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Immunogenetic diversity and transcriptomic response to fibropapillomatosis in Hawaiian green sea turtles

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ABSTRACT: Sea turtles are one taxon of high conservation concern that encounter many pathogens, but their disease ecology is understudied, hindering our ability to predict impacts of disease on population viability. Fibropapillomatosis (FP) is a neoplastic tumor-forming disease that has been documented in all sea turtle species, with an especially high prevalence in green turtles *Chelonia mydas.* Here, we use Hawaiian green turtles (honu) as a study system to examine the roles of immunogenetic diversity and transcriptional modulation in sea turtle disease responses. Specifically, we quantified gene expression profiles associated with FP and characterized host diversity of major histocompatibility complex class I (MHCI) immune loci. We found 65 genes differentially expressed in blood between clinically healthy (n = 5) and FP-afflicted turtles (n = 5) with enriched biological processes of the innate immune system, aligned with expectations of reptilian immune systems and active disease resistance. Our results also suggest a role for disease tolerance in response to FP, as evidenced by enriched biological processes related to regulation of immune and metabolic homeostasis, increase in cellular detoxification, and increased tissue repair mechanisms. Honu (n = 89) had 23 unique MHCI alleles belonging to 3 distinct functional supertypes, but none were significantly associated with FP; this could be a result of intrinsic demographic properties of the population or reflect a lesser/differing role of the reptilian adaptive immune system. Our study advances the understanding of reptilian disease response and evolutionary mechanisms underlying immunogenetic diversity, both of which are important for promoting the adaptive potential of species vulnerable to extinction.

KEY WORDS: Major histocompatibility complex · MHC · Genetics · Adaptation · Disease ecology

1. INTRODUCTION

Disease can alter ecosystem dynamics and contribute to wildlife population decline, particularly in taxa facing other anthropogenic threats (Daszak et al. 2000, Blehert et al. 2009, Schloegel et al. 2010, Tompkins et al. 2011, Cunningham et al. 2017). However, predicting the extent and magnitude of disease effects in wild populations is often difficult due to the complex interplay between regional evolutionary

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history, host–pathogen relationships, environmental co-factors, and pathogen dynamics (Vander Wal et al. 2014, Stephens et al. 2016). Further, although variation in host physiology and genetics are known to affect population persistence in the face of pathogenic agents (Beldomenico & Begon 2010, Morris et al. 2015), most disease ecology studies focus on characterization of pathogens or environmental co-factors without considering the role of the host in disease emergence and spread. Our understanding of disease dynamics is particularly limited in marine systems (Mccallum et al. 2004, Lafferty 2017) and within some vertebrate clades such as reptiles (Zimmerman 2020) that are highly impacted by anthropogenic threats known to affect disease prevalence (e.g. habitat loss and climate change; Janzen 1994, Harvell 2002, Böhm et al. 2016).

Sea turtles are highly migratory marine reptiles that are circumglobally distributed and exhibit substantial variation among the 7 extant species in life history characteristics, physiology, and threats to population sustainability (Fuentes et al. 2023). Diseases of concern for sea turtles include fibropapillomatosis (FP), ocular spirorchiidiosis, fungi-induced pneumonia, and bacterial ulcers or gastritis (Jerdy et al. 2019, Mashkour et al. 2020). FP is a neoplastic, tumor-forming disease, and while individuals can recover from the disease or survive with it, it can become fatal when tumors obstruct vision, airways, or impact organ functioning (Work et al. 2004). FP is considered epizootic and has been documented in all extant species, but has been of particular concern in green sea turtles *Chelonia mydas* (Herbst 1994, Williams et al. 1994, Jones et al. 2016), especially in Hawai'i and Florida, US populations where the disease has historically been highly prevalent (Work et al. 2004, Hirama & Ehrhart 2007). FP was first formally documented in 1938 but became the focus of scientific study largely after prevalence rapidly increased in the 1980s across multiple locations (Smith et al. 1938, Hirama & Ehrhart 2007). Prevalence has since declined for some initially impacted populations but is currently increasing in populations that previously had few documented infections (Patricio et al. 2011, Page-Karjian et al. 2015, Hargrove et al. 2016, Suárez-Domínguez 2020). There is now a large body of literature describing putative viral and environmental co-factors of FP that has advanced our understanding of how and why certain sea turtle species and populations are more susceptible or resistant to FP (Jones et al. 2016, Duffy & Martindale 2019). However, multiple aspects of FP etiology remain unclear, and the aforementioned co-factors of the disease do not fully explain its highly differential prevalence across space and time.

Incorporating host immunogenetics into studies of disease can help explain otherwise perplexing patterns of disease dynamics (Doeschl-Wilson et al. 2011, Tompkins et al. 2011). However, for sea turtle diseases, and FP in particular, the role of immunogenetics has only very recently begun to be investigated and is still poorly understood (Duffy et al. 2018, Duffy & Martindale 2019, Yetsko et al. 2021, Martin et al. 2022). Two key ways that host genetics can influence disease susceptibility and capacity for recovery are through (1) functional diversity of immune genes and (2) modulation of genes in key physiological pathways in response to pathogenic agent exposure and disease progression.

First, high levels of standing genetic variation, particularly in functional loci, can aid the potential of populations and species to evolve tolerance or resistance to pathogens (Boots et al. 2009, Mable 2019). The major histocompatibility complex (MHC) is one genomic region widely recognized for its crucial role in the vertebrate adaptive immune response; multiple selection pressures maintain high inter-genic diversity across taxa which allows for broad pathogen recognition by hosts (Kaufman 2018). There are 2 classes of MHC involved in antigen presentation pathways, but MHC class I (MHCI) is of particular interest for its role in detection of intracellular pathogens like viruses, including herpesviruses (Hewitt 2003). Studies characterizing MHC in natural populations across taxa have broadly demonstrated the importance of its genetic diversity in disease resistance, susceptibility, and overall fitness (Sommer 2005, Stervander et al. 2020). However, many gaps remain in our understanding of the reptilian immune system, particularly the adaptive immune system and genes like MHC (Zimmerman 2022).

Second, transcriptomic studies comparing diseased and healthy individuals have revealed how immune genes such as MHC — along with other key genes associated with physiological pathways — are involved in disease resistance, recovery, and health impacts in species of conservation concern (Larsen et al. 2014, Campbell et al. 2018). Transcriptomics can also provide a snapshot of whole-organism function or tissue-specific function as well as provide insight into mechanisms of tolerance and resistance in disease and cancer contexts (Rao & Ayres 2017). For example, tumor cells may migrate through blood and endothelial cells to invade tissue, and because FP tumors are highly vascularized, we might expect that circulating blood contains tumor-derived components (Haber &

Velculescu 2014). The mechanism of FP tumor development is currently unclear; therefore, characterizing the blood transcriptome in relation to FP is an essential component to understanding the etiology and establishment of internal tumor formation in conjunction with tumor-specific transcriptomics (Duffy et al. 2018, Duffy & Martindale 2019, Blackburn et al. 2021, Yetsko et al. 2021).

Multi-omic approaches to understanding immunity in wildlife species have thus far been understudied in large part due to limited genomic resources, costs of sequencing, and complexity of genomic regions like MHC; however, technological improvements and decreased costs have recently enabled high-quality genomic resources to facilitate such analyses in species of conservation concern, including sea turtles (Brandies et al. 2019, Rhie et al. 2021, Bentley et al. 2023). Characterizing immunogenetic variation and physiological pathways regulated in response to FP could therefore provide insight into the mechanisms of susceptibility and resilience to disease in sea turtles.

The Hawaiian green sea turtle (honu) population, is an evolutionarily distinct management unit inhabiting the main and Northwestern Hawaiian Islands (Dutton et al. 2008). Prior to Endangered Species Act protections, this population was heavily exploited for commercial harvest and suffered a strong decline, but has since been increasing at a nearly constant annual rate (Balazs et al. 2015, Seminoff et al. 2015, Staman et al. 2020). FP has been documented throughout the Hawaiian Islands and contributes to mortality and stranding in the region. FP prevalence is spatially and temporally variable (Work et al. 2004, Van Houtan et al. 2010), but generally has been decreasing since its peak of 50% in the 1990s to 9.4% in 2007 (Chaloupka et al. 2009, Hargrove et al. 2016). Since 2018, the percentage of stranded honu with FP listed as a primary threat was <15% (Clemans & Murakawa 2023), suggesting that current FP prevalence remains lower than during its peak but is still present in the population. This population has been extensively monitored across decades for multiple demographic metrics, and much of the pioneering work on potential environmental co-factors of FP was conducted in Hawai'i (Landsberg et al. 1999, Keller et al. 2014, Work et al. 2015, preprint doi: 10.7287/peerj.preprints.539v1, Jones et al. 2016), but there have been no immunogenetic studies to date. Thus, this well-studied population offers an ideal study system to investigate immunogenetic diversity and physiological modulation associated with FP because it can be integrated with knowledge of historical demography and environmental co-factors to advance our understanding of the disease.

Here, we add to a burgeoning body of work quantifying the role of host immunogenetics in resistance and tolerance to a disease of significant concern for sea turtles using the honu population as a study system. We first compare gene expression profiles in blood of tumor-free versus FP-affected turtles to identify the genes and biological pathways involved in response to disease, with particular emphasis on immune pathways. We then quantify inter- and intra-host diversity in the MHCI region to compare to other populations and test for allelic associations with disease susceptibility, both of which may serve as a foundation for future studies assessing adaptive potential. Collectively, this work advances our understanding of immunological mechanisms of reptiles at both genomic and transcriptomic levels and contributes to advancing the critical but understudied aspect of host genetics and physiology in disease ecology.

2. MATERIALS AND METHODS

2.1. Study system and sample collection

Blood samples (n = 10) were collected from free-ranging honu across 3 years (2012, 2013, and 2015) as part of ongoing monitoring efforts conducted by the Pacific Islands Fisheries Science Center (PIFSC) and National Institute of Standards and Technology Biorepository (NMFS permits #1581-01 and #15685). Environmental factors that may vary over space and time in natural systems can influence gene expression, so we used a matched sample design such that samples collected from turtles in different locations, years, and seasons occurred in roughly equal proportions (Supplement 1 at www.int-res.com/articles/suppl/n057p059_supp/; for all supplements). Collection sites included Kailua, located on Oahu, and Kapoho, located on the island of Hawai'i, where FP prevalence at the time of sampling in free-ranging honu was 8% and 38%, respectively (Keller et al. 2014). For gene expression analyses, we collected 2.5 ml (with the exception of Turtle21 which had slightly less than 2.5 ml) of whole blood from the dorsal cervical sinus using 21-gauge and 3.8 cm needles (Owens & Ruiz 1980) into 10 ml PAXgene™ Blood RNA tubes (Qiagen; cat# 762165) from 5 tumor-free individual turtles and 5 FP-afflicted individuals (n = 10 total). After blood collection, samples were kept at room temperature for 1 h, moved to −20°C for 24 h, and finally transferred to −80°C or liquid nitrogen storage until RNA extraction. The 5 FP-afflicted individuals had tumor scores ranging from 2 to 3, while non-tumor individuals all had

tumor scores of 0; tumors were scored as described in Work & Balazs (1999). We also recorded emaciation scores and body condition indices of individuals at the time of sampling, as described in Keller et al. (2014).

For MHCI diversity genotyping, skin biopsies (n = 90) were accessed from the PIFSC sample archive from previously collected individuals sampled at foraging and nesting grounds throughout the main and Northwestern Hawaiian Islands across 3 years (2019, 2020, and 2021; Supplement 1); individuals were confirmed to be unique based on passive integrated transponder tag number. Because not all sampled individuals in the sample archive are assessed for FP, and sampling efforts can be biased, the best estimate of FP prevalence for the population at the time of sample collection is <15% of stranded honu that had FP listed as a primary threat (Clemans & Murakawa 2023). Skin biopsies were collected in saturated salt and stored at room temperature or 2°C until DNA extraction, as described in Stewart et al. (2013). Individuals who were skin-biopsied (2019–2021) are independent of those used for blood collection.

2.2. Total RNA extraction, library preparation, and RNA sequencing from blood samples

Total RNA was extracted from blood samples according to Townsend et al. (2020) within 1–4 yr post sample collection, followed by mRNA isolation and library preparation as described in Banerjee et al. (2021). Only samples with RNA integrity number (RIN) >7.5 and high-quality RNA confirmed with qualitative visual inspection by gel electrophoresis were used for library preparation. Briefly, we used the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs), followed by library preparation using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina and the NEBNext Multiplex Oligos for Illumina (New England Biolabs) for dual indexing, followed by quality assessment and quantification using a Fragment Analyzer Standard Sensitivity RNA kit (Agilent Technologies). We sequenced a pool of individually indexed libraries from 10 individuals across 3 lanes of 150 bp paired-end reads at Novogene Corporation on an Illumina HiSeq 4000 sequencer.

2.3. Genomic DNA extraction and MHC amplicon sequencing from skin samples

Genomic DNA was extracted from skin samples of 90 individuals using an AMPure magnetic bead-based isolation (Beckman Coulter) custom protocol (https://github.com/MolEcolConsLab/Wet-Lab-Protocols), and we quantified DNA quality and concentration with a Fragment Analyzer Standard Sensitivity Large Fragment Analysis kit (Agilent Technologies). We prepared targeted amplicon sequencing libraries using a 2-step PCR protocol and primers adapted from Stiebens et al. (2013) with modification for Illumina sequencing as detailed in Martin et al. (2022). Briefly, we first amplified the MHCI exon 3 region using primers as described in Stiebens et al. (2013), and assessed amplification quality using agarose gels; only samples that produced bands of expected size (220 bp) were used for the second-step PCR. We then used unique combinations of forward and reverse custom fusion primers (Eurofins Genomics) containing Illumina adaptor sequences, 8-mer indexes differing by ≥2 bp, and the MHC amplicon primers for the second PCR step (Martin et al. 2022). Successful adaptor ligation was confirmed using agarose gels, and amplicons were pooled into 3 groups based on visual assessment of band intensity. We cleaned each pool using AMPure beads and quantified the concentration and confirmed amplicon size using the Fragment Analyzer Standard Sensitivity Large Fragment Analysis kit. We then combined the pools based on relative concentrations and sample size of each pool to approximately normalize sequence depth per sample. The final pooled library was sequenced on the Illumina MiSeq platform, with a MiSeq Reagent Nano 500 cycle kit and 2 × 176 PE chemistry at the University of Massachusetts Amherst Genomics Core Facility.

2.4. Identification of differentially expressed genes in blood

For RNA-sequencing (RNA-Seq) data, we trimmed sequences for adaptors and quality as described in Banerjee et al. (2021), mapped to the *Chelonia mydas* reference genome (Bentley et al. 2023; NCBI ID# GCF_015237465.2) using the STAR splice-aware aligner (2.7; Dobin et al. 2013), and then quantified expression at the gene level using RSEM (1.3; Li & Dewey 2011). Importantly, RSEM uses an Expectation-Maximization algorithm to assign multi-mapping reads to genes, in contrast to other methods that discard these reads; given that we expect immune genes to be important to FP and that many of these are known to be duplicated in the genome, we wanted to retain multi-mapping reads. To evaluate whether individuals were infected with chelonid alphaherpesvirus 5 (ChHV5) at the time of sampling, we used STAR

(Dobin et al. 2013) to align trimmed reads to the ChHV5 partial genome (NCBI ID# HQ878327.2) and retained alignments with minMapQ = 20 and unique mappings to the ChHV5 genome. To compare differential gene expression between tumor-free and FP-afflicted individuals, we used edgeR with GLM functionality (v.3.42.4; Robinson et al. 2010) implemented in R (v.4.0.3; R Core Team 2020). We used transcript per million (TPM) counts from RSEM as input for edgeR. Genes with <1 count per million in ≥5 samples (the smallest group size) were removed from analysis. We performed trimmed mean of M-values (TMM) normalization on counts in edgeR to account for between-sample differences and library size. Because our primary research focus was to identify differences in expression between tumor-free individuals compared to FP-afflicted individuals, we used a simple GLM model for analysis, with presence or absence of tumors as the single model variable. We used the glmQLFit function to fit the model, followed by a likelihood ratio test to identify differentially expressed genes between the 2 groups. Genes were considered differentially expressed between groups at a false discovery rate (FDR) <0.05. We visualized individual patterns of gene expression using the EnhancedVolcano (v.1.18.0; Blighe et al. 2018) and ggplot2 (v.3.5.1; Wickham 2016)) R packages. Although we used a high-quality, annotated reference genome, some genes were not annotated with gene symbols but rather a location in the genome (e.g. 'LOC102938741'). For genes identified as differentially expressed that were not annotated with a gene symbol, we manually extracted gene identifier information from the associated genome CDS file.

2.5. Identification of biological processes association with FP

As a first pass analysis, we summarized differentially expressed genes as higher-level Gene Ontology (GO) terms using GOnet and mapping to human GO terms (Pomaznoy et al. 2018). To identify GO biological processes that are significantly different between FP-afflicted and tumor-free groups, we conducted gene set enrichment analysis using a Kolmogorov-Smirnov test implemented in the topGO (v.2.52.0; Alexa & Rahnenfuhrer 2019) R package and visualized data using the GOplots (v.1.0.2; Walter et al. 2015) R package. We used all genes expressed in this dataset as the gene universe, and FDR as the gene rank. This test differs from overrepresentation tests in that it uses all expression data and allows for identification of pathways important to a condition without being limited to significantly differentially expressed genes; therefore, pathways can be enriched even when they only contain a few significantly differentially expressed genes (Subramanian et al. 2005, Maleki et al. 2020). We focused on biological process GO terms to characterize functional differences in gene expression profiles between the tumor-free and FP turtle groups. We report raw p-values for this analysis rather than corrected p-values following the recommendation of the package authors, because the observations are not independent, and thus corrected p-values may be misleading (see Supplement 4; Alexa & Rahnenfuhrer 2019). To further characterize biological processes relevant to FP, we conducted a gene set analysis using Reactome pathways in the ReactomePA R package (v.1.32; Yu & He 2016), with genes at Benjamini-Hochberg adjusted $p < 0.05$ as the genes of interest, compared to a background set of all expressed genes in the dataset, as suggested by the package developers.

2.6. MHCI variant identification

We demultiplexed and trimmed raw sequencing reads for adapter contamination and quality using the Sickle (v.1.33; Joshi & Fass 2011) and Scythe packages (v.0.994; Buffalo 2014), respectively. To identify unique MHCI sequence variants, we used the Dada2 package (v.1.28.0; Callahan et al. 2016) implemented in R (v.4.0.3). Within this package, we filtered reads with stringent maximum error rates (MaxEE = 0.1), removed primer sequences, merged reads, removed chimeric sequences, and assigned sequence variants. One sample was removed from further analysis due to most of the forward and reverse reads failing to merge, such that there were an insufficient number of reads (min read depth = 1000) to retain confidence in downstream analyses. We set additional filters to retain only those variants matching the expected amplicon length, present in ≥5% of individuals in the dataset, and meeting a per-individual minimum allele depth of 5%, which led to the removal of 10 additional individuals. (See 'Data accessibility' for details and access to all code used for analyses.)

To determine if MHCI alleles were shared between our RNA-Seq and MHCI amplicon sequencing datasets, we ran a BLAST search querying the MHCI regions from our RNA-Seq reads against the MHCI nucleotide sequences identified in our Dada2 analysis. Because the RNA-Seq reads were shorter and provided less sequencing depth for confident de novo identification of MHCI alleles, we filtered our search alleles in the RNA-Seq data by the alleles identified

in our targeted amplicon sequencing data. Briefly, we first mapped the filtered and trimmed RNA-Seq reads to the green sea turtle reference genome (rCheMyd1.pri.v2; accessed from NCBI), extracted reads matching MHCI genes, and converted bam files to fasta format using the Samtools package (v.1.14; Danecek et al. 2021). We then queried the sample-specific fasta files against the 23 MHCI nucleotide sequences using BLAST (E-value < 1×10^{-5}) and filtered results to retain hits with 100% identity and >150 bp matching between target and query sequence to ensure each sequence matched only one unique MHCI variant.

2.7. Quantification of MHCI sequence diversity

To quantify nucleotide diversity of MHCI variants within and between samples, we calculated pairwise sequence nucleotide diversity and mean sequence p-distances per sample using the MHCtools package in R and the CalcDist function (method = 'P') (v.1.5.3; Roved et al. 2022). We then performed a network analysis of the final set of MHC haplotypes using Pegas to determine the relationship of the unique sequence variants to each other (v.1.3; Paradis 2010). The haplotype network was built using an infinite site model of DNA sequences and pairwise deletion of missing data. To assess peptide sequence diversity, we identified the longest open reading frame (ORF) using TransDecoder (v.5.5.0; Haas & Papanicolaou 2018). ORF3, which contained no stop codons and resulted in a full ORF, was used for all sequences to calculate amino acid residue conservation scores using the full set of MHCI variant sequences. We obtained conservation scores for each residue using ScoreCons (model type = valdar01, otherwise default parameters; accessed June 2023), which we used to calculate the measure of diversity between sequences at each amino acid residue (Valdar 2002). The measure of diversity per residue is on a scale of 0–1, with 0 indicating that the amino acid residue is the same in all sequences, and 1 indicating difference in every sequence.

2.8. Identification of phylogenetically conserved MHCI alleles

We reconstructed a Bayesian gene tree to assess the relationships between MHCI alleles in our population as well as those identified in previous studies of MHCI in sea turtles. First, we created an alignment of the unique alleles recovered in this study (n = 11) and from studies in other populations of green and loggerhead turtles (n = 124 alleles) (Stiebens et al. 2013, Martin et al. 2022). The alignment was created in Geneious software v.9.0.5 (Kearse et al. 2012) using the Clustal Omega alignment algorithm using default parameters (Sievers et al. 2011). To find a model of evolution that best fit the nucleotide alignment, we used jmodeltest2 v.2.1.10 (Darriba et al. 2012) on the CIPRES server (Miller et al. 2010) and chose the best model according to the Akaike information criterion corrected for small sample sizes (AICc). The best model was the General Time Reversible model with a gamma distribution across sites (GTR + G). We reconstructed a Bayesian gene tree in MrBayes v.3.2.7a (Ronquist et al. 2012) on the CIPRES server with MHCI sequences from *Gallus gallus* (KF032390.1) and *Tymphanuchus cupido* (KF466478.1) as outgroups. The analysis consisted of 2 independent runs of 2×10^7 generations and 4 chains each, with the first 2×10^5 generations discarded as burn-in. Using Tracer v.1.7 (Rambaut et al. 2018), we checked that the Markov chain Monte Carlo chains converged and that the posterior distribution of both runs was adequately sampled (effective sample size of each parameter > 200). We also reconstructed a maximum likelihood tree in IQTree v.1.6.8 (Nguyen et al. 2015) with 1000 bootstrap replicates to compare to the topology of the Bayesian gene tree.

2.9. Testing for selection acting on MHCI

To determine if selection was acting on specific amino acid residues within the set of MHCI alleles present in honu, we used HyPhy (Kosakovsky Pond et al. 2020) accessed on the Datamonkey server (Weaver et al. 2018; accessed Feb 2022) for selection tests. We first used Genetic Algorithm for Recombination Detection (GARD) (Kosakovsky Pond et al. 2006) to detect any recombination breakpoints in our sequences which would inform subsequent analyses, supplying a Bayesian tree generated as described above but restricted to only the alleles recovered in the present study. We ran the following site-specific analyses to detect selection acting on individual codons: FEL (Fixed Effects Likelihood), SLAC (Single Likelihood Ancestry Counting) (Kosakovsky Pond & Frost 2005), FUBAR (Fast Unconstrained Bayesian AppRoximation) (Murrell et al. 2013), and MEME (Mixed Effects Model of Episodic Diversifying Selection) (Murrell et al. 2012). We also ran BUSTED (Branch-site Unrestricted StatisticlTest for Episodic Diversification) (Murrell et al. 2015) to test for signa-

tures of selection on branches of the phylogeny, and aBSREL (adaptive Branch-Site Random Effects Likelihood) (Smith et al. 2015) to test for branch-wide evidence of selection in our dataset. Statistical significance was inferred at alpha ⩽ 0.05 or posterior probability ≥ 0.95, as appropriate for the selection test used.

2.10. Functional characterization of MHCI alleles

In order to functionally characterize MHCI alleles, we grouped alleles into supertypes using descriptor values for physiochemical properties of amino acid residues (Sandberg et al. 1998). We generated a matrix of these values for each amino acid residue in every variant that served as input for a discriminant analysis of principal components (DAPC) using the adegenet package in R (v.2.1.10; Jombart & Ahmed 2011). We evaluated *k*-means clusters over a range of 23 values, repeating each 10 times. We then used a 2-step validation step with each value of *k* and the *xvalDAPC* function to identify the optimal number of principal components to retain for the DAPC.

2.11. Association of MHCI alleles with FP

To determine if any individual alleles or supertypes were associated with FP status, we first conducted random forest (RF) modeling to allow for simultaneous incorporation of many variables (i.e. individual alleles; Brieuc et al. 2018). The explanatory variables we used to predict FP occurrence included individual alleles, year, capture location, total number of MHC alleles per individual, and the presence of each MHC supertype. Samples that did not have a confirmed tumor score were excluded, leaving 54 individuals in the analysis. We stratified the random forests using a sample size of 10 (recommended 2/3 of the underrepresented group of FP-positive individuals) and used optimized values for the *mtry* and *ntree* parameters and a randomly separated training dataset (70% [n = 37] for test dataset; 30% [n = 17] for training dataset) to build the final model. We assessed the relative importance of each predictor based on mean decrease in accuracy and mean decrease in Gini impurity. Secondly, we conducted a relative risk ratio analysis to identify factors associated with the disease. Because relative risk ratio analysis is sensitive to small sample size (Lemeshow et al. 1988), we limited the analysis to alleles present in ≥7 individuals using a subset of individuals (n = 32); FFS samples, which all had tumor scores of 0, were excluded to balance the study design and avoid confounding location variables. We conducted the analysis in R with the epiR package (v.2.0.62; Stevenson & Sergeant 2023) using 95% confidence intervals and Bonferroni correction for multiple comparisons.

3. RESULTS

3.1. Differential gene expression between tumor-free and FP-afflicted turtles

We generated an average of 33.8 million (SD = 3.8 million) raw reads per sample and retained 33.5 million (SD = 3.8 million) reads per sample after trimming (Supplement 1). We identified a total of 65 genes (Supplement 2) that were significantly differentially expressed, including 38 genes that were downregulated and 27 that were upregulated in FP-afflicted turtles relative to the tumor-free group (Fig. 1, Fig. S1 in Supplement 3). The 10 most differentially expressed genes (Fig. 2A) included 1 known adaptive immune gene: MHC class II (MHCII; gene symbol = 'HLA-DRA'). There were multiple genes with known immune functions that were significantly differentially expressed between groups (Fig. 2B), including 2 MHCII genes, complement C3a receptor 1, chitinase 1, and interferon alpha inducible protein 27. We did not detect any ChHV5 transcripts in the samples in this study.

3.2. Key physiological pathways are enriched in FP-afflicted turtles

A total of 76 biological process GO terms were enriched in the FP-afflicted group relative to the tumor-free group (Supplement 4). At the summarized GOslim level, differentially expressed genes were most commonly associated with transport (18.5%), vesicle-mediated transport (12.3%), immune system processes (12.3%), and response to stress (12.3%; Supplement 5).

From the GO term gene set enrichment analysis , we found evidence of active immune response to disease in the tumor group. Within the immune system processes, gamma-delta T-cell activation, positive regulation of interferon-gamma production, and negative regulation of cytokine-mediated signaling were in the top 10 most significantly enriched processes (Fig. 3, Supplement 4). Innate immune processes in particular were significantly enriched, and genes associated with this process were upregulated (*Z*-score = 1.07) in the tumor group relative to the tumor-free group (e.g.

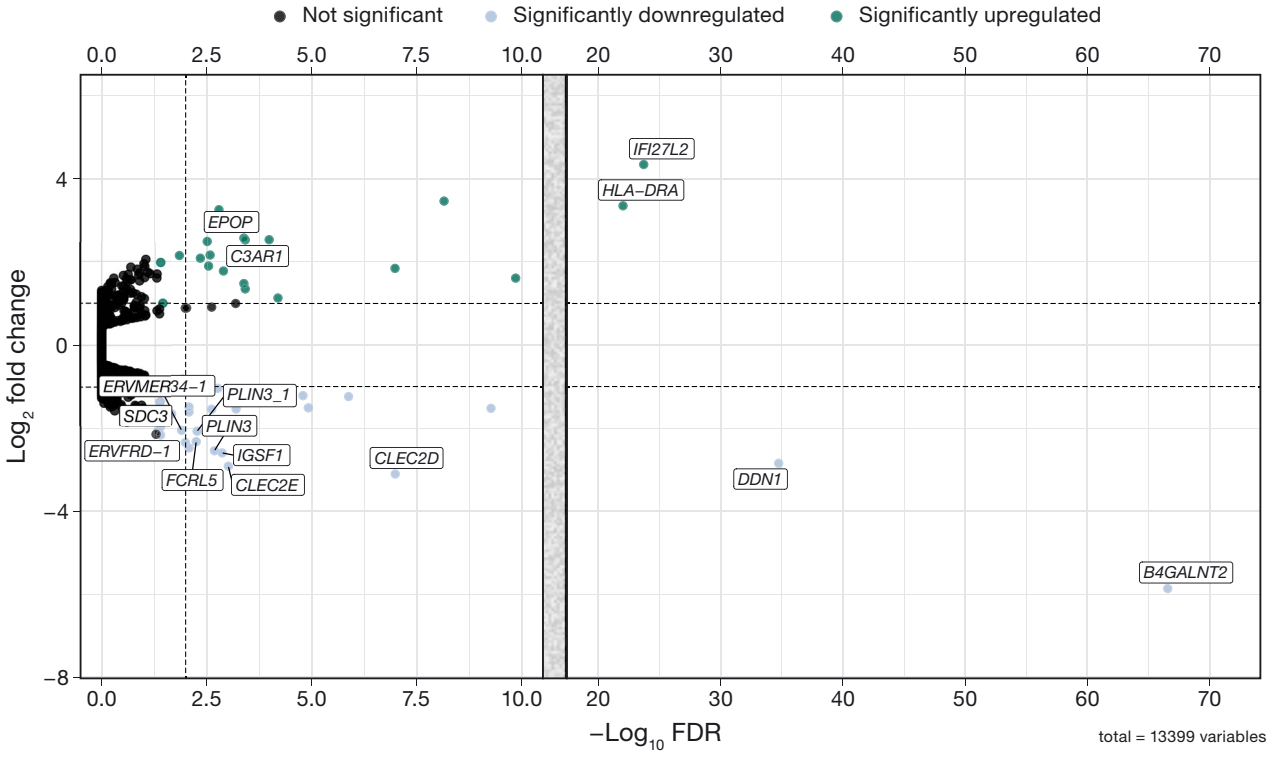


Fig. 1. Volcano plot of all differentially expressed genes. The *y*-axis indicates log fold change (logFC) in gene expression in the tumor group compared to the tumor-free group. Teal points: genes significantly differentially expressed between these 2 groups (false discovery rate [FDR] < 0.05) and with a logFC > 1. Light blue points: genes significantly differentially expressed between the 2 groups (FDR < 0.05) but with logFC < −1. Black points: genes that are not significantly differentially expressed. Labeled genes have FDR< 0.05 and absolute logFC > 2 and have a gene annotation. The grey bar in the middle of the plot indicates a breakpoint in the plot to visualize outlier genes; a version of this plot without the *x*-axis break can be found in Fig. S1 in Supplement 3

cellular response to cytokine stimulus, complement activation, negative regulation of interleukin-2 production). Inhibition of viral genome replication was significantly enriched in the tumor group, and genes related to this process were generally upregulated (*Z*-score = 1.88). Our Reactome GSEA (Supplement 6) also indicated multiple significant processes related to viral infection enriched in the tumor group. However, the adaptive immune system may be slightly suppressed overall (*Z*-score = −0.77), including significant downregulation of genes associated with gamma-delta T-cell activation (*Z*-score = −2.49).

Pathways outside of disease resistance mechanisms were also enriched, including processes related to cytoplasmic translation, cell–cell adhesion, and metabolism (Fig. 3). Xenobiotic metabolism genes (within the broader GO category of cellular detoxification) were upregulated in the tumor group relative to the tumor-free group (*Z*-score = 1.45). Biological processes for tissue development, regulation of tissue remodeling, and animal organ development were all significantly enriched and upregulated (*Z*-score > 0) in the tumor group relative to the tumor-free group, which may be indicative of active tissue repair. Finally, our results suggest decreased protein synthesis in the tumor group; cytoplasmic translation was the most significantly enriched GO biological process in the tumor group and was downregulated (*Z*-score = −3.48).

3.3. Characterization of MHCI diversity

We identified 23 unique MHCI alleles from 79 individual honu using the targeted amplicon sequencing data. Only 13 of the 23 alleles were detected in the RNA-Seq data, but given the limited sample size of 10 individuals, differences in sequencing strategy, and variability of RNA expression, we did not expect to recover all possible alleles in that dataset. With the combined datasets, individuals possessed 1–7 unique alleles (mean = 4.3; Supplement 7), and alleles Chmy02, Chmy01, and Chmy10 were the most common, occurring in ≥58% (n > 51) of individuals, while the remaining 20 alleles were each found in <30% (n < 27) of indi-

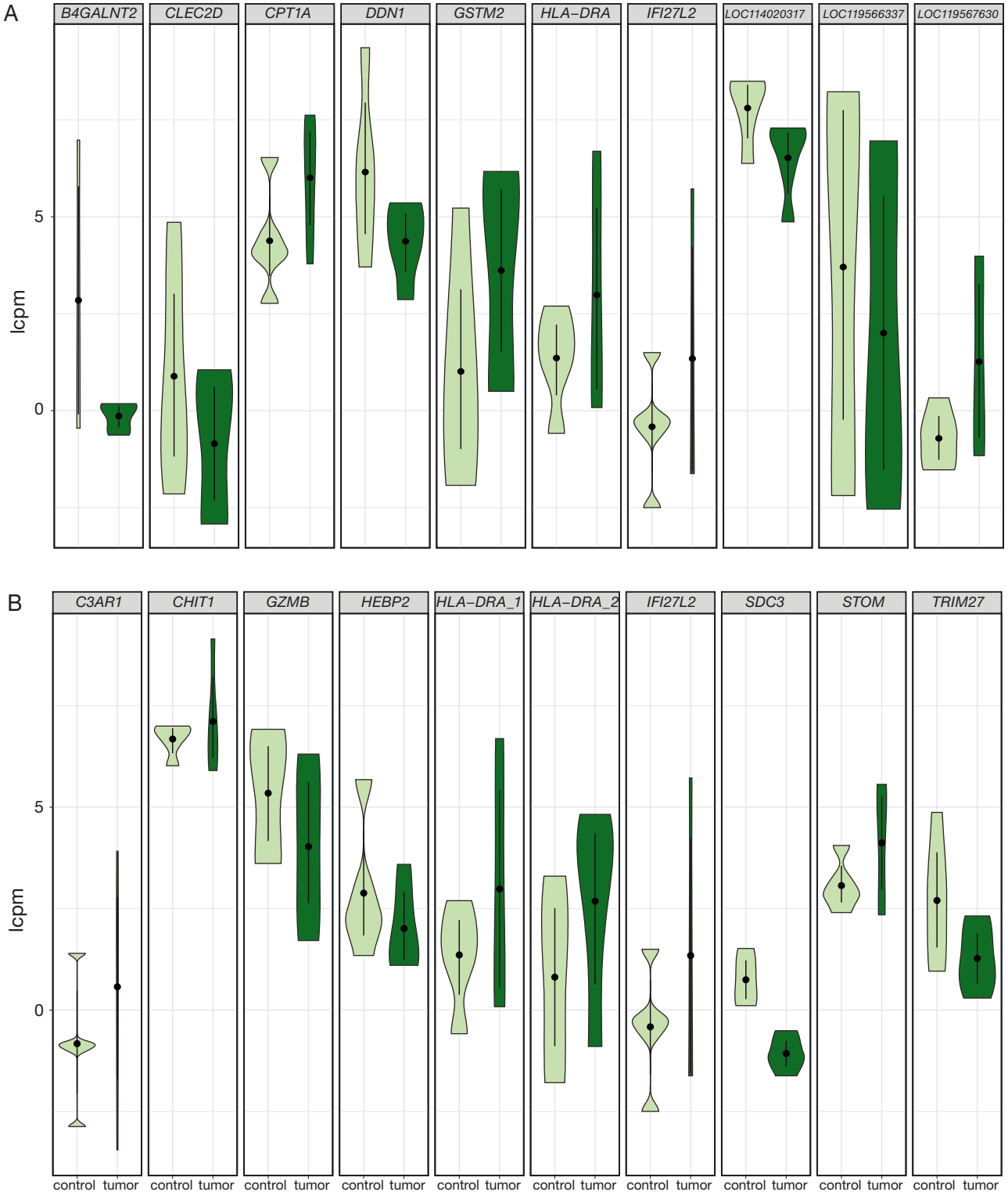
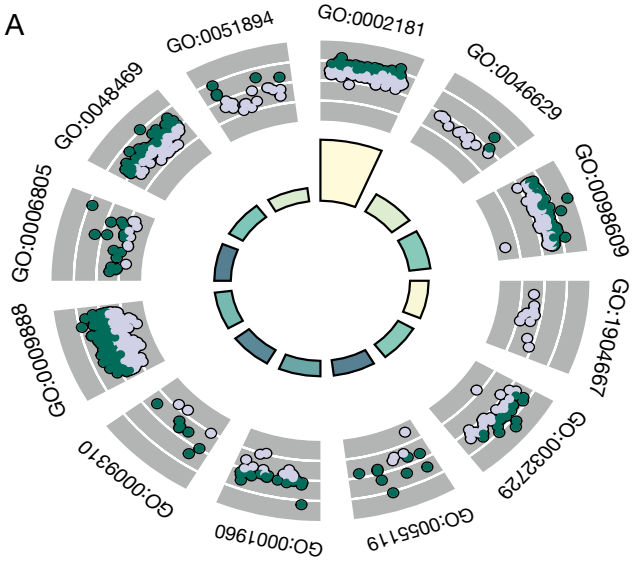
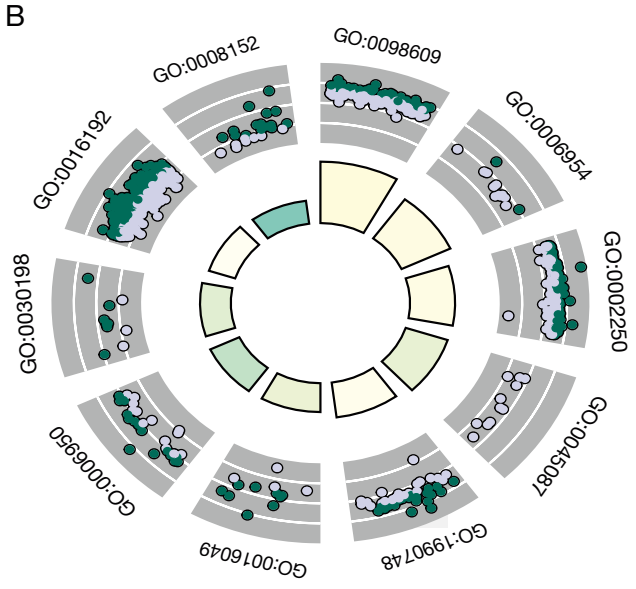
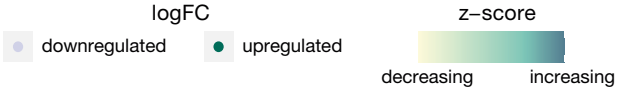


Fig. 2. Violin plots indicating average lcpm (conversion of the raw counts of a gene per individual to log-transformed count per million) of a given gene in the tumor group (dark green) and the tumor-free group (light green). The shape of the violin in each plot indicates the distribution of the data using a density curve. Gene symbol identifiers are shown in the grey bars, with additional gene information provided in Supplement 3. (A) The top 10 most significantly differentially expressed genes and (B) 10 significantly differentially expressed genes with known immune function. HLA-DRA genes include 2 differentially expressed gene copies; the second copy has appended modifier HLA-DRA_1 to distinguish between 2 copies



ID	Description
GO:0002181	cytoplasmic translation
GO:0046629	gamma-delta T cell activation
GO:0098609	cell-cell adhesion
GO:1904667	negative regulation of ubiquitin protein ligase
GO:0032729	positive regulation of interferon-gamma production
GO:0055119	relaxation of cardiac muscle
GO:0001960	negative regulation of cytokine-mediated signalling
GO:0009310	amine catabolic process
GO:0009888	tissue development
GO:0006805	xenobiotic metabolic process
GO:0048469	cell maturation
GO:0051894	positive regulation of focal adhesion assembly





ID	Description
GO:0098609	cell-cell adhesion
GO:0006954	inflammatory response
GO:0002250	adaptive immune response
GO:0045087	innate immune response
GO:1990748	cellular detoxification
GO:0016049	cell growth
GO:0006950	response to stress
GO:0030198	extracellular matrix organization
GO:0016192	vesicle-mediated transport
GO:0008152	metabolic process

Fig. 3. Circle plots representation of enriched biological process Gene Onotology (GO) terms in the fibropapillomatosis (FP) group relative to the healthy group. The outer ring of the circle plot displays scatter plots for each GO term. Green and purple dots in scatterplots represent log fold change (logFC) of individual genes associated with a GO term, with color indicating up- or downregulation in turtles with FP compared to healthy turtles. The number of genes associated with each GO term is indicated by the number of dots in the scatterplot. The inner ring of the circle plot indicates *Z*-score of the GO term, based on the overall pattern of gene logFC associated with the GO term, with increasing *Z*-score indicating a pattern of upregulation across all genes associated with the GO term. *Z*-score is calculated as the number of upregulated genes minus the number of downregulated genes divided by the square root of the count, and indicates the general trend of up- or downregulation for the genes in that GO term. Bar height of the inner circle indicates the significance of the GO term. (A) The top 12 most significantly enriched GO terms, and (B) 10 pathways of interest in disease tolerance and resistance. These terms individually are not all significantly enriched, but displayed to show patterns of gene regulation in key terms of interest in disease and cancer response

viduals (Supplement 7). We identified some MHCI alleles that were shared between very divergent populations (Hawaiian and Florida green sea turtles), as well as trans-species polymorphisms (Florida loggerhead turtles *Caretta caretta*; Fig. 4). Our phylogenetic trees supported that alleles do not cluster by species or population, though a few Hawai'i-specific alleles formed their own distinct shallow clade (Fig. 4). Twenty of the 23 sequenced MHCI alleles were functionally unique based on amino acid sequences (Supplement 7). Amino acid residues varied at each codon in our ScoreCons

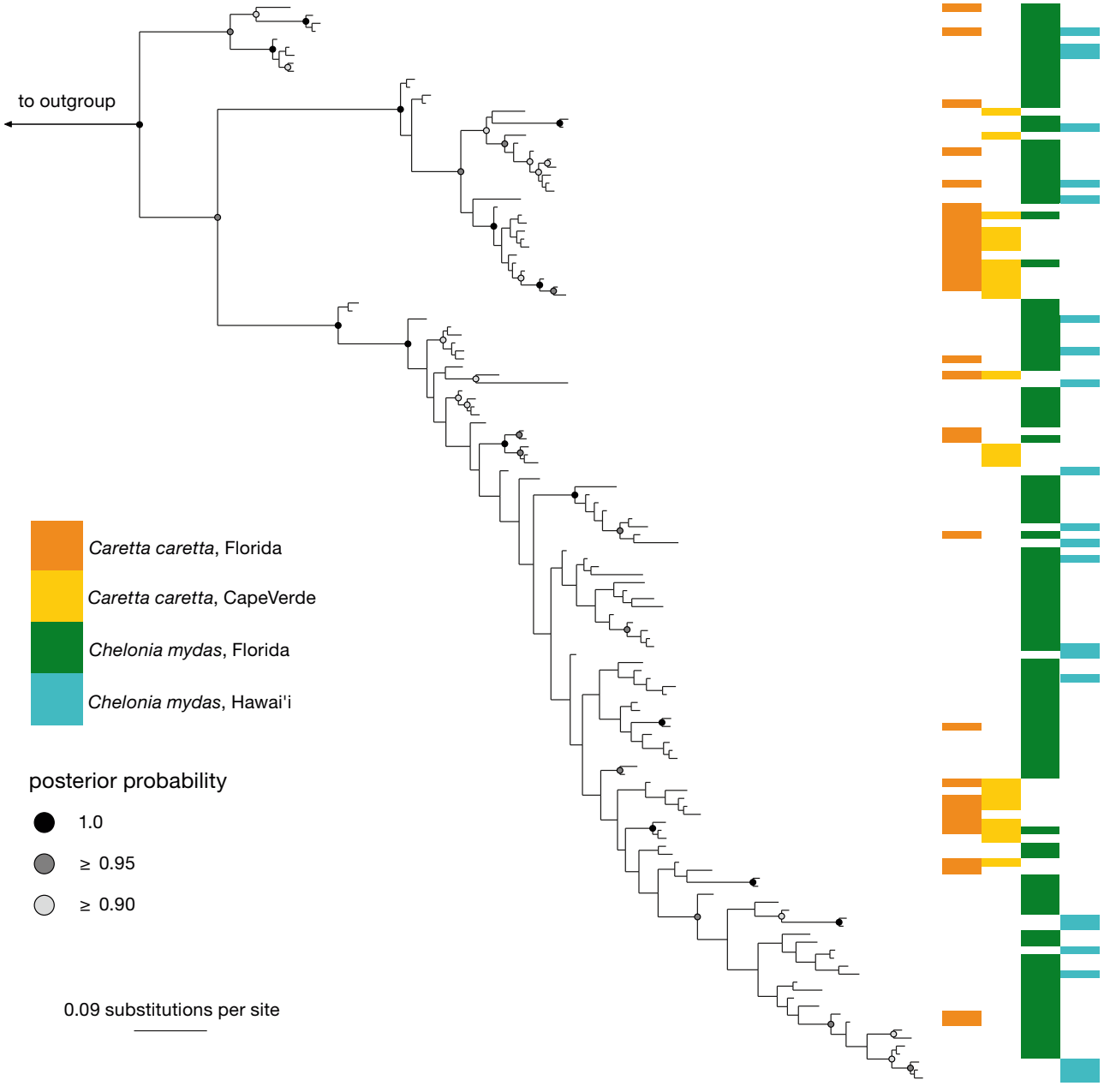


Fig. 4. Phylogenetic reconstruction of MHCI alleles including *Chelonia mydas* Florida and Hawaii populations, as well as *Caretta caretta* Florida and Cape Verde populations. Colors at the branch tips indicate which of the 4 sea turtle populations the allele was recovered in. Branch tips with multiple colors indicate an allele is found in multiple population. Branch nodes are shaded by posterior probability, with black circles indicating a posterior probability of 1.0

analysis, with only 15 (28.3%) of the 53 residues completely conserved across MHCI variants (Fig. 5A), indicating high protein diversity.

The optimal number of groups (k) was 3, with 5 principal components retained to best explain the variance in the dataset of MHCI alleles using DAPC. With these parameters, every sequence variant was assigned with >99% probability to its respective group, supporting 3 unique supertypes in the population (Fig. 5B). Supertype 3 was the most common, with 94.9% of individuals having ≥1 supertype 3 allele; 91.1% of individuals possessed ≥1 supertype 1 allele, while only 31.6% of individuals had supertype 2 alleles. Individuals generally had a heterogenous mixture of supertype alleles, most often split between supertype 1 and 3 alleles (Supplement 8).

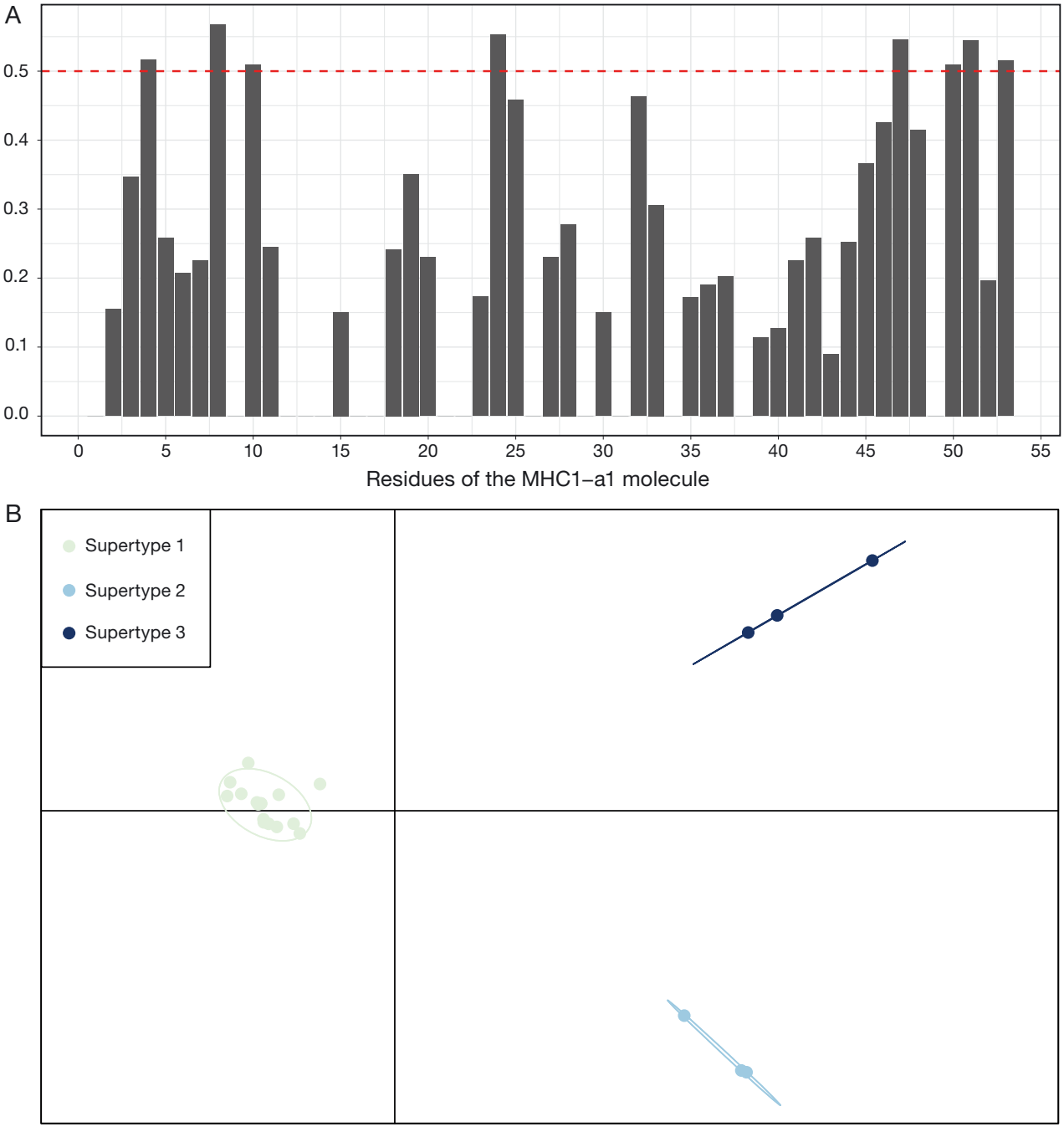


Fig. 5. (A) Levels of variation (*y*-axis) at each amino acid residue of the MHCI region from the 79 amplicon-sequenced individuals in the dataset. Variation is on a scale of 0–1, with 0 indicating that amino acid residue is exactly the same in every sequence (no diversity), and 1 indicating a residue is different in every sequence (residue not conserved at all). The *x*-axis represents the amino acid residue in the order it occurs in the sequence. (B) Supertypes of MHCI alleles. Each dot represents a single MHCI allele. The 23 alleles clustered into 3 supertypes, with optimal number of PCs retained = 5. Alleles are separated into supertype group (1–3) by color

We determined that alleles of the same supertype are also closely related evolutionarily, with 48 nucleotide mutations separating the most closely related supertype 1 and 2 alleles, and 48 mutations separating the most closely related supertype 2 and 3 alleles (Fig. 6).

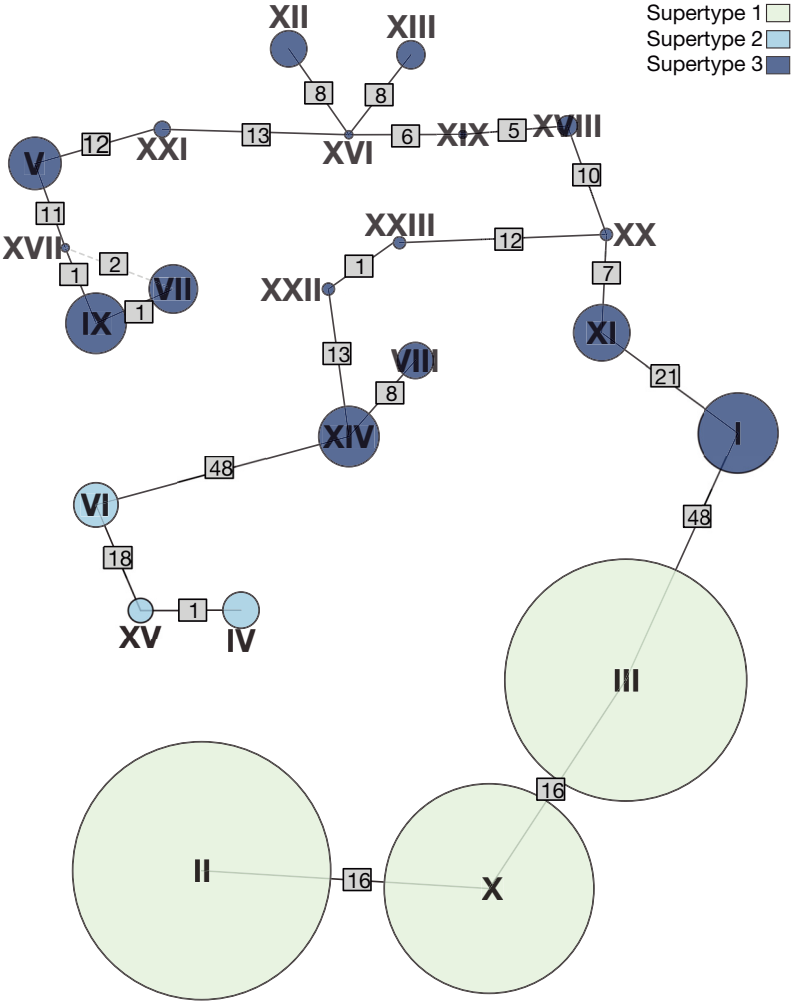


Fig. 6. Network analysis of the 23 MHCI alleles recovered in Hawai'i *Chelonia mydas*. Roman numerals correspond to unique alleles, and circle size is proportional to the frequency of that allele in the dataset (i.e. the total number of individuals possessing that allele). Numbers in grey boxes: the number of nucleotide mutations between alleles. Alleles of the same supertype are more closely related to each other

3.4. MHCI is under selection but alleles are not predictive of FP status

We detected no evidence of recombination in the MHCI alleles found in honu. Within the amino acid sequences composing our MHCI alleles, we identified 6 individual codons under selection with ≥1 site-specific test, 1 codon under selection in 3 site-specific tests (codon position 53, Supplement 9), but no codons under selection in all 4 site-specific tests. When looking at selection at the gene level rather than at individual codons, BUSTED analysis indicated evidence of episodic diversifying selection in the dataset, and aBSREL found evidence for diversifying selection in branches leading to allele Chmy17, and branches leading to node 20 (containing Chmy96 and Chmy100) and node 30 (containing Chmy97, Chmy90, Chmy103, and Chmy104) (Supplement 9).

We found no statistically significant relationships attributing individual alleles or supertypes to risk of developing FP using risk ratio analysis (Supplement 10). Our trained random forest model with 10 000 trees and mtry = 1 had an out-of-bag error rate of 29.4% and predictive accuracy of 72.9%. Location was the most important predictor in the model for FP occurrence, and the mean decrease accuracy as well as mean decrease Gini were marginal for the model (Fig. S2 in Supplement 3); therefore, none of the variables tested can be stated as clear indicators of FP status.

4. DISCUSSION

Though host physiology and genetics are well known to affect population persistence in the face of pathogenic agents, many disease ecology studies to date have focused on characterization of pathogens or environmental co-factors without considering the role of the host in disease emergence and spread. Capitalizing on recently developed genomic tools for sea turtles, our results demonstrate that green sea turtles modulate key physiological processes in response to FP, with particular emphasis on maintenance of homeostasis and innate immune response. The enriched physiological processes indicate both active immune response and regulation of homeostasis in turtles afflicted with FP. Additionally, although MHCII gene expression was significantly upregulated in FP-afflicted turtles, we did not observe any significant difference in MHCI expression or allelic association to FP. While the lack of allelic association to this disease was counter to our expectations, given that genomic diversity of MHCI in other taxa affords fitness benefits (Sommer 2005), genes involved in innate immunity and/or disease tolerance may be better predictors of health outcome in this population (Seal et al. 2021). Given that our current understand-

ing of reptile and amphibian immune function is limited, it is also possible that MHCII has a greater role than MHCI in viral response compared to mammals, consistent with recent findings for frogs (Savage et al. 2019). Finally, the lower levels of standing MHCI diversity in honu compared to other sea turtle populations could suggest low adaptive potential (Radwan et al. 2010); however, further work is needed to understand their risk and resiliency to new disease stressors. Collectively, our study provides baseline immunogenetic data for honu that may contribute to our understanding of the physiological and evolutionary processes involved in sea turtle resistance and tolerance to FP, and more broadly adds to the limited knowledge of reptilian immune systems.

4.1. Modulation of physiological processes in response to FP

In disease response, organisms can utilize mechanisms of resistance and/or tolerance; resistance entails directly reducing pathogen burden, whereas tolerance limits the negative impacts of infection without necessarily reducing pathogen load (Rao & Ayres 2017). If there is an association between FP and ChHV5, we would expect a physiological response to viral pathogens and active disease resistance, which could be indicated by differential expression of interferon and cytokine-associated genes (Pereira et al. 2023). Based on prior transcriptomic studies of FP tumors, we expected to see activation of immune system pathways in FP-afflicted turtles, particularly of leukocyte and cytokine-associated processes (Blackburn et al. 2021, Kane et al. 2021, Yetsko et al. 2021). In line with these hypotheses, we identified multiple pathways indicative of active resistance to disease in the tumor group, including significant enrichment of the inhibition of the viral genome replication process; genes related to this process are generally upregulated, suggesting a direct resistance to a viral pathogen. While we found few adaptive immune genes differentially expressed, 2 copies of MHCII were upregulated in the tumor group, which suggests at least a small role of the adaptive immune response. Innate immune processes, which are a critical first line of defense to viral pathogen infection and are robust in reptiles (Zimmerman 2020), were significantly enriched and upregulated in the tumor group relative to the tumor-free group (e.g. cellular response to cytokine stimulus, complement activation, regulation of interleukin-2 production). These processes have been implicated in viral pathogen response and disease resistance across taxa, further supporting our hypothesis of active disease resistance in FP-afflicted turtles (Pereira et al. 2023). Gamma-delta T-cell activation was one of the most highly enriched processes in our study, and genes in this pathway were strongly downregulated in the tumor group. The downregulation of these processes was surprising, as gamma-delta T cells are characterized as innate immune cells, and are important to tumor immunology (Park & Lee 2021). However, prolonged or dysregulated innate immune processes in response to pathogens or chronic inflammation can lead to exhaustion of T-cells, tissue damage, and loss of homeostasis (e.g. acute respiratory distress syndrome associated with COVID-19; Ayres 2020, Medzhitov 2021). Neutrophilia alongside additional biochemical and haematological markers of chronic inflammation have been documented in turtles with FP (Aguirre et al. 1995). It is therefore possible that the innate immune response we observed is due to a combination of active disease resistance and chronic inflammation; determining the relative contribution of each may be important in FP treatment.

Tolerance to disease may co-occur with active resistance or constitute the primary mechanism by which an organism maintains fitness when challenged with disease. Tissues such as the epidermis or epithelial tissues — which are the primary tumor sites in FP — have a high tolerance capacity due to inherently high rates of cell turnover and tissue renewal, thus pathogen tolerance may be favored (Medzhitov et al. 2012). In a scenario of tolerance, there are 4 key mechanisms by which fitness can be maintained without altering pathogen burden, including: (1) the regulation and dampening of immune response that can damage surrounding tissue; (2) an increase in detoxification processes; (3) increased tissue repair mechanisms; and (4) regulation of metabolic homeostasis (Rao & Ayres 2017). Our results suggest that all 4 of these mechanisms may be occurring in FP-afflicted turtles. First, the increased expression of innate immune genes in the tumor group described above could reflect pathogen resistance and/or underlying inflammation, but the overall dampened expression of adaptive immune genes in the tumor group may be an indicator of disease tolerance. Prior work aligns with this result in suggesting that immunosuppression occurs as a result of FP (Work et al. 2001). Next, we found some evidence of an increase in detoxification processes, including significantly enriched xenobiotic metabolism genes upregulated in the tumor group relative to the healthy group, with one significantly upregulated gene in particular — glutathione-S-transferase 1 — in-

volved in detoxification of carcinogenic and toxic compounds and associated with human cancers (Al-Achkar et al. 2017). Individuals in the tumor group increased tissue repair mechanisms; GO terms for tissue development, regulation of tissue remodeling, type 2 immune response, and animal organ development were all significantly enriched and upregulated in the tumor group relative to the tumor-free group. Finally, our results suggest an attempt to regulate homeostasis, in line with the tolerance hypothesis. First, we observed that protein synthesis is decreased in FP-afflicted turtles; translational processes are essential to maintaining homeostasis but also can become dysregulated in disease and cancer states (Hershey et al. 2012, Knight et al. 2020). Additionally, multiple pathways involved in cellular transport and protein catabolism were significantly enriched, likely reflecting alterations in pathways to maintain overall homeostasis. The innate immune genes identified in our study may also play dual roles both in disease resistance and homeostatic regulation related to disease tolerance (Medzhitov 2021). Our results suggest that FP-afflicted turtles exhibit signs of disease tolerance in addition to resistance, though experimental studies clarifying mechanisms of disease tolerance in model reptilian species would help support this claim.

While a mechanism of tolerance can be more advantageous to sustained fitness, high or overactive tolerance in cancerous disease ultimately leads to tumor promotion and decreased fitness over the long term (Rao & Ayres 2017). Tolerance mechanisms utilizing sustained tissue repair require increased cell proliferation and differentiation as well as extracellular matrix remodeling, and can result in pathologies such as tumors (Medzhitov et al. 2012). Epithelial cells — including those lining blood vessels which we might expect to detect when working with blood samples — can transition into a proliferative state when stimulated to maintain organ homeostasis, such as under conditions of chronic inflammation or disease, and can contribute to tumor development and metastasis (Lei et al. 2023). Our enrichment analysis suggests this proliferative epithelial cell state may be occurring in FP-afflicted turtles, as we identified pathway alterations in the tumor group including downregulation of cell—cell adhesion and loss of regulation of extracellular matrix, which includes bicellular tight junction assembly and focal adhesion assembly. Within these processes, carnitine palmitoyltransferase 1A and interferon alpha-inducible protein 27-like genes are significantly upregulated in the FP-afflicted group; these genes are also upregulated in multiple human cancers where they are markers of a proliferative epithelial cell state and can be expressed as oncogenes (Ullah et al. 2021, Wang et al. 2021). Upregulation of MHCII genes would typically suggest an adaptive immune response, as these genes are primarily expressed by antigen-presenting immune cells, but recent work has demonstrated that MHCII expression by endothelial-like cancer cells can promote metastasis and immune system evasion (Lei et al. 2023). Therefore, upregulation of MHCII in FP-afflicted turtles may not actually be an indicator of an active immune response, and instead may be a marker of tumor formation due to overexpressed tolerance mechanisms. Gamma-delta T-cell activation is one of the most significantly enriched processes and is downregulated in the tumor group in this study; as activated T-cells inhibit tumor growth within the tissue microenvironment, inhibition of their activation would allow for tumor progression (Jongsma et al. 2021). While there is a substantial body of work connecting sustained innate and inflammatory immune response to human cancers (Hibino et al. 2021), only recently has the concept of disease tolerance been applied to cancers in model systems (Dillman & Schneider 2015). Taken together, our results suggest fibropapilloma formation and growth may be a consequence of sustained or dysregulated disease tolerance, though additional studies are needed to confirm this hypothesis.

Though our study's constraints limit a full mechanistic understanding of the molecular and physiological processes involved in FP susceptibility and resilience, it provides key insight to generate hypotheses that can be tested in the future with larger sample sizes in both *in situ* and *ex situ* studies. Because gene expression profiles can be tissue-specific and temporally sensitive, components of the FP response are likely not captured in our dataset. It is likely that the differences in expression observed between groups are a direct response to FP rather than a reflection of overall poor health due to tumor burden (e.g. due to reduced foraging capacity), given that turtles at the time of sampling were not emaciated and body condition scores were normal (Supplement 1). Our dataset demonstrates that blood captures important components of the response that otherwise could not be studied in species of conservation concern such as sea turtles. Moreover, we identified multiple genes and pathways that may serve as biomarkers of FP via the use of minimally invasive blood sampling, including differential expression of interferon alpha-inducible protein 27 that has been previously suggested as a putative FP tumor biomarker (Yetsko et al. 2021). We also did not have temporal data available for the turtles sampled, so we could not assess physiolog-

ical signatures of disease progression or regression, which would clarify the pathways involved in FP susceptibility and recovery. Future studies that can track and sample individual turtles over time to link transcriptomic profiling and organismal responses (e.g. tumor regression and/or the onset of morbidity due to succumbing to the disease) would provide key insight into understanding the relationships between FP tolerance, resistance, and resilience. Finally, coupling gene expression data with established assays to detect inflammation would also help to disentangle the role of innate immune genes in pathogen resistance and damaging inflammation. A mechanistic understanding of the relative contributions of tolerance and resistance to FP will ultimately clarify the capacity of sea turtles to adapt to chronic stressors such as disease.

4.2. Standing immunogenetic variation in honu

Genetic diversity, particularly in functional regions like immune genes, is crucial to a species' ability to adapt to changing environments, including introduction of novel pathogens. High levels of polymorphism in the MHC gene family are documented across all vertebrate taxa, with higher MHC diversity evolving in species occupying more pathogen-rich environments (e.g. passerines; O'Connor & Westerdahl 2021). The level of MHCI allelic diversity we observed in honu is substantially lower than what has been frequently observed in avian species but is still higher than the allelic diversity found in some endangered amniotes (Miller et al. 2008, Castro-Prieto et al. 2011, Babik et al. 2012, Minias et al. 2019). MHCI allelic diversity in honu is lower than documented levels in green sea turtles in Florida and loggerhead turtles in Florida and Cape Verde (Stiebens et al. 2013, Martin et al. 2022). These differences could be due to the evolutionary and/or demographic history of these populations, as colonization and migration events can drive evolutionary patterns of immune genes (O'Connor et al. 2018). The Florida green sea turtle population includes migrants from other North Atlantic green sea turtle population segments and is roughly 3× larger than the genetically distinct honu population where immigration is rare, in line with this hypothesis. Alternatively, honu underwent a drastic population decline in the early 20th century that could have produced a bottleneck effect and reduced MHCI diversity; current neutral genetic diversity is slightly lower than other Pacific and Atlantic green sea turtle populations (Jensen et al. 2019, Roden et al. 2023). Loss of MHC variation both within and among individuals has been documented following population bottlenecks in other avian and reptilian species (Eimes et al. 2011, Elbers et al. 2017), though it can also be maintained despite declines (Minias et al. 2019, Stervander et al. 2020). The extent of polymorphism retained post-decline depends on factors like pathogen selection, bottleneck severity, and host generation time; rapid rebounds in the population size may, however, mitigate loss of diversity (Ejsmond & Radwan 2011). This suggests that for the honu population, a combination of long generation time and relatively rapid conservation intervention may have buffered against substantial loss of MHC polymorphism, though it's likely that the current level of diversity is still lower than it was pre-decline, given the extent of nesting site extirpation (Kittinger et al. 2013, Staman et al. 2023). Quantifying MHCI diversity prior to the population decline is needed to test this hypothesis, which could be possible through analysis of historical samples; alternatively, continued future sampling could indicate whether MHCI diversity is maintained regardless of fluctuations in population size.

While MHCI diversity is lower in this population relative to some others, our results suggest there are likely multiple evolutionary mechanisms maintaining or promoting allelic diversity. First, we found alleles in our population shared with loggerhead turtles. These species diverged approximately 35–46 million yr ago and have slow rates of mutation; thus, it is possible that long-term balancing selection rather than convergent evolution is maintaining shared polymorphisms (Vilaça et al. 2021). MHC alleles have also been shown to be conserved across primate lineages with similar divergence times (Azevedo et al. 2015). Given that this is a closed, genetically distinct population with no known evidence of hybridization between species, introgression is unlikely to play a role in the maintenance of these alleles (Seminoff et al. 2015). Additionally, alleles were shared between honu and Florida green sea turtles, which inhabit an entirely separate ocean basin and diverged approximately 2.83 million yr ago (Jensen et al. 2019). This suggests either long-term maintenance of these shared alleles due to balancing selection or shared selective pressures maintaining the same alleles in both populations. Phylogenetic reconstruction of allelic similarity did not reveal clear species groupings, supporting ancient balancing selection at these loci (Gao et al. 2015). Finally, aBSREL identified positive diversifying selection on 3 branches of our phylogeny, which encompassed 7 of the 23 alleles recov-

ered in honu; 5 of these were alleles found in Hawai'i but not in Florida, suggesting that local selection pressures are likely contributing to the maintenance of allelic diversity in honu.

Many studies of the MHC have posited that pathogen-mediated selection maintains the high level of allelic diversity within these genes, though clarifying the form(s) of selection underlying this maintenance is challenging (Spurgin & Richardson 2010). Given the putative association of FP with ChHV5 and long-term co-evolution of this pathogen with its sea turtle host (Herbst et al. 2004), pathogen-mediated balancing selection is a plausible explanation for the evidence of positive selection we observed acting on this gene. While we cannot state with certainty which mechanism(s) of pathogen-mediated selection could be at play in this population, we suggest that the rare-allele advantage and fluctuating selection hypotheses either alone or synergistically may explain allelic diversity. The majority of alleles identified in honu occur at low frequency, which could support either hypothesis (Spurgin & Richardson 2010). Alternatively, (1) these rare alleles may have arisen due to genetic drift following the recent population contraction and rebound over the last few decades, or (2) expression of alleles rather than genomic diversity could be more important to immunological fitness in sea turtles, as suggested in avian species (O'Connor & Westerdahl 2021). To clarify the current mechanism(s) of selection, future studies should (1) increase sample sizes and statistical power in the random forest models to identify associations with FP, as the present implementation may have been underpowered due to sample size, and (2) continue population monitoring to determine whether allele frequencies fluctuate cyclically (rare-allele advantage) or directionally (fluctuating selection). Ultimately, understanding evolutionary mechanisms underlying immune gene diversity is important, as climate change and habitat fragmentation can alter pathogen regimes, thereby affecting selection pressures and resulting in population-level effects on phenotypes (Borghans et al. 2004, Van Oosterhout et al. 2006).

Finally, if mechanisms of tolerance are utilized over resistance in response to FP, the lack of significant association between MHCI alleles and FP we observed might be expected. In a system with high disease tolerance, we should observe strong directional selection for tolerance genes rather than resistance genes like the MHC (Seal et al. 2021). ChHV5 has co-evolved with its marine turtle host over millions of years (Herbst et al. 2004), and if the virus predominantly remained in its latent state with very few periods of reactivation, it may have been evolutionarily advantageous for turtles to utilize mechanisms of disease tolerance rather than resistance (Seal et al. 2021). Additionally, recent studies have suggested that while many of the conventional adaptive immune system components (e.g. T- and B-cells, MHC) are present in reptiles, the adaptive immune system may serve as an accessory response to the broad and robust innate immune system (Zimmerman 2022). The lack of significant association between FP and MHCI alleles could therefore be due to an altogether different functional role of the adaptive immune system in reptiles resulting in a lower likelihood for selection to act on adaptive immune genes like MHC. It is also important to note when looking for allelic associations to FP that this dataset does not look at individuals longitudinally, therefore we cannot say whether an individual has had FP in the past even if it was negative at the time of sampling. Continued genetic monitoring of this population with emphasis on the MHC region alongside putative tolerance and innate immune genes we identified in our transcriptomic dataset would help clarify the mechanisms driving maintenance of MHC allelic diversity.

4.3. Anthropogenic and disease stress in honu and beyond

Understanding whether and how sea turtles can respond to disease is increasingly important, considering the multitude of threats to their survival (Fuentes et al. 2023), which can increase the likelihood of disease being a co-driver of extinction (Heard et al. 2013). Temperature and climate variability will likely further alter the pathogen—host landscape in nuanced ways, possibly increasing rates of disease transmission and altering host immune response (Lafferty 2017, Rohr & Cohen 2020). Maintaining or increasing genome-wide and functional genetic diversity (e.g. MHC genes) is therefore crucial for the adaptive potential of wildlife populations (Funk et al. 2018); indeed, a recent meta-analysis found that across taxa, high population genetic diversity reduced parasite impacts (Ekroth et al. 2019). FP prevalence in honu has been declining in recent years (Clemans & Murakawa 2023), which could result from a variety of mechanisms, including positive selection on immunogenetic loci leading to resilience. However, ongoing monitoring of immunogenetic diversity continues to be necessary to determine the cause of FP decline, and more broadly, the resilience of this population to emerging stressors. Honu are a case study in successful population recovery following

concerted multi-agency conservation efforts, but genetic diversity may still be lost following declines, even as population size rebounds. While the historical nesting distribution for honu spanned the entire Hawaiian archipelago, recent studies have found that >80% of historical nesting locations have been extirpated in this region and >90% of the current population utilizes just one low-lying atoll (Kittinger et al. 2013). This atoll is already at risk of degradation or loss due to climate change-driven sea level rise and increasing sand temperature (Baker et al. 2020, Staman et al. 2020, 2023), leaving the population vulnerable to impacts of other stressors. Continued monitoring of diversity and expression patterns in putative resistance and tolerance genes would help determine the adaptive capacity of honu confronted with disease stress.

5. CONCLUSION

Moving toward a mechanistic understanding of FP is important, given the increasing prevalence of FP globally and the multitude of threats to sea turtle survival (Dujon et al. 2021, Whilde et al. bioRxiv 2024.06.06.597728; doi: https://doi.org/10.1101/2024.06.06.597728). There is already a robust body of work pertaining to FP in honu, and therefore integrating immunogenetic data into this framework has the potential to advance our knowledge of FP susceptibility and resilience in Hawai'i and globally. To this aim, we identified biological pathways and individual genes associated with response to FP in blood of honu. These results suggest that mechanisms of both disease tolerance and resistance may be contributing to disease phenotype, and that the innate immune system plays a significant role in the response to FP. We also described the first baseline diversity metrics for a functional immune gene, MHCI, in this population, finding overall low levels of genomic diversity in contrast to other sea turtle populations and taxonomic groups. While we did not find a clear association between genomic diversity in this adaptive immune gene and FP presence, future work should focus on expanding the dataset to include more individuals with FP, as well as sequencing of additional genes associated with disease tolerance and resistance. Only recently have researchers considered the framework of tolerance and resistance in wildlife disease, and few have explored the ramifications of such a framework in the case of tumor-forming disease; utilizing such a framework may provide novel insights into disease etiology and treatments and should be explored further. Finally, reptilian immune systems are relatively understudied compared to other taxa; thus, these findings contribute to a deeper understanding of physiological and evolutionary response to disease and the nuanced roles of innate and adaptive immunity.

Data accessibility. Scripts and input files for all analyses are available at https://github.com/jlastoll/Immunogenetics-of-FP-2024. RNA-sequencing reads are available under SRA Accession no. PRJNA660024. Targeted amplicon sequencing reads are available under SRA Accession no. PRJNA1146005.

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