species and genera due to balancing selection (i.e., trans-species and trans-generic polymorphism; Takahata 1990; Klein et al. 1998). Within an individual, harboring a diverse array of class I and class II pathogen-recognition molecules is critical for disease resistance, while among-individual diversity improves population persistence in the presence of a pathogen (Hedrick 1998).

Understanding infectious disease risk is particularly important for amphibian hosts. Amphibians are declining globally (Houlahan et al. 2000; Stuart et al. 2004) with disease often cited as an important causative agent of decline and extinction (Alroy 2015). While the fungal disease chytridiomycosis, caused by *Batrachochytrium dendrobatidis* (*Bd*), has devastated amphibians globally (Wake and Vredenburg 2008; Alroy 2015), two other amphibian pathogens have emerged in recent decades, the Irridoviridae viral lineage *Ranavirus* (Chinchar 2002; Gray et al. 2009) and the alveolate parasite *Perkinsea* (Chambouvet et al. 2015; Karwacki et al. 2018). Recent assessment of 247 wild anuran mortality events in the USA from 1999 to 2015 found that *Ranavirus* was the most common pathogen associated with mortality, accounting for 37% of all cases (Isidoro-Ayza et al. 2017), which highlights that *Ranavirus* may be particularly devastating in certain regions of the world. Furthermore, *Ranavirus* lineages can readily cross species barriers and infect a wide array of ectothermic vertebrates, including fish, reptiles, and amphibians (Gray et al. 2009; Chinchar et al. 2011). Frog virus 3 (FV3) is the type species of the genus *Ranavirus* and is the most common and widespread viral lineage in amphibians and other ectothermic vertebrates (Chinchar and Waltzek 2014). Understanding *Ranavirus* susceptibility in amphibian hosts and how to mitigate infection risk is of primary interest for developing strategies to mitigate disease-associated population decline.

In amphibians, MHC genes are significantly associated with host responses to pathogen infection. For instance, susceptibility to chytridiomycosis is significantly associated with MHC class II genetic variants in a variety of host species and populations (Savage and Zamudio 2011; Bataille et al. 2015; Savage and Zamudio 2016; Kosch et al. 2016; Hu et al. 2017). However, *Ranavirus* susceptibility has been studied most extensively in the context of MHC class I genetic variation and gene expression in the model frog genus *Xenopus*. *Xenopus laevis* has one classical MHC class I locus (i.e., a single class Ia gene; Shum et al. 1993) that contributes to FV3 susceptibility (Gantress et al. 2003). Non-classical class I (class Ib) loci also contribute to *X. laevis* FV3 susceptibility based on reduced survival times of experimentally-infected larvae with a transgenically non-functional class Ib gene (Edholm et al. 2013). Adult *X. laevis* are readily infected with FV3, but the infection is localized to the kidneys where it can be rapidly cleared via antibody and cytotoxic T cell responses that are likely stimulated by MHC class Ia antigen presentation (Morales et al. 2010). In contrast, larval *X. laevis*, which cannot express MHC class Ia genes (Flajnik et al. 1987), have reduced innate immune responses to FV3 (Andino et al. 2012) and develop systemic infections that often cause mortality (Robert et al. 2011). MHC class I associations with FV3 susceptibility have been investigated in one additional frog species, the highly FV3-susceptibile *Rana temporaria*, and class I genotypes were significantly associated with FV3 infection status (Teacher et al. 2009). Due to the traditional role of class Ia molecules in generating antiviral immunity and evidence in support of this process in adult frogs, it is unsurprising that investigations into MHC class II genotype relationships to *Ranavirus* susceptibility are lacking. However, because larval amphibians are the life stage most susceptible to *Ranavirus* and can only express MHC class II genes to stimulate acquired immune responses (Flajnik et al. 1987; Rollins-Smith 1998), quantifying immunogenetic relationships between MHC class II alleles and *Ranavirus* infection dynamics in larval anurans can establish the role of acquired immune responses in larval susceptibility to viral pathogens.

Wood frogs (*Rana sylvatica*) show the highest *Ranavirus* susceptibility among 19 North American amphibian species tested in FV3 lab exposures (Hoverman et al. 2011). *Rana sylvatica* is one of the best amphibian models for *Ranavirus* infection experiments due to its broad distribution, amenable life history, and high larval susceptibility (Lee-Yaw et al. 2008; Lesbarrères et al. 2012). Consequent studies have revealed that *R. sylvatica* susceptibility to FV3 is dose-dependent (Forzán et al. 2015), that variation in host susceptibility drives larval FV3 transmission (Brunner et al. 2017), and that ecological and epidemiological factors are associated with ranavirosis (Price et al. 2017). However, the relationship between immunogenetic diversity and *Ranavirus* susceptibility has not been evaluated within the *R. sylvatica* model system.

Here, we investigate whether host immunogenetic factors contribute to *Ranavirus* susceptibility within natural populations of larval *R. sylvatica* by sequencing the peptide-binding region (PBR) of a constitutively-expressed MHC class IIβ locus (Kiemnec-Tyburczy et al. 2010; Mulder et al. 2017). This locus has previously been associated with amphibian chytridiomycosis susceptibility in several amphibian hosts (reviewed in Fu and Waldman 2017), but has not been evaluated in the context of *Ranavirus* susceptibility in natural anuran populations. We measure *Bd* and *Ranavirus* infection prevalence and infection intensity across 17 ponds and 2 years, characterize individual- and population-level MHC class IIβ polymorphism, test for evidence of positive selection in driving MHC gene evolution, and assess whether immunogenetic diversity predicts *Ranavirus* infection prevalence and intensity. Understanding the relationship between MHC class IIβ variation and larval susceptibility to *Ranavirus* will help to clarify the role of MHC class II-mediated acquired immunity for defense against viral pathogens in an amphibian life stage that lacks MHC class I immune function. Improved knowledge of larval immune function will also improve management strategies for amphibians threatened with infectious diseases.

Methods

Field sampling

We sampled a random set of ephemeral ponds to estimate the prevalence of *Ranavirus* at the Patuxent Research Refuge, Maryland, USA. At each site and sampling date, we captured up to 12 *R. sylvatica* tadpoles using dip nets and collected tail tips (< 5 mm) using sterilized scissors. Tail samples are a non-lethal sampling technique used to test for *Ranavirus* and have high detection probability for the pathogen (Mosher et al. in press). To sample for the presence of *Bd*, we swabbed the mouthparts of each individual using cotton-tipped swabs, rubbing around their oral disc 10 times. Each swab and tail tip were placed into a sterile 1.5 ml microcentrifuge tube with 70% ethanol and kept frozen until analysis. Individual samples were allocated randomly to plates for molecular diagnostics of *Ranavirus* loads. To quantify MHC alleles in these populations, we randomly selected four 96-well plates, comprising 381 samples collected in March and April 2012 and in June 2013. This resulted in an uneven sampling among sites and years, but because it was random, allows us to make inference to the entire Patuxent Research Refuge population of wood frogs.

Molecular pathogen analyses

We quantified pathogen infection intensity for *Bd* and *Ranavirus* using qPCR. We extracted DNA from mouthpart swabs and tail clips using the Qiagen Biosprint kit following the tissue extraction protocol. We tested swabs for *Bd* in singlicate following Boyle et al. (2004) with slight modifications as follows: KlearKall Mastermix was used, primer concentrations were 0.6 μM, BSA was added at 0.4 μg/μl (Garland et al. 2010), and qPCR conditions included an initial denaturation at 95 °C for 10 mins followed by 45 cycles of 95 °C for 15 s and 60 °C for 45 s. Negative PCR controls and *Bd* positive standards (100, 10, 1, and 0.1 zoospore genomics equivalents created from the Maine *Bd* isolate JEL 404) were run with each set of *Bd* qPCR reactions. We tested tail clips for *Ranavirus* in singlicate using the methods outlined by Brunner and Collins (2009) with slight modifications as follows: KlearKall Mastermix was used, BSA was added at 0.4 μg/μl, 25 ul reactions consisted of 20 μl mastermix and 5 μl of DNA and qPCR conditions were initial denaturation at 95 °C for 15 mins then 40 cycles of 95 °C for 15 s, 54 °C for 30 s, and 72 °C for 15 s. The primers and probes target a 70-bp region within the major capsid protein (MCP) of *Ranaviruses* (Brunner and Collins 2009). Negative controls and *Ranavirus* positive standards (10^7 virus copies/μl to 10^2 virus copies/μl; obtained from Jesse Brunner) were run with each set of *Ranavirus* qPCR reactions. The *Ranavirus* positive control was a plasmid bearing the MCP gene from FV3. For a subset of samples, we performed replicate qPCR reactions on the same DNA extracts ($n = 52$) and replicate qPCR reactions on replicate tissue DNA extracts ($n = 23$) to validate our methods for *Ranavirus* detection and quantification.

We sequenced a 321 bp fragment of the MCP gene from 18 *Ranavirus* positives from three ponds (pond 7: $n = 6$, pond 11: $n = 8$, pond 12: $n = 4$). We used PCR to amplify *Ranavirus* DNA using the primers M151F and M152R (Marsh et al. 2002). PCR conditions were carried out in 25 μl reaction containing 5 μl of DNA, 2.5 μl of 10x Amplitaq Gold PCR buffer, 0.8 μM of each primer, 0.2 mM of DNTPs, 2 mM of $MgCl_2$, BSA at 0.4 μg/μl, and 0.2 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR conditions consisted of denaturing at 95 °C for 15 min then 45 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 40 s; with a final extension of 72 °C for 5 min. We then cleaned amplified PCR products with ExoSAP-IT (United States Biochemical), and sequenced the cleaned amplicons using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Inc). The sequenced products were column-filtered, dried down, rehydrated with 10 μl of HPLC purified formamide, and then analyzed on an Applied Biosystems 3130xl DNA Analyzer. Individual sequences were assembled and edited in a Sequencher 5.1.

MHC sequencing and bioinformatics

We used 454 amplicon chemistry to sequence the MHC class IIβ locus to high coverage ($n = 381$). Barcoded fusion primers were previously designed to amplify only a single locus by targeting the flanking introns of the exon 2 (Mulder et al. 2017). We used 25 forward and 8 reverse primers to multiplex all 381 samples on two parts of one 454 FLX run. Amplicons were amplified using AmpliTaq Gold (Thermo Fisher Scientific, Waltham, MA) following manufacturer guidelines in 25 μl reactions and adding 2X BSA and 0.12 μM of each primer. PCRs were run using a touchdown of 10 cycles (95 °C, 55 °C to 50 °C, 72 °C for 45 s each) and 30 additional cycles at an annealing temperature of 50 °C and adding an initial 5 min of 95 °C and a final extension of 10 min at 72 °C. We used agarose gels stained with Gel-Red (Biotium Fremont, CA) to pool all samples in groups of 12 based on band intensity. We also included 15 μl of all PCR negatives for quality control. Pools were cleaned with 2X Sera-Mag beads and quantified using the dsDNA HS assay kit on a Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA) to produce final library pools, each with unique barcodes for sequencing. Libraries were quantified using qPCR and run on our in-house 454 FLX (Center for Conservation Genomics, Smithsonian Institution) using Lib-A chemistry and split over the two parts of the plate.

Reads were demultiplexed and primers were removed using jMHC version 1.0 (Stuglik et al. 2011). Many MHC amplicon studies follow the two PCR criterion, requiring an allele to be found in at least two independent PCRs for it to be considered a true allele (Babik 2010). As we only ran one PCR per individual, but also amplified only a single locus, we defined alleles as true alleles if they were recovered from at least two individuals at over 20% Minor Allele Frequency (MAF) or in one individual at 30% MAF. We considered an individual a heterozygote if it had at least 10% MAF of one of our defined true alleles. To avoid negatively biasing the number of homozygotes in our dataset, we excluded any individual that had a unique potential allele that we could not confirm as real because the MAF was between 10 and 30% and it did not occur in any other individual. Second, we excluded any individual that had fewer than 50 total preassigned reads or more than three alleles at 10% MAF. After exclusions of these individuals, 334 sampled individuals had robust genotypes that were included in the final dataset. We also prepared a second dataset that excluded any individuals that had a unique allele. We used this dataset of 331 individuals to ensure that our statistical analyses were not biased by these rare alleles that were not confirmed by a second PCR. All true alleles were aligned using 16 iterations of MUSCLE as implemented in a Geneious 9.1 (Kearse et al. 2012) adding *Xenopus laevis* (GenBank ID: D50039.2) as an outgroup. We assigned exon-intron boundaries using *X. laevis* as the reference, identified PBR codons following the Brown et al. (1993) model, and identified the conserved codons from Kaufman et al. (1994), all following the approach described in Mulder et al. (2017).

MHC supertyping

Alleles were converted into functional supertypes by analyzing physiochemical binding properties of the 13 codon positions within our MHC class IIβ gene alignment. Functional supertypes are known to affect peptide-binding capabilities of human class II MHC alleles (Jones et al. 2006). We characterized each of the 13 codon sites based on five physiochemical descriptor variables, including: z1 (hydrophobicity), z2 (steric bulk), z3 (polarity), z4, and z5 (electronic effects; Sandberg et al. 1998). We then conducted a discriminant analysis of principle components (DAPC) analysis, which implements a *k*-means clustering algorithm using the Bayesian information criterion (BIC), to define functional clusters using the adegenet 1.4–0 package in R (Jombart et al. 2010). Our threshold for identifying the optimal number of clusters was $\Delta BIC \leq 2$, and all alleles within clusters based on this threshold were collapsed into one PBR supertype.

Genealogy reconstruction and population genetics

All subsequent phylogenetic and selection analyses were performed on the coding region of the alignment. Nucleotide diversity statistics and Tajima D were calculated in MEGA7 (Kumar et al. 2016). Hardy-Weinberg Equilibrium (HWE) and population genetic differentiation (pairwise F_{ST} among all populations) were calculated using Genepop (Rousset 2008) implemented with the R package "genepop" (https://CRAN.R-project.org/package=genepop). We used the Markov chain method (1000 batches, 100 iterations per batch) to generate the HWE Fisher exact test *P*-values for all *R. sylvatica* populations with sufficient sampling ($N \geq 5$ individuals). We used the PartitionFinder 2 (Lanfear et al. 2016) to find the best fitting substitution model for our alignment using linked branch lengths, the corrected Akaike Criterion (AICc) and allowing the alignment to be partitioned across all three codon positions. To reconstruct the genealogy, we ran MrBayes 3.2 (Ronquist et al. 2012) using the optimized partitions and substitution schemes for 10 million generations, sampling every 500 generations and excluding a burn in of 25%.

Selection analyses

We used the full alignment and our reconstructed Bayesian genealogy to conduct selection analyses using the program "Hypothesis testing using Phylogeny" (HyPhy; Kosakovsky Pond et al. 2005) implemented on the Datamonkey server (Delport et al. 2010). Because intragenic recombination can

bias selection results, we tested for recombination using GARD (genetic algorithms for recombination detection) prior to conducting selection analyses (Kosakovsky Pond et al. 2005; Kosakovsky Pond et al. 2006). We tested for positive/diversifying selection (excessive nonsynonymous (dN) relative to synonymous (dS) substitution rates, or significantly positive dN-dS ratios) and negative/purifying selection (significantly negative dN-dS ratios) using the three methods currently recommended by Datamonkey that test for codon-based positive and negative selection: FEL (fixed effects likelihood), SLAC (single likelihood ancestry counting), and FUBAR (fast unconstrained Bayesian approximation; Kosakovsky Pond and Frost 2005; Murrell et al. 2013). We considered *P*-values lower than 0.05 to indicate significant evidence of positive or negative selection, except when using the Bayesian approach in FUBAR for which we considered a posterior probability of at least 0.98 as significant. To test for diversifying selection along individual branches of the genealogy, we also implemented the branch-site random effects model (aBSREL), which uses models of different complexity for different branches in the phylogeny to allow variation in selective pressures among both codon sites and individual branches (Smith et al. 2015). The significance threshold was set at a likelihood ratio test *P*-value lower than 0.05.

Statistical analyses

We determined if *Ranavirus* infection status and pathogen load changed in relation to host immunogenetics using a hurdle model (function hurdle in R package "pscl": Jackman 2017). Hurdle models are two-part models that first model infection status as a binomial process (uninfected versus infected), and then model pathogen load as a count process (either poisson or negative binomial). Larval MHC genetic variation was measured using three metrics of MHC class IIβ diversity: (i) allelic heterozygosity (homozygote or heterozygote), (ii) supertype PBR heterozygosity and (iii) supertype PBR genotype (the combination of supertypes present within an individual). We included all individuals in the analyses with robust genotypes and with supertype genotypes that were represented by more than five individuals ($N = 301/381$ individuals and 7/18 supertype genotypes). We were unable to include allelic genotype in our statistical analyses due to the high diversity of genotypes with many genotypes represented by few individuals. In the final dataset of 301 individuals, 229 samples were collected in 2012, and 72 samples were collected in 2013. We included allelic heterozygosity, supertype PBR heterozygosity and supertype PBR genotype as explanatory variables and sampling year as a covariate in the hurdle model with infection status and load as response variables in both submodels. We found that the hurdle model with a negative binomial distribution provided the best fit for the data compared to a hurdle model with a Poisson distribution or a zero-inflated negative binomial model (function zeroinfl in package "pscl") based on Akaike's information criterion (AIC). We determined significance of variables using the summary function and the Anova function in the R package "car" (Fox and Weisberg 2011) using the *F* test as the test statistic. For explanatory variables that were significant, as a second step we then fit hurdle models with that sole explanatory variable to identify how infection status or pathogen load changed across the range of that covariate. We exponentiated the coefficients of the two-part hurdle model to interpret odds ratios (binomial model) and multipliers (count model).

Results

We tested 381 individual *R. sylvatica* larvae for *Bd* and *Ranavirus* from 17 ponds sampled over 2 years (Figs. 1 and 2; Online Resource 1). At two ponds (16 and 17; Fig. 1), we collected fewer than 5 total individuals; therefore, we excluded them from population-level comparisons (Fig. 3). We did not detect *Bd* on any individual. We detected *Ranavirus* on 97/381 of individuals (26% overall prevalence; Fig. 2), with an average pathogen load of 6.3×10^7 virus copies ($SE \pm 3.5 \times 10^7$). Replicate *Ranavirus* assays produced similar measures of viral copy number (Online Resource 2). We detected 2/24 individuals as false negatives. We did not detect any false positives, and replicate qPCR values for infected individuals ($n = 51$) were on average 1.4x different between the two values ($SE \pm 0.2x$). The *Ranavirus* strain we detected was most likely FV3, as Sanger sequences from 18 *Ranavirus* positive samples produced a 321 bp consensus sequence of the MCP gene which matched the MCP gene in the FV3 genome (Genbank: AY548484.1) at 100% sequence similarity (*E*-value, $9E^{-166}$) as well as similar 100% matches to other FV3 isolates (e.g., GenBank: FJ601916.1, GQ144408.1, FJ459783.1). We detected no disease signs or evidence of *Ranavirus* outbreaks during either sampling year.

We generated 293,433 high-quality sequences of the MHC class IIβ locus across all 381 individuals, including the complete exon sequence (261 bp) and 14 bp of flanking intron. Among individuals with robust genotypes ($N = 334$), no individual had more than 2 alleles, and only these individuals were retained in the analyzed MHC dataset. We recovered 20 true MHC class IIβ alleles (GenBank accession numbers MK372646-MK372665), of which 17 alleles differed in the coding regions and three pairs varied only within intronic regions (*Rasy-2* and *Rasy-7*; *Rasy-6* and *Rasy-8*; *Rasy-4* and *Rasy-12*; Fig. 3). These 20 alleles represent 7 functional supertypes based on physiochemical binding properties of the PBR amino acids (Fig. 3; Online Resource 3). None of the alleles had stop codons, suggesting functional, expressed alleles. Nine populations showed significant deviations from HWE based on observed MHC class IIβ genotype

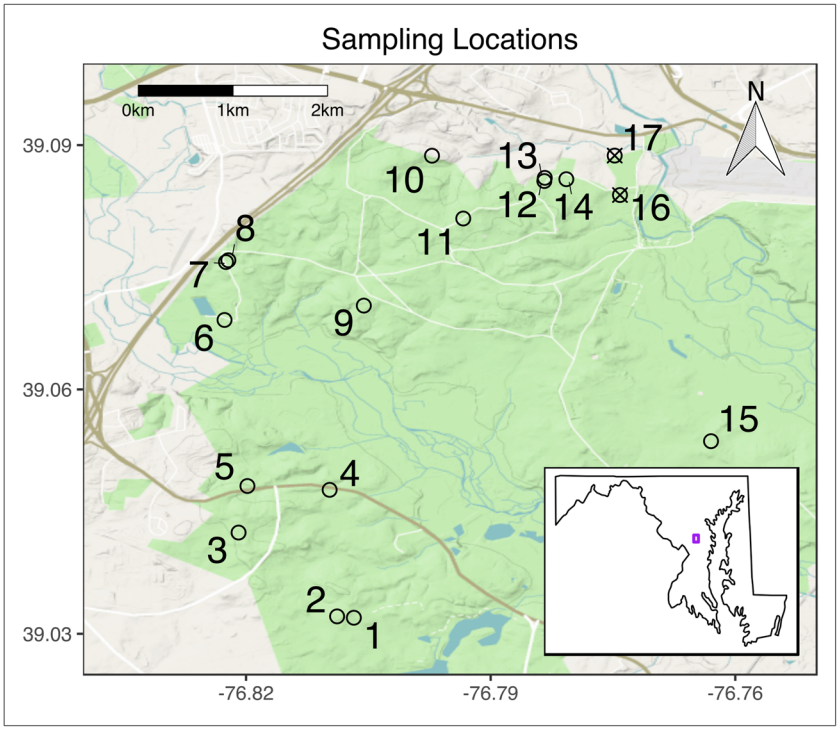

Fig. 1 Map of ponds sampled for *Rana sylvatica* larvae and their *Ranavirus* infection intensities in 2012 and 2013 in Patuxent State Park, Maryland, USA. Numbers 1–15 represent each robustly sampled pond ordered clockwise west to east and are consistent with pond supertype frequencies shown in Fig. 2. Numbers 16 and 17 represent the two ponds where sample sizes were insufficient ($N < 5$) for population assessments. The inset shows the state of Maryland with the location of our sampling area indicated in purple

frequencies (Online Resource 4). MHC class IIβ pairwise population differentiation was variable among population pairs, but three populations (8, 9, and 15; Figs. 1 and 3) showed consistently high levels of differentiation compared to all other populations ($F_{ST} > 0.05$; Online Resource 5).

We detected intermediate levels of nucleotide diversity when looking across the entire exon (Table 1). When restricting the analyses to the codons representing the peptide-binding region of the protein, nucleotide diversity and Tajima's D increased, consistent with balancing selection acting on the PBR. Conversely, when restricting analyses to codons in the Kaufman model, Tajima D decreased but was still higher than 1. This is most likely a combination of balancing selection acting on the exon as a whole, but certain

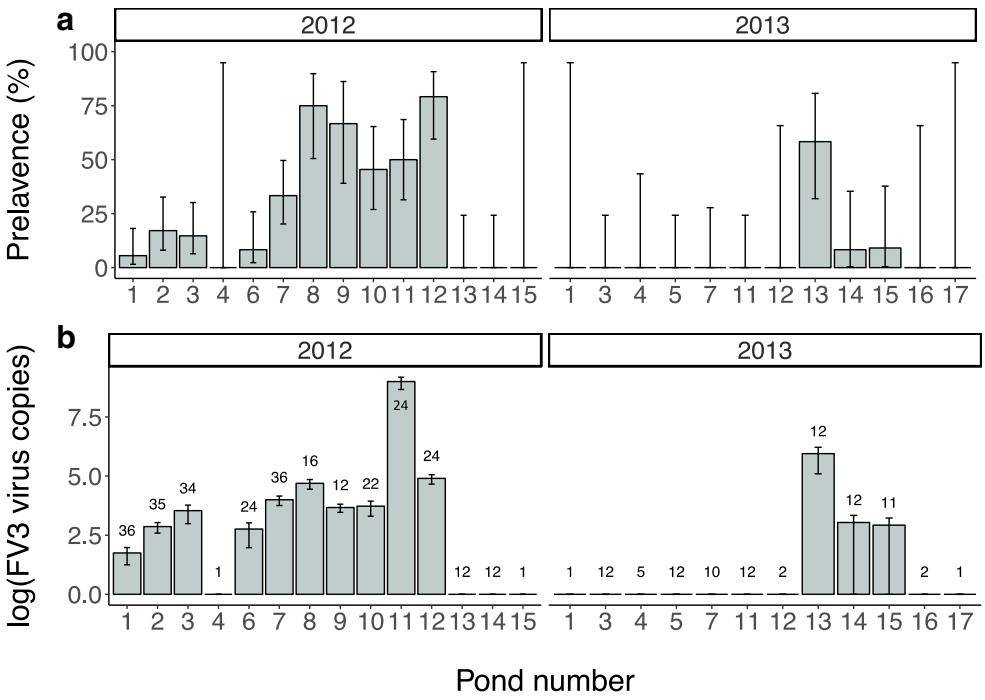

Fig. 2 Bar plots showing the spatial and temporal distribution of *Ranavirus* (A) prevalence with 95% binomial confidence intervals and (B) load ± SE among 381 individuals sampled across 17 ponds. Only ponds that were sampled in a given year are shown. Sample size per pond are shown above the bars in panel B

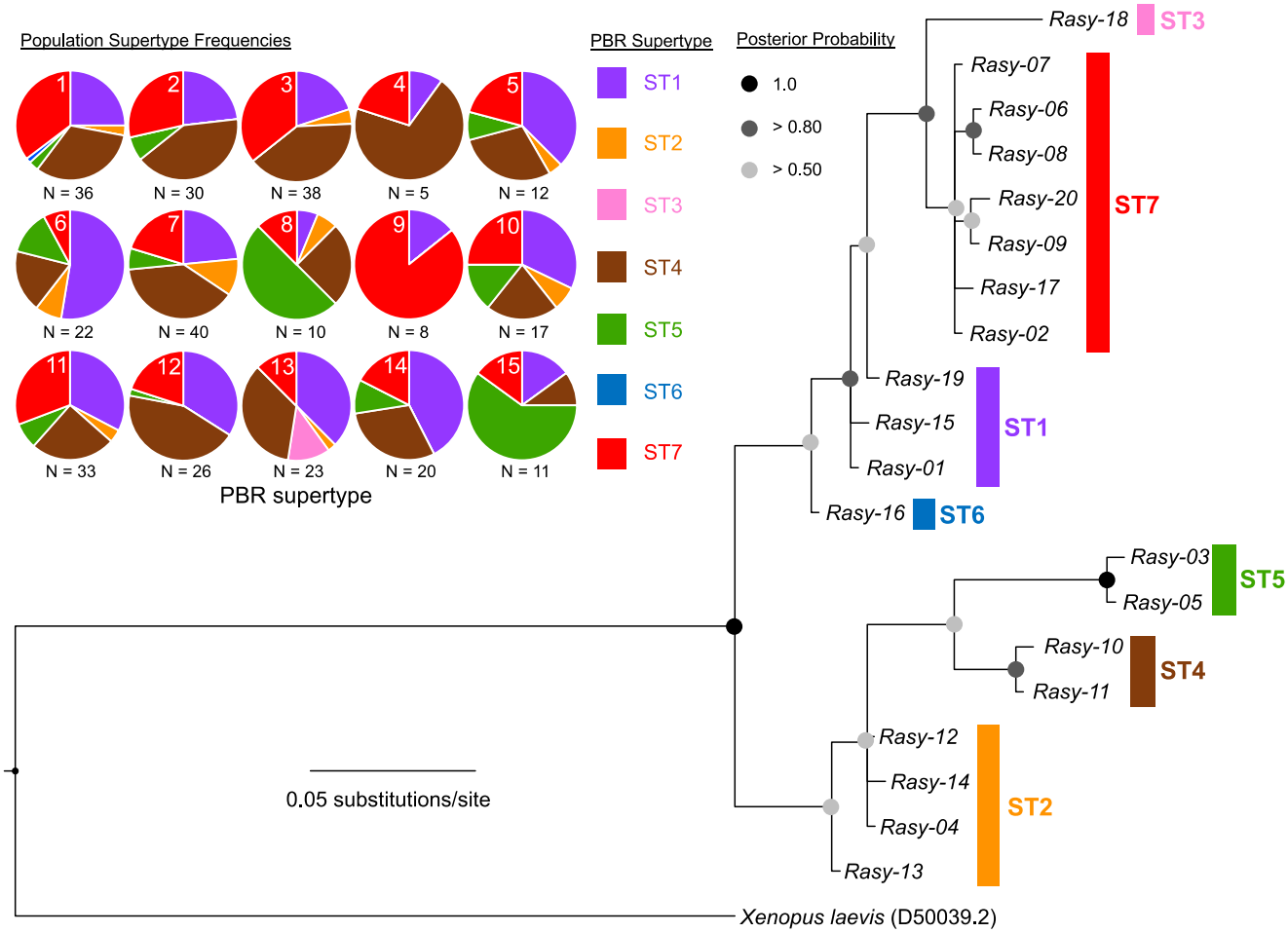

Fig. 3 Phylogenetic reconstruction of the 20 recovered *Rana sylvatica* MHC class IIβ alleles, seven corresponding PBR supertypes (ST1-ST7), and supertype frequencies across ponds. Labels 1–15 represent sampled ponds numbered clockwise west to east, corresponding to Fig. 1. Numbers below each pie chart indicate the number of individuals genotyped per pond

configurations being selected against because they interrupt the stable and functional structure of the protein. Codon 19 was found to be under significant positive selection (dN-dS = 4.19) by both FEL ($P = 0.032$) and FUBAR (posterior probability = 0.99), but not by the more conservative SLAC model ($P = 0.22$). Codon 62 was under negative selection (dN-dS = −6.41) using all three methods (SLAC, $P = 0.032$; FEL, $P = 0.016$; FUBAR, posterior probability = 0.99). No other codons were found under significant positive or negative selection using more than one method.

In the alignment of all 20 *R. sylvatica* alleles and the *X. laevis* outgroup allele, we identified three different substitution models for the codon partitions (codon 1: SYM + G, codon 2: F81 + G, codon 3: HKY). Phylogenetic analysis grouped class IIβ alleles into two large clades, with considerable variation in branch lengths among allelic lineages (Fig. 3). Three supertypes clustered phylogenetically (ST4, ST5, and ST7), two were represented by single alleles (ST3 and ST6), and two were paraphyletic (ST1 and ST2), although allelic relationships within these two supertypes had low posterior support (Fig. 3). Using aBSREL, only the branch leading to the ST4 and ST5 clades was under significant diversifying selection, although weaker evidence of diversifying selection was present along terminal branches leading to several alleles in ST1 and ST7, suggesting very recent selection (Online Resource 6). Supertypes ST1 and ST7 occurred in larvae from all 15 sampled ponds and ST4 occurred in larvae from all except one pond. ST2 and ST5 were also present in more than half of the sampled ponds. In contrast, ST3 and ST6 were each only recovered from larvae in a single pond (Fig. 3).

Of 301 individuals with robust and common supertypes ($N = 229$ in 2012 and $N = 72$ in 2013), *Ranavirus* was detected on 81 individuals, with an average pathogen load of 7.9×10^7 virus copies (SE ± 4.4×10^7). Among *Ranavirus*-infected individuals, pathogen load was significantly influenced by PBR supertype (Fig. 3a; hurdle count model, $F = 5.3$, df = 6, $P < 0.001$) and allelic heterozygosity (Fig. 3b; hurdle count model, $F = 16.8$, df = 1, $P < 0.001$; Online Resource 7). These results remained significant even when removing the three individuals that had unique MHC alleles recovered only

Table 1 Nucleotide diversity statistics across the 20 unique alleles as calculated by MEGA 7. The data was additionally sub-divided by the previously defined PBR regions, non-PBR regions, and conserved regions. S = Segregating sites, ps = segregating sites out of total sites, Θ = Watterson estimator, π = nucleotide diversity, Tajima's *D* = Tajima's test statistic

MHC subset	Size (bp)	No. codons	S	ps	Θ	π	Tajima's *D*
Whole exon	261	87	38	0.15	0.041	0.059	1.72
PBR	72	24	20	0.28	0.078	0.13	2.48
Non-PBR	189	63	18	0.10	0.027	0.032	0.71
Conserved codons (Kaufmann)	60	20	4	0.067	0.19	0.027	1.31

once. *Ranavirus* infection intensity was lowest in larvae with MHC supertype genotype ST1/ST7 (hurdle count model; pairwise $P < 0.001$). Compared to the ST1/ST7 baseline (Fig. 4A), infection intensity increased by at least 1.8×10^3 (ST4/ST7) to as high as 5.6×10^5 (ST7/ST7). *Ranavirus* infection intensity was similar among all other MHC supertype genotypes, except that the homozygote supertype genotype ST7/ST7 had significantly higher average infection intensities compared to the heterozygote supertype genotype ST4/ST7 (hurdle count model; $P = 0.01$). Average *Ranavirus* infection intensity was 2.2×10^7 virus copies for all MHC heterozygotes and 4.4×10^8 virus copies for all MHC homozygote genotypes. MHC homozygotes had a 23-fold increase in *Ranavirus* infection intensity compared to heterozygotes (Fig. 4B). We did not find an association between allelic heterozygosity, PBR heterozygosity or PBR supertype, and *Ranavirus* prevalence (hurdle binomial model, $P > 0.05$).

Discussion

MHC-linked associations with disease susceptibility have been documented in humans (Trowsdale 2011) and a variety of wildlife taxa (Acevedo-Whitehouse and Cunningham 2006), including amphibians (Fu and Waldman 2017). Here, we find that MHC class II genotypes are associated with variation in *Ranavirus* infection intensity in free-ranging populations of wood frogs. In contrast, none of the measured variables explained the observed variation in *Ranavirus* prevalence, suggesting that other ecological factors may influence occurrence of the pathogen. This two-step process of disease progression (i.e., the probability of initial infection, followed by the rate of pathogen replication within infected individuals), with different variables driving outcomes of each process, is also observed in *Bd* disease dynamics (Raffel et al. 2015). However, an opposite pattern of genetic associations is frequently recovered from studies of *Bd*-MHC associations: *Bd* prevalence tends to be associated with host genotype, while infection intensity is best explained by climatic factors such as temperature (Savage et al. 2015; Horner et al. 2017). The pattern we identified for FV3 disease dynamics, where probability of infection may be linked to (unmeasured) environmental variation, while infection intensity is predicted by host genotype, is likely explained by viral biology. FV3 infection intensity is positively associated with mortality (Miller et al. 2011) and varies among hosts and host genotypes in controlled exposure experiments conducted in several frog and salamander systems (Rojas et al. 2005; Price et al. 2018; Ariel et al. 2009), including *R. sylvatica* (Echaubard et al. 2014; Brand et al. 2016). Thus, infection intensity is a robust

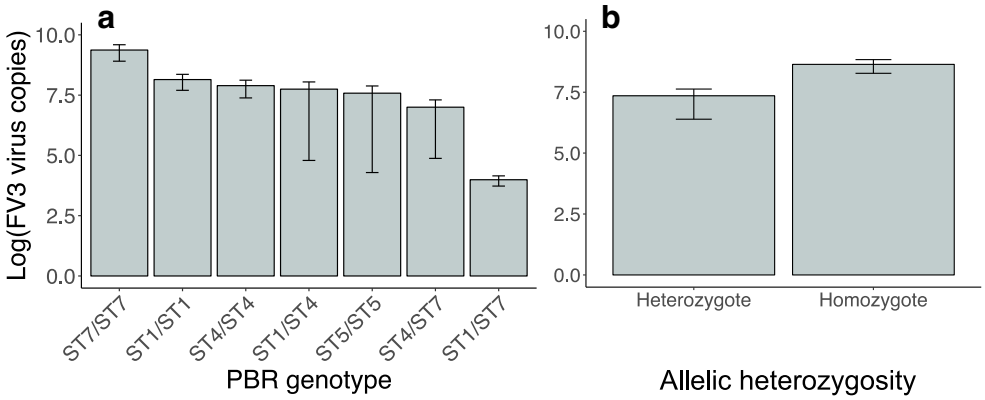

Fig. 4 Mean *Ranavirus* infection intensity among FV3-infected individuals based on (A) PBR genotype and (B) MHC allelic heterozygosity. PBR genotypes are measured as the combination of functional MHC supertypes (ST) based on physiochemical binding properties of PBR amino acids, while allelic heterozygosity is whether an individual has one or two MHC alleles at the characterized locus. For supertype-allele relationships, see Fig. 2

predictor of FV3 susceptibility in amphibians, whereas *Bd* susceptibility is more complex, with numerous species maintaining high *Bd* intensity and remaining asymptomatic (Garner et al. 2006; Rollins-Smith et al. 2015; Savage and Zamudio 2016).

The nature of the relationship between immune gene diversity and response to pathogens in natural populations of vertebrates is highly variable. At the population level, some studies find evidence of negative frequency-dependent selection driving high allelic polymorphism, while others reveal directional selection for particular alleles (Bernatchez and Landry 2003). At the individual level, diverse patterns are also observed. Even when considering the amphibian-*Bd* system only, empirical support exists for heterozygote advantage (Savage and Zamudio 2011), homozygote advantage (Savage et al. 2017), and specific alleles that are associated with differential pathogen susceptibility (Bataille et al. 2015). In our particular *Ranavirus*-larval *R. sylvatica* study system, we found weak evidence for site-specific positive selection, with one codon showing elevated nonsynonymous to synonymous rate ratios. Although this codon does not sit in the canonical PBR as found by Brown and Tong, it has been found to be under positive selection in numerous studies that have used a variety subsets of amphibian species (Mulder et al. 2017; Bataille et al. 2015; Kiemnec-Tyburczy et al. 2010; Lillie et al. 2015) and has been hypothesized by Mulder et al. (2017) to be an important codon for amphibian-specific pathogen-recognition.

We found evidence for balancing selection acting on MHC allelic diversity at the population level in the PBR compared to non-PBR regions as indicated by positive Tajima's *D* values. One caveat is that demographic processes can also produce Tajima's *D* values that mimic those expected under directional or balancing selection (Nielsen 2005). At the individual level, we recovered a more striking and definitive pattern of heterozygote advantage, with MHC heterozygotes showing a 23-fold reduction in *Ranavirus* infection intensity. The importance of individual MHC molecules or allele combinations relative to heterozygosity arising from any combination of alleles remains unclear in our study and in others (Acevedo-Whitehouse and Cunningham 2006). However, the variation in pairwise MHC genetic differentiation among populations and in whether a population showed significant MHC deviations from HWE suggests that different alleles may be beneficial across sampled populations, potentially in response to the variable pathogen pressures observed within and among years.

The significant associations between MHC genotypes and *Ranavirus* infection intensity we observed in larval *R. sylvatica* provides evidence that MHC class II variants are indeed contributing to antiviral immunity in amphibian populations. However, the role of MHC class II gene products—which would reveal an understanding of a heritable mechanism for population susceptibility—in amphibian antiviral immunity are poorly understood. Extensive work on immunity in the *X. laevis* amphibian model system demonstrates that larval anurans do not express MHC class I gene products (Rollins-Smith 1998), which are the central regulators of acquired antiviral immune function across vertebrates (Matsumura et al. 1992). Indeed, *X. laevis* larval susceptibility to *Ranavirus* is significantly higher than adult susceptibility, which may be driven by a lack of MHC class I gene products stimulating CD8 T cell restricted immune function. However, larval *X. laevis* exhibit significant variation in *Ranavirus* susceptibility among individuals (Chen and Robert 2011), suggesting that variation in other immune loci may drive susceptibility. MHC class II loci are expressed and can generate acquired immune responses in pre-metamorphic *X. laevis* individuals by stimulating CD4 T cells (Rollins-Smith 1998; Rollins-Smith 2001). Processes such as autophagy can also cause MHC class II molecules to present intracellular antigens such as viruses on the surface of antigen-presenting cells (Dengjel et al. 2005). Thus, CD4 T cell-restricted acquired immunity may be more important for larval amphibian antiviral immunity than the traditional dogma of MHC class restriction would suggest (Cresswell et al. 2005). As genome editing becomes increasingly feasible in non-model animal systems, conducting functional genomics experiments in diverse amphibian lineages will allow us to better understand the importance of different immune genes in driving susceptibility to global pathogens such as *Ranavirus* and *Bd*.

Our data on free-ranging amphibian populations demonstrate that MHC class IIβ functional genetic variation may be an important component of *Ranavirus* susceptibility in wood frogs. We recovered typically high levels of MHC polymorphism and heterozygosity, both within and among ponds. Therefore, we encourage future comparative studies of MHC diversity relative to overall population genetic variation to assess the specific importance of functional PBR variants for *Ranavirus* susceptibility. Replicating this study design across a range of larval host amphibians and *Ranavirus* strains, as well as conducting experimental exposure trials across MHC genotypes, will be important next steps in assessing how general this association is relative to other factors such as temperature (Brand et al. 2016), additional immune genes (Teacher et al. 2009), or host microbiome (Campbell et al. 2018). *Rana sylvatica* is among the most *Ranavirus*-susceptible ranid frogs in North America (Hoverman et al. 2011) despite high levels of class II PBR diversity and heterozygosity within the small spatial scale investigated here, suggesting that the presence of particular alleles within individuals may be more important for *Ranavirus* susceptibility than overall immunogenetic diversity. Broader characterization of MHC class II functional variation among species using rapid amplicon sequencing approaches developed for ranid frogs (Mulder et al. 2017)

can further elucidate allele-supertype-heterozygosity relationships across a variety of anurans with a history of exposure to *Ranavirus*. Ultimately, understanding whether natural selection favoring MHC or other genetic variants in natural amphibian populations can lead to the evolution of *Ranavirus* resistance can inform intervention strategies, such as genetically-informed captive breeding or gene editing resistance alleles, for affected populations and reduce disease-associated global amphibian declines.

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Author contributions AES and KPM conceived the study design. EHCG and RCF provided samples. AES, EHCG, and RCF provided funds and laboratory space. KPM and CRMW performed all the lab works. AES, KPM, CRMW, and EHGC analyzed the data. AES wrote the manuscript with input from all other authors.

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Compliance with ethical standards

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References

Acevedo-Whitehouse K, Cunningham AA (2006) Is MHC enough for understanding wildlife immunogenetics? Trends Ecol Evol 21(8): 433–438

Aguilar A, Roemer G, Debenham S, Binns M, Garcelon D, Wayne RK (2004) High MHC diversity maintained by balancing selection in an otherwise genetically monomorphic mammal. Proc Natl Acad Sci U S A 101(10):3490–3494

Allendorf FW, Hohenlohe PA, Luikart G (2010) Genomics and the future of conservation genetics. Nat Rev Genet 11(1):697–709

Alroy J (2015) Current extinction rates of reptiles and amphibians. Proc Natl Acad Sci U S A 112(42):13003–13008

Andino F, Chen G, Li Z, Grayfer L, Robert J (2012) Susceptibility of Xenopus laevis tadpoles to infection by the Ranavirus frog virus 3 correlates with a reduced and delayed innate immune response in comparison with adult frogs. Virology 432(2):435–443

Ariel E, Nicolajsen N, Christophersen MB, Holopainen R, Tapiovaara H, Jensen BB (2009) Propagation and isolation of ranaviruses in cell culture. Aquaculture 294(3–4):159–164

Babik W (2010) Methods for MHC genotyping in non-model vertebrates. Mol Ecol Resour 10(2):237–251

Bataille A, Cashins SD, Grogan L, Skerratt LF, Hunter D, McFadden M, Scheele B, Brannelly LA, Macris A, Harlow PS, Bell S (2015) Susceptibility of amphibians to chytridiomycosis is associated with MHC class II conformation. Proc R Soc Lond B 282(1805): 20143127

Bernatchez L, Landry C (2003) MHC studies in nonmodel vertebrates: what have we learned about natural selection in 15 years? J Evol Biol 16(3):363–377

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Dis Aquat Org 60(2):141–148

Brand MD, Hill RD, Brenes R, Chaney JC, Wilkes RP, Grayfer L, Miller DL, Gray MJ (2016) Water temperature affects susceptibility to Ranavirus. EcoHealth 13(2):350–359

Brown JH, Jardetzky TS, Gorga JC, Stern LJ, Urban RG, Strominger JL, Wiley DC (1993) Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature 364(6432):33–39

Brunner JL, Collins JP (2009) Testing assumptions of the trade-off theory of the evolution of parasite virulence. Evol Ecol Res 11(8):1169–1188

Brunner JL, Beaty L, Guitard A, Russell D (2017) Heterogeneities in the infection process drive ranavirus transmission. Ecology 98(2):576–582

Campbell LJ, Hammond SA, Price SJ, Sharma MD, Garner TWJ, Birol I, Helbing CC, Wilfert L, Griffiths AGF (2018) A novel approach to wildlife transcriptomics provides evidence of disease-mediated differential expression and changes to the microbiome of amphibian populations. Molec Ecol 27(6):1413–1427

Chambouvet A, Gower DJ, Jirků M, Yabsley MJ, Davis AK, Leonard G, Maguire F, Doherty-Bone TM, Bittencourt-Silva GB, Wilkinson M, Richards TA (2015) Cryptic infection of a broad taxonomic and geographic diversity of tadpoles by Perkinsea protists. Proc Natl Acad Sci U S A 112(34):E4743–E4751

Chen G, Robert J (2011) Antiviral immunity in amphibians. Viruses 3(11):2065–2086

Chinchar VG (2002) Ranaviruses (family Iridoviridae): emerging cold-blooded killers. Arch Virol 147(3):447–470

Chinchar VG, Waltzek TB (2014) Ranaviruses: not just for frogs. PLoS Path 10(1):e1003850

Chinchar VG, Yu KH, Jancovich JK (2011) The molecular biology of frog virus 3 and other iridoviruses infecting cold-blooded vertebrates. Viruses 3(10):1959–1985

Cooke GS, Hill AV (2001) Genetics of susceptibility to human infectious disease. Nat Rev Genet 2:967–977

Cresswell P (1994) Assembly, transport, and function of MHC class II molecules. Ann Rev Immunol 12(1):259–291

Cresswell P, Ackerman AL, Giodini A, Peaper DR, Wearsch PA (2005) Mechanisms on MHC class I-restricted antigen processing and cross-presentation. Immunol Rev 207(1):145–157

Delport W, Poon AFY, Frost SDW, Kosakovsky Pond SL (2010) Datamonkey 2010: a suite of phylogenetic analysis tools for evolutionary biology. Bioinformatics 26(1):2455–2457

Dengjel J, Schoor O, Fischer R, Reich M, Kraus M, Müller M, Kreymborg K, Altenberend F, Brandenburg J, Kalbacher H, Brock R (2005) Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. Proc Natl Acad Sci U S A 102(22):7922–7927

Echaubard P, Leduc J, Pauli B, Chinchar VG, Robert J, Lesbarreres D (2014) Environmental dependency of amphibian–ranavirus

genotypic interactions: evolutionary perspectives on infectious diseases. Evol Appl 7(7):723–733

Edholm ES, Saez LMA, Gill AL, Gill SR, Grayfer L, Haynes N, Myers JR, Robert J (2013) Nonclassical MHC class I-dependent invariant T cells are evolutionarily conserved and prominent from early development in amphibians. Proc Nat Acad Sci U S A 110(35):14342–14347

Flajnik MF, Kasahara M (2001) Comparative genomics of the MHC: glimpses into the evolution of the adaptive immune system. Immunity 15(3):351–362

Flajnik MF, Hsu E, Kaufman JF, Du Pasquier L (1987) Changes in the immune system during metomorphosis of *Xenopus*. Immunol Today 8(2):58–64

Forzán MJ, Jones KM, Vanderstichel RV, Wood J, Kibenge FS, Kuiken T, Wirth W, Ariel E, Daoust PY (2015) Clinical signs, pathology and dose-dependent survival of adult wood frogs, Rana sylvatica, inoculated orally with frog virus 3 Ranavirus sp., Iridoviridae. J Gen Virol 96(5):1138–1149

Fox J, Weisberg S (2011) An {R} companion to applied regression, 2nd edn. Sage, Thousand Oaks. URL: http://socserv.socsci.mcmaster.ca/jfox/Books/Companion. Accessed 1 Sept 2018

Fu M, Waldman B (2017) Major histocompatibility complex variation and the evolution of resistance to amphibian chytridiomycosis. Immunogenetics 69(8–9):529–536

Gantress J, Maniero GD, Cohen N, Robert J (2003) Development and characterization of a model system to study amphibian immune responses to iridoviruses. Virology 311(2):254–262

Garland S, Baker A, Phillott AD, Skerratt LF (2010) BSA reduces inhibition in a TaqMan® assay for the detection of *Batrachochytrium dendrobatidis*. Dis Aquat Org 92(2–3):113–116

Garner TW, Perkins MW, Govindarajulu P, Seglie D, Walker S, Cunningham AA, Fisher MC (2006) The emerging amphibian pathogen *Batrachochytrium dendrobatidis* globally infects introduced populations of the North American bullfrog, *Rana catesbeiana*. Biol Lett 2(3):455–459

Gray MJ, Miller DL, Hoverman JT (2009) Ecology and pathology of amphibian ranaviruses. Dis Aquat Org 87(3):243–266

Hedrick PW (1998) Balancing selection and MHC. Genetica 104(3):207–214

Horner AA, Hoffman E, Hether T, Tye M, Savage AE (2017) Cryptic chytridiomycosis linked to climate and genetic variation in amphibian populations of the southeastern United States. PLoS One 12(4): e0175843

Houlahan JE, Findlay CS, Schmidt BR, Meyer AH, Kuzmin SL (2000) Quantitative evidence for global amphibian population declines. Nature 404(6779):752–755

Hoverman JT, Gray MJ, Haislip NA, Miller DL (2011) Phylogeny, life history, and ecology contribute to differences in amphibian susceptibility to ranaviruses. EcoHealth 8(3):301–319

Hu W, Dong B, Kong S, Mao Y, Zheng R (2017) Pathogen resistance and gene frequency stability of major histocompatibility complex class IIB alleles in the giant spiny frog *Quasipaa spinosa*. Aquaculture 468(1):410–416

Hughes AL, Nei M (1989) Nucleotide substitution at major histocompatibility complex class II loci: evidence for overdominant selection. Proc Natl Acad Sci U S A 86(3):958–962

Isidoro-Ayza M, Lorch JM, Grear DA, Winzeler M, Calhoun DL, Barichivich WJ (2017) Pathogenic lineage of Perkinsea associated with mass mortality of frogs across the United States. Sci Rep 7(1): 10288

Jackman S (2017) pscl: classes and methods for R developed in the political science computational laboratory. United States Studies Centre, University of Sydney. Sydney, New South Wales, Australia. R package version 1.5.2. URL https://github.com/atahk/pscl/. Accessed 20 Aug 2018

Jombart T, Devillard S, Balloux F (2010) Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genet 11(1):94

Jones EY, Fugger L, Strominger JL, Siebold C (2006) MHC class II proteins and disease: a structural perspective. Nat Rev Immunol 6(4):271–282

Karwacki EE, Atkinson MS, Ossiboff RJ, Savage AE (2018) Novel quantitative PCR assay specific for the emerging Perkinsea amphibian pathogen reveals seasonal infection dynamics. Dis Aquat Org 129(2):85–98

Kaufman J, Salomonsen J, Flajnik M (1994) Evolutionary conservation of MHC class I and class II molecules - different yet the same. Semin Immunol 6(6):411–424

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T (2012) Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12):1647–1649

Kiemnec-Tyburczy KM, Richmond JQ, Savage AE, Zamudio KR (2010) Selection, trans-species polymorphism, and locus identification of major histocompatibility complex class IIβ alleles of New World ranid frogs. Immunogenetics 62(11–12):741–751

Klein J, Sato A, Nagl S, O'hUigín C (1998) Molecular trans-species polymorphism. Annu Rev Ecol Syst 29(1):1–21

Kosakovsky Pond SL, Frost SD (2005) Not so different after all: a comparison of methods for detecting amino acid sites under selection. Mol Biol Evol 22(5):1208–1222

Kosakovsky Pond SL, Frost SD, Muse SV (2005) HyPhy: hypothesis testing using phylogenies. Bioinformatics 21(1):676–679

Kosakovsky Pond SL, Posada D, Gravenor MB, Woelk CH, Frost SDW (2006) GARD: a genetic algorithm for recombination detection. Bioinformatics 22(24):3096–3098

Kosch TA, Bataille A, Didinger C, Eimes JA, Rodríguez-Brenes S, Ryan MJ, Waldman B (2016) Major histocompatibility complex selection dynamics in pathogen-infected túngara frog (*Physalaemus pustulosus*) populations. Biol Lett 12(8):20160345

Kumar S, Stecher G, Tamura K (2016) MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33(7):1870–1874

Lanfear R, Frandsen PB, Wright AM, Senfeld T, Calcott B (2016) PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. Mol Biol Evol 34(3):772–773

Lee-Yaw JA, Irwin JT, Green DM (2008) Postglacial range expansion from northern refugia by the wood frog, *Rana sylvatica*. Mol Ecol 17(3):867–884

Lesbarrères D, Balseiro A, Brunner J, Chinchar VG, Duffus A, Kerby J, Miller DL, Robert J, Schock DM, Waltzek T, Gray MJ (2012) Ranavirus: past, present and future. Biol Lett 8(4):481–483

Lillie M, Grueber CE, Sutton JT, Howitt R, Bishop PJ, Gleeson D, Belov K (2015) Selection on MHC class II supertypes in the New Zealand endemic Hochstetter's frog. BMC Evol Biol 15(1):63

Marsh IB, Whittington RJ, O'Rourke B, Hyatt AD, Chisholm O (2002) Rapid differentiation of Australian, European and American ranaviruses based on variation in major capsid protein gene sequence. Mol Cell Probes 16(2):137–151

Matsumura M, Fremont DH, Peterson PA, Wilson IA (1992) Emerging principles for the recognition of peptide antigens by MHC class I molecules. Science 257(5072):927–934

Miller D, Gray M, Storfer A (2011) Ecopathology of ranaviruses infecting amphibians. Viruses 3(11):2351–2373

Morales HD, Abramowitz L, Gertz J, Sowa J, Vogel A, Robert J (2010) Innate immune responses and permissiveness to ranavirus infection of peritoneal leukocytes in the frog *Xenopus laevis*. J Virol 84(10): 4912–4922

Mosher BM, Grant EHC, Wiewel AW, Miller DAW, Gray MJ, Miller D (in press) Estimation of disease presence and prevalence of amphibian ranavirus. J Wild Dis

Mulder KP, Cortazar-Chinarro M, Harris DJ, Crottini A, Grant EHC, Fleischer RC, Savage AE (2017) Evolutionary dynamics of an expressed MHC class IIβ locus in the Ranidae (Anura) uncovered by genome walking and high-throughput amplicon sequencing. Dev Comp Immunol 76(11):177–188

Murrell B, Moola S, Mabona A, Weighill T, Sheward D, Kosakovsky Pond SL, Scheffler K (2013) FUBAR: a fast, unconstrained Bayesian approximation for inferring selection. Mol Biol Evol 30(5):1196–1205

Nielsen R (2005) Molecular signatures of natural selection. Ann Rev Genet 39(1):197–218

Price SJ, Ariel E, Maclaine A, Rosa GM, Gray MJ, Brunner JL, Garner TW (2017) From fish to frogs and beyond: impact and host range of emergent ranaviruses. Virology 511(11):272–279

Price SJ, Leung WT, Owen C, Sergeant C, Cunningham AA, Balloux F, Garner TW, Nichols RA (2018) Temperature is a key driver of a wildlife epidemic and future warming will increase impacts. bioRxiv:272369

Raffel TR, Halstead NT, McMahon TA, Davis AK, Rohr JR (2015) Temperature variability and moisture synergistically interact to exacerbate an epizootic disease. Proc R Soc B Biol Sci 282(1801): 20142039

Robert J, George E, Andino FDJ, Chen G (2011) Waterborne infectivity of the Ranavirus frog virus 3 in *Xenopus laevis*. Virol 417(2):410–417

Rojas S, Richards K, Jancovich JK, Davidson EW (2005) Influence of temperature on Ranavirus infection in larval salamanders *Ambystoma tigrinum*. Dis Aquat Org 63(2–3):95–100

Rollins-Smith LA (1998) Metamorphosis and the amphibian immune system. Immunol Rev 166(1):221–230

Rollins-Smith LA (2001) Neuroendocrine-immune system interactions in amphibians: implications for understanding global amphibian declines. Immunol Res 23(2–3):273–280

Rollins-Smith LA, Reinert LK, Burrowes PA (2015) Coqui frogs persist with the deadly chytrid fungus despite a lack of defensive antimicrobial peptides. Dis Aquat Org 113(1):81–83

Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst Biol 61(3):539–542

Rousset F (2008) genepop'007: a complete re-implementation of the genepop software for Windows and Linux. Molec Ecol Resour 8(1):103–106

Sandberg M, Eriksson L, Jonsson J, Sjöström M, Wold S (1998) New chemical descriptors relevant for the design of biologically active peptides. A multivariate characterization of 87 amino acids. J Med Chem 41(14):2481–2491

Savage AE, Zamudio KR (2011) MHC genotypes associate with resistance to a frog-killing fungus. Proc Natl Acad Sci U S A 108(40): 16705–16710

Savage AE, Zamudio KR (2016) Adaptive tolerance to a pathogenic fungus drives major histocompatibility complex evolution in natural amphibian populations. Proc R Soc Lond B 283(1827):20153115

Savage AE, Becker CG, Zamudio KR (2015) Linking genetic and environmental factors in amphibian disease risk. Evol Appl 8(6):560–572

Savage AE, Mulder KP, Torres T, Wells S (2017) Lost but not forgotten: MHC genotypes predict overwinter survival despite depauperate MHC diversity in a declining frog. Con Gen 19(2):309–322

Schaschl H, Aitman TJ, Vyse TJ (2009) Copy number variation in the human genome and its implication in autoimmunity. Clin Exp Immunol 156(1):12–16

Scholthof KBG (2007) The disease triangle: pathogens, the environment and society. Nat Rev Microbiol 5(2):152–156

Shum BP, Avila D, Du Pasquier L, Kasahara M, Flajnik MF (1993) Isolation of a classical MHC class I cDNA from an amphibian : evidence for only one class I locus in the Xenopus MHC. J Immunol 151(1):5376–5386

Siddle HV, Marzec J, Cheng Y, Jones M, Belov K (2010) MHC gene copy number variation in Tasmanian devils: implications for the spread of a contagious cancer. Proc R Soc Lond B 277(1690): 2001–2006

Smith KF, Acevedo-Whitehouse K, Pedersen AB (2009) The role of infectious diseases in biological conservation. Anim Cons 12(1): 1–12

Smith MD, Wertheim JO, Weaver S, Murrell B, Scheffler K, Kosakovsky Pond SL (2015) Less is more: an adaptive branch-site random effects model for efficient detection of episodic diversifying selection. Mol Biol Evol 32(5):1342–1353

Sommer S (2005) The importance of immune gene variability (MHC) in evolutionary ecology and conservation. Front Zool 2(1):16

Spielman D, Brook BW, Briscoe DA, Frankham R (2004) Does inbreeding and loss of genetic diversity decrease disease resistance? Con Gen 5(4):439–448

Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues AS, Fischman DL, Waller RW (2004) Status and trends of amphibian declines and extinctions worldwide. Science 306(5702):1783–1786

Stuglik MT, Radwan J, Babik W (2011) jMHC: software assistant for multilocus genotyping of gene families using next-generation amplicon sequencing. Mol Ecol Res 11(4):739–742

Takahata N (1990) A simple genealogical structure of strongly balanced allelic lines and trans-species evolution of polymorphism. Proc Natl Acad Sci U S A 87(7):2419–2423

Teacher AG, Garner TW, Nichols RA (2009) Evidence for directional selection at a novel major histocompatibility class I marker in wild common frogs (*Rana temporaria*) exposed to a viral pathogen (Ranavirus). PLoS One 4(2):e4616

Trowsdale J (2011) The MHC, disease and selection. Immunol Lett 137(1–2):1–8

Wake DB, Vredenburg VT (2008) Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proc Natl Acad Sci U S A 105(Suppl 1):1466–11473