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Immune genotypes, immune responses, and survival in a wild bird population

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Abstract

Individuals vary in their immune genotype, inbreeding coefficient *f*, immune responses, survival to adulthood, and adult longevity. However, whether immune genes predict survival or longevity, whether such relationships are mediated through immune responses, and how *f* affects immune genotype remain unclear. We use a wild song sparrow (*Melospiza melodia*) population in which survival to adulthood, adult longevity, and *f* were measured precisely, and in which immune responses have previously been assessed. We investigate four toll-like receptor (TLR) and the major histocompatibility complex (MHC) class IIB exon 2 genes. We test whether immune genes predict fitness (survival to adulthood or adult longevity); whether immune genes predict immune response; whether immune response predicts fitness and whether fitness, immune responses, or immune genotypes are correlated with *f*. We find that survival to adulthood is not associated with immune gene variation, but adult longevity is decreased by high MHC allele diversity (especially in birds that were relatively outbred), and by the presence of a specific MHC supertype. Immune responses were affected by specific immune genotypes. Survival to adulthood and adult longevity were not predicted by immune response, implying caution in the use of immune response as a predictor for fitness. We also found no relationship between *f* and immune genotype. This finding indicates that immune gene associations with longevity and immune response are not artefacts of *f*, and suggests that pathogen-mediated selection at functional loci can slow the loss of genetic variation arising from genetic drift and small population size.

KEYWORDS
immune response, immunoecology, MHC class II B exon 2, PHA, toll-like receptor

1 | INTRODUCTION

Immune systems vary greatly among classes, populations, and individuals, and influence multiple fitness components (Kamiya et al., 2014; Liston et al., 2021; Spielman et al., 2004). Quantifying among-individual variation in immune status typically involves examining immune gene sequence variation or assays of immune response (e.g., degree of inflammation or antibody titre; Viney et al., 2005). These metrics are widely linked to variation in individual fitness and population viability, but are also vulnerable to inbreeding depression, and it can be difficult to separate the effects of heterozygosity at candidate genes from those of genome-wide heterozygosity (Grueber et al., 2017; Norris & Evans, 2000; O'Connor et al., 2019; Reid et al., 2007). In addition, precisely estimating individual fitness, immune function, and inbreeding coefficient (*f*) for a representative fraction of individuals in wild populations is extremely challenging. We overcame these challenges through use of a wild passerine population in which individual fitness and *f* have been precisely measured, and individuals have been sampled for immune response. Here, we conduct a comprehensive assessment of empirical relationships between individual variation in immune response, immune gene variation, survival to adulthood, and adult longevity while controlling statistically for the potential effects of inbreeding on immune status and fitness.

The toll-like receptor (TLR) and the major histocompatibility complex (MHC) gene families are essential components of the vertebrate immune system. Toll-like receptors are membrane-bound proteins that recognize pathogen-associated molecular patterns (PAMPs), evolutionarily conserved structures present in all major micro-organism classes; different TLR genes encode proteins that recognize different PAMPs (Beutler, 2004; Downing et al., 2010). Given recognition of a pathogen, TLRs activate an acute inflammatory response that is the first line of innate host defence (Netea et al., 2012) and thereby play an essential and conserved role in the immune systems of vertebrate and invertebrate animals (reviewed in Nie et al., 2018). MHC genes comprise a highly polymorphic component of the immune system of jawed vertebrates (Janeway et al., 2001) that encode molecules that bind pathogen-derived antigens and present them to T lymphocytes to initiate specific adaptive immune responses (Klein & Figueroa, 1986). Two structurally and functionally distinct MHC gene subgroups, class I and class II, present antigens from pathogens that are located intracellularly and extracellularly, respectively (Minias et al., 2018), and thus play an essential role in the adaptive immunity of vertebrates.

Greater variability in immune gene sequences is thought to confer better resistance to parasites (Radwan et al., 2020). The coevolution of hosts and parasites has likely been fundamental in shaping MHC polymorphism; hosts may benefit via rare MHC alleles (negative frequency-dependent selection), the largest number of MHC alleles ("heterozygote advantage"), or the optimal number of MHC alleles ("optimality hypothesis") (Acevedo-Whitehouse & Cunningham, 2006; Doherty & Zinkernagel, 1975; Nowak et al., 1992; Takahata & Nei, 1990; Wegner, 2003). Support for both negative frequency-dependent selection and heterozygote advantage has been found in various populations of mammals, fish, and birds (reviewed in (Radwan et al., 2020)). Investigations of individual MHC parameters and survival in wild populations of passerine birds have so far revealed that specific MHC alleles and/or higher individual MHC diversity can improve survival (Table 1). Avian TLR genes were characterized relatively recently (Alcaide & Edwards, 2011); avian TLRs experience mainly purifying selection to conserve sequence identity, though some amino acid residues experience positive selection (e.g., Grueber et al., 2014; Nelson-Flower et al., 2018). Correlations of TLR variation or specific TLR alleles with survival have been investigated in only a few wild avian populations, and there is little consensus regarding effects on survival (Table 1; Minias & Vinkler, 2022).

In addition to immune genes themselves, the overall functioning of an individual's immune system or immune response can also affect fitness (Møller & Saino, 2004). Individual variation in immune response is often characterized by measuring the outcome of a standardized "challenge" to a novel antigen; the use of such novel antigens allows evaluation of individual de novo responses and not those caused by a previous infection (Norris & Evans, 2000; Viney et al., 2005). For example, Reid et al. (2007) inoculated wild sparrows with inactivated tetanus toxoid antigen to measure primary antibody titres indicative of the humoral acquired immune response. A second type of immune challenge uses swelling at the injection site of the lectin phytohemagglutinin (PHA) (Norris & Evans, 2000; Reid et al., 2003) to estimate sensitivity in the innate immune system's inflammatory response, and potentially the T cell response, a major component of adaptive cell-mediated immunity (Strandin et al., 2018; Viney et al., 2005; Vinkler et al., 2014). These tests can be done in the field and represent a standardized assay of immune response, something that can be difficult to achieve in wild populations (Norris & Evans, 2000).

Variation in immune genes, individual responses to immune challenge, and connections between these are widely reported in wild populations (Gaigher et al., 2019, Table 2). However, the exact mechanistic role of immune genotypes (TLR heterozygosity, MHC allele diversity, and specific alleles in both MHC and TLRs) in immune responses remains unclear. For example, while PHA is a T lymphocyte mitogen, and historically PHA response was thought to indicate T-cell activation and proliferation (Strandin et al., 2018), timescales of typical PHA response assays do not allow for extensive T cell proliferation (Rekdal et al., 2021). PHA responses may represent a general increase in cytokine expression, inflammation and both innate and adaptive immune cell recruitment to the injection site (Martin II et al., 2006; Vinkler et al., 2014). Overall, connections between MHC and TLR genotypes and PHA responses are likely to be mechanistically indirect. Similarly, there is no direct link between TLR activation and humoral acquired immunity (antibody response), though most activated TLRs produce inflammatory cytokines that provide overall stimulus to the immune system (Nie et al., 2018). On the other hand, MHC is more directly involved in the antibody response

TABLE 1 A recent sample of studies investigating associations between immune genotype (MHC class I exon 3, MHC class II B exon 2, and/or TLR genes) and survival in wild bird populations.

Species	Loci	Predictor tested	What affects survival?	References
Great tits (*Parus major*)	MHC class I	MHC supertypes, MHC diversity	A specific MHC I supertype increased survival	Sepil et al., 2013
House sparrows (*Passer domesticus*)	MHC class I	MHC alleles, MHC diversity	A specific MHC I supertype decreased survival	Lukasch, Westerdahl, Strandh, Knauer, et al., 2017
Common yellowthroats (*Geothlypis trichas*)	MHC class I, MHC class II	MHC I and II alleles, MHC I and II diversity	MHC class II diversity improved survival; A specific MHC class I allele decreased survival	Dunn et al., 2013
Seychelles warblers (*Acrocephalus sechellensis*)	MHC class I	MHC alleles, MHC diversity	Both MHC diversity and an MHC allele increased survival	Brouwer et al., 2010
Seychelles warblers (*Acrocephalus sechellensis*)	TLR3	Specific TLR3 alleles	A specific TLR3 allele decreased survival	Davies et al., 2021
Attwater's prairie-chicken (*Tympanuchus cupido attwateri*)	MHC class I, MHC class II, TLR1LB, TLR15	MHC I and II alleles, MHC heterozygosity, TLR alleles, TLR heterozygosity	Specific TLR1LB allele decreased survival, a specific MHC class II allele increased survival	Bateson et al., 2016
Paleheaded brushfinch (*Atlapetes pallidiceps*)	TLR1LA, TLR1LB, TLR4, TLR5, TLR21	TLR heterozygosity	TLR heterozygosity decreased survival	Hartmann et al., 2014
Stewart Island robin (*Petroica australis rakiura*)	TLR1LA, TLR1LB, TLR2B, TLR4, TLR5, TLR15, TLR21	TLR heterozygosity, specific TLR alleles	A specific TLR allele increased survival	Grueber et al., 2013

TABLE 2 A recent sample of studies examining the association between immune genotype (MHC class I exon 3, MHC class II B exon 2, and/or MHC class II DRB) and immune responsiveness in wild animal populations.

Species	Locus	Predictor	Outcome	References
House sparrows (*Passer domesticus*)	MHC class I	MHC alleles, MHC diversity	Antibody responses increased with specific MHC alleles; PHA primary response (swelling after first injection) not affected	Bonneaud et al., 2005, 2009; Lukasch, Westerdahl, Strandh, Knauer, et al., 2017
Bluethroat nestlings (*Luscinia svecica*)	MHC class II	MHC supertypes	An intermediate number of supertypes predicted maximal PHA response	Rekdal et al., 2021
Lesser kestrel nestlings (*Falco naumanni*)	MHC class II	MHC heterozygosity	Did not affect PHA response	Rodríguez et al., 2014
Montane water vole (*Arvicola scherman*)	MHC class II	MHC heterozygosity, MHC genetic distance	PHA responses increased with specific MHC alleles	Charbonnel et al., 2010
California sea lion (*Zalophus californianus*)	MHC class II	MHC diversity, MHC alleles	PHA responses decreased with specific MHC alleles	Montano-Frías et al., 2016
Talas tuco-tuco (*Ctenomys talarum*)	MHC class II	MHC heterozygosity, MHC alleles	Antibody responses increased with specific MHC alleles	Cutrera et al., 2011

via its activation of B-lymphocytes which go on to produce antibodies (Janeway et al., 2001).

Immune responsiveness is costly and is expected to affect fitness, including survival (reviewed by Møller & Saino, 2004; Norris & Evans, 2000). Few studies have tested whether adult PHA response predicts survival using reliable estimates of adult longevity in wild bird populations. Adult PHA response is positively correlated with survival during temporary captivity in wild adult house sparrows (Gonzalez et al., 1999) and adult male zebra finches (*Taeniopygia guttata*; Birkhead et al., 1999). The avian PHA response is easily studied in nestlings, but nestling PHA response reflects not only individual immune response but also maternal effects and the early environment (Reid et al., 2003). Humoral immunity assays (antibody titres) predict survival to the next breeding season in some wild populations such as barn swallows (*Hirundo rustica*; Saino et al., 1997). However, the relationship between antibody titres and fitness may not be linear; intermediate antibody levels may be optimal, because mounting an immune response is thought to be costly (reviewed in Graham et al., 2011). For example, in blue tits (*Parus caerulus*), individuals with very high or very low primary antibody responses to diphtheria toxoid returned to the breeding site at lower rates the following year, suggesting that these individuals experienced reduced survival compared to "intermediately responsive" individuals (Råberg & Stjernman, 2003).

Most studies investigating immune gene diversity and fitness in wild populations are constrained by the inability to disentangle effects of heterozygosity at candidate genes from those of genome-wide heterozygosity. Individual inbreeding coefficient (*f*) reflects relatedness between an individual's genetic parents, can been estimated from a long-term genetic pedigree, and has been shown in song sparrows (*Melospiza melodia*) to reflect heterozygosity at neutral genetic markers (Keller, 1998; Nietlisbach et al., 2017; Reid et al., 2014). In song sparrows, individuals with higher *f* are less likely to survive to adulthood, though adult longevity is not affected by *f* (Keller, 1998; Nietlisbach et al., 2017; Reid et al., 2014; Taylor et al., 2010; Wolak et al., 2018). High-*f* individuals also show reduced PHA response and reduced tetanus antibody titres (Reid et al., 2003, 2007). While *f* is not associated with TLR heterozygosity (Nelson-Flower et al., 2018), it remains unclear whether *f* is associated with MHC diversity.

We investigate the potential links between immune gene variability, immune response, and survival in a wild bird population, the song sparrows of Mandarte Island. The immune genes considered were TLR1LB, TLR3, TLR4, TLR15 and MHC class II B exon 2. We use data on individual demography, immune genotype, and response to experimental immune challenges to test: (a) whether TLR or MHC genotype or variation predict survival to adulthood or adult longevity. We then test whether any such relationships can be explained because (b) TLR or MHC genotype or variation predicts immune response and/or (c) immune response predicts longevity. We also examine (d) whether survival to adulthood, adult longevity, immune responses, and TLR or MHC genotypes are correlated with *f*.

2 | MATERIALS AND METHODS

2.1 | Study system

Mandarte Island, BC, Canada, (~6 ha; 48.6335° N, 123.2871° W) hosts a nonmigratory song sparrow population (4–71 pairs) that has been monitored from 1975 to the present (Smith et al., 2006). Briefly, all individuals are colour-banded soon after hatch (3–8 days of age) or after arriving as adult immigrants. Since 1993, all individuals have been genotyped at 13 highly polymorphic microsatellite loci, facilitating construction of a precise genetic pedigree from which lifetime reproductive success and *f* are estimated with high precision (Nietlisbach et al., 2015, 2017; Reid et al., 2021; Sardell et al., 2010).

Immigrants to Mandarte (~1 per year on average) are assumed to be unrelated to existing residents, and their offspring are assigned $f = 0$ (Dickel et al., 2021; Germain et al., 2016; Wilson & Arcese, 2008). Emigration appears to be rare; an annual re-sighting probability of ≥0.99 ensures that local survival is assessed precisely based on annual censuses conducted in late April (Wilson et al., 2007; Wilson & Arcese, 2008). This socially monogamous but genetically polygynandrous (~28% of chicks have extra-pair sires) population is moderately inbred with mean f~0.06 (range in our data set: 0–0.31); f of 0.06 is equivalent to being an offspring of a mating between first cousins (Keller & Arcese, 1998; Reid et al., 2014; Sardell et al., 2010; Smith et al., 2006). In Mandarte Island song sparrows, variation in f has wide-ranging fitness consequences, with links to survival to adulthood, hatching success, and reproductive output (Keller, 1998; Nietlisbach et al., 2017; Reid et al., 2014; Taylor et al., 2010), particularly in challenging environmental conditions (Marr et al., 2006).

We genotyped MHC and TLR genes in individuals for whom immune response was previously measured experimentally (tetanus toxoid antibody response and PHA inflammation response; Reid et al., 2003, 2007). However, many of these individuals were adults when tested for immune response, and the majority (~73%) of song sparrows that reach independence in this population on Mandarte do not survive to adulthood (Reid et al., 2021; Smith et al., 2006; Wolak et al., 2018). To minimize bias, we first genotyped all individuals that were measured as adults or juveniles for immune response, then genotyped one additional broodmate at random for comparison, regardless of survival. A greater number of individuals were genotyped at TLR than at MHC due to budgetary constraints involved in MHC genotyping. Overall, we genotyped 376 individuals at TLR loci, and 156 individuals at MHC class II B exon 2 (Table 3).

2.2 | Genetic analysis of toll-like receptor (TLR) loci

We previously sequenced TLR1LB, TLR3, TLR4, and TLR15 in 32 Mandarte Island song sparrows and thereby identified 19 nonsynonymous single nucleotide polymorphisms (SNPs) (Nelson-Flower et al., 2018). We chose the 11 most common nonsynonymous SNPs for further analysis. For each, we designed Custom TaqMan SNP Genotyping Assays using the Custom TaqMan Assay Design Tool (Applied Biosystems; see Table S1 in the Supporting Information for context sequences used to design custom assays). DNA was extracted from 376 avian blood samples following Sardell et al. (2010); 10 ng of DNA was aliquoted per well in 384 well plates and allowed to dry. We performed the qPCR in a total volume of 5 μL which included 0.25 μL of Custom TaqMan SNP Genotyping Assay, 2.5 μL of Master Mix and 2.25 μL of dH_2O per well (Applied Biosystems). qPCR was performed using the ViiA 7 Real-Time PCR System (ThermoFisher Scientific) with an initial step of 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. qPCR results were analysed using the TaqMan Genotyper Software (Applied Biosystems), allowing heterozygotes and homozygotes for each SNP to be determined. TLR heterozygosity (H_{TLR}) per individual was calculated by dividing the number of nonsynonymous heterozygous TLR SNPs by the total number of nonsynonymous TLR SNPs. We identified the alleles present in each individual for each of the TLR genes via phasing in DNAsp version 5.10.01 (Librado & Rozas, 2009) using 10,000 iterations, with a thinning interval of 100 and 10,000 burnin iterations. This process resulted in a total of 17 alleles across four genes: three for TLR1LB, four for TLR3, three for TLR4 and seven for TLR15. TLR alleles TLR1LB_2 and TLR15_2 were removed from analyses testing the effects of specific alleles as these alleles were invariable in this data set.

2.3 | Genetic analysis of major histocompatibility complex (MHC class II B exon 2)

In passerine birds, gene duplication and conversion has increased MHC copy number; next-generation sequencing techniques can allow researchers to correctly determine the number of MHC alleles present per individual (Zagalska-Neubauer et al., 2010). We

TABLE 3 Details of models designed to test for an effect of immune gene variability on survival. Div_{MHC}, MHC allele diversity; Div_{MHC}^2, quadratic term for MHC allele diversity; f, individual inbreeding coefficient; H_{TLR}, individual TLR heterozygosity; H_{TLR}^2, quadratic term for individual TLR heterozygosity.

Sample	All individuals genotyped for TLR (N = 376 from 227 nests over 17 years)	Individuals genotyped for TLR that lived at minimum 1 year (N = 144 from 122 nests over 17 years)	All individuals genotyped for MHC (N = 156 from 103 nests over 9 years)	Individuals genotyped for MHC that lived at minimum 1 year (N = 63 from 56 nests over 9 years)
Response variable	Survival to adulthood	Adult longevity (years)	Survival to adulthood	Adult longevity (years)
Model	1A	2A	3A	4A
explanatory variables	f, H_{TLR}, f x H_{TLR}, H_{TLR}^2, f x H_{TLR}^2	f, H_{TLR}, f x H_{TLR}, H_{TLR}^2, f x H_{TLR}^2	f, Div_{MHC}, f x Div_{MHC}, Div_{MHC}^2, f x Div_{MHC}^2	f, Div_{MHC}, f x Div_{MHC}, Div_{MHC}^2, f x Div_{MHC}^2
Model	1B	2B	3B	4B
explanatory variables	f, 15 TLR alleles	f, 15 TLR alleles	f, 9 MHC supertypes	f, 9 MHC supertypes

performed PCR to amplify MHC class II B exon 2 from 156 individuals. We used a degenerate forward primer SospMHCint1f (5'-AGY GGG GAY CCG GGG TGG-3′) (Slade et al., 2016) and the reverse primer Int2r.1 (5'-CCG AGG GGA CAC GCT CT-3′ (Edwards et al., 1998)) to bind within introns 1 and 2, respectively. Each primer included an adaptor sequence for the Illumina MiSeq platform, four wobble bases, and a unique barcode of eight bases. This barcode allowed us to pool our library and assign the recovered sequences to individuals.

We performed PCR in a total volume of 35 μL containing 12.5 μL of GoTaq Hot Start Green Master Mix (Promega), 0.2 μM of each primer, and 10–60 ng of genomic DNA. The thermocycling profile consisted of 3 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 62°C, and 45 s at 72°C, and a final extension step of 10 min at 72°C. We confirmed amplification (expected product size = 480–500 bp) by agarose gel electrophoresis. Negative controls were included, and showed no amplification. We then pooled products into a library that was sent for next-generation sequencing using 300 bp paired-end reads (265 cycles) on an Illumina Miseq at the London Regional Genomics Centre. After sequencing, we used a custom pipeline (Gloor et al., 2010) to assign sequences to individuals and collapse sequences into clusters of identical reads. Based on prior cloning work in this system to identify the frequency of sequences probaby due to PCR errors (e.g., chimeras), we established a stringent threshold error rate of 1% (Grieves et al., 2019; Slade et al., 2016). Sequences comprising less than 1% of an individual's reads were thus considered to have resulted from PCR and/or sequencing errors and were discarded (mean ± SE retained reads per individual = 38,447 ± 830). Using a custom R script (Supporting Information), we aligned nucleotide sequences and trimmed intron sequences based on comparison to other song sparrow sequences in GenBank. Sequences with premature stop codons or frameshift mutations were removed and excluded from further analysis. Trimming resulted in alleles of 86 amino acids, corresponding to the entire putative second exon. Sequences were queried against our database (unpublished data) and using the Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990) in GenBank, and were either assigned to a pre-existing allele or named as a new allele, resulting in the identification of 126 MHC class IIB alleles. Sequences were deposited in GenBank (72 previously reported sequences: accession numbers can be found in the Supporting Information; 54 new sequences: accession numbers pending). The number of MHC alleles per individual was 14.61 (± 0.51 SEM; range: 1–36); previous studies in other song sparrow populations found 18.47 ± 0.41 (Slade et al., 2016) and 15.5 ± 0.5 amino acid alleles per individual (Grieves et al., 2019). Div_{MHC} was found by summing the number of MHC alleles for each individual.

Large numbers of MHC alleles and small sample sizes can limit the utility of statistical approaches investigating the effects of individual alleles; classification of MHC alleles into functionally similar MHC "supertypes" has therefore been recommended (Schwensow et al., 2007; Sepil et al., 2012). MHC supertypes were assigned following established protocols (Doytchinova & Flower, 2005; Sepil et al., 2012). First, the peptide-binding region (PBR) of the passerine MHC class II B exon 2 was identified as 18 amino acid residues experiencing positive (diversifying) selection in passerines (Minias et al., 2018). Similar methods have used to identify the PBR of MHC molecules in several species for which MHC structure is not well resolved (Radwan et al., 2012; Schwensow et al., 2007; Sepil et al., 2012). For each amino acid in the PBR, 5 z-scores encoding amino acid traits were added to a matrix (Sandberg et al., 1998). Five of the 126 MHC class II alleles contained amino acid deletions in the PBR; such deletions are not uncommon and do not necessarily preclude the functionality of the allele (Minias et al., 2018), therefore these alleles remained in the data set and the z-scores for the missing amino acids were set at 0. The z-score matrix was used with the R package adegenet (Jombart & Ahmed, 2011) to identify a set of MHC supertypes using k-means clustering and discriminant analysis of principal components (DAPC). Following the methods reported in Jombart and Collins (2015), we used 20 principal components and seven discriminant functions to classify all 126 MHC alleles into functional genetic clusters (supertypes). Overall, we identified nine supertypes (mean ± SEM; 6.67 ± 0.13 per individual; range: 1–9).

2.4 | Immune gene variability and survival or longevity

We tested for statistical associations between *f*, immune gene variability in TLR and MHC genes, and survival using mixed effects Cox models (Table 3). We first examined the survival of chicks to adulthood (aged 1 year or older), then examined adult longevity (in years) among those surviving individuals. Variation in the number of individuals genotyped for immune genes led to variation in sample size among tests. For all samples, we first tested for correlations in overall immune gene variability (Div_{MHC} and H_{TLR}) and survival or longevity, and included quadratic terms for Div_{MHC} and H_{TLR} in these analyses because intermediate levels of variability may be beneficial via the optimality hypothesis (Nowak et al., 1992). We also tested for effects of 15 specific TLR alleles and 9 MHC supertypes on survival or longevity. The inbreeding coefficient *f* is strongly associated with decreases in survival of juvenile but not adult song sparrows (Keller, 1998; Nietlisbach et al., 2017; Reid et al., 2014; Taylor et al., 2010; Wolak et al., 2018). To control for the influence of *f* in analyses involving survival to adulthood (models 1A, 1B, 3A, and 3B), we included *f* in all models, including null models (models containing the intercept plus variables previously found to be influential). Random effects in all Cox mixed models included brood identity, nested within the natal year.

2.5 | Immune response assays

Immune response assays were carried out and first reported by Reid et al. (2003, 2007). For humoral immunity assays (Reid et al., 2007), sparrows were mist-netted in September 2004 and September 2005

and approximately 100 μL blood was collected by brachial venipuncture. Individuals were vaccinated with 70 μL human diptheria-tetanus vaccine in the pectoral muscle and released. To measure primary antibody responses, sparrows were recaptured 10–12 days after vaccination, blood-sampled, and released. Blood samples were centrifuged for 4 min at 700*g* within 5 h, and plasma was separated and frozen at −20°C within 72 h. Enzyme-linked immunosorbent assays (ELISAs) were subsequently used to quantify tetanus and diphtheria antibody titres in pre- and post-vaccination plasma samples (Hasselquist et al., 1999; Owen-Ashley et al., 2004). Each individual's primary antibody response to tetanus toxoid was estimated as the difference between post- and prevaccination standardized antibody titres.

For phytohemagglutinin (PHA) assays, sparrows were assayed in 2002 and 2003 and patagium (wing-web) thickness was measured three times; mean thickness was used in analyses. 30 μL of 2 mg/mL PHA in phosphate buffered saline (PBS) was injected into the right patagium and 30 mL plain PBS into the left patagium. Left and right patagial thickness was remeasured approximately 18 h after injection. PHA response was estimated as the difference in increase in thickness between right and left patagia over the experimental period. Nestlings were assayed in May in 2002 and 2003; independent juveniles and adults were assayed after being trapped in mist-nets in February 2002, September 2002 and September 2003. Nestling data from 2003 are previously unpublished (nestlings: $N = 58$ in 2002, $N = 58$ in 2003), but followed the protocols in Reid et al. (2003).

2.6 | Immune gene variability and immune response assays

To test whether variability at TLR or MHC loci affected immune response, we used tetanus antibody titre as a response variable in generalized linear models (GLMs) assuming a negative binomial distribution of errors. PHA response was similarly used in linear models after transformation by square-root.

Antibody titres for the tetanus response were measured over 2 years from 45 individuals genotyped for MHC and 66 for TLR. Twelve individuals were assayed as chicks for PHA response and then assayed again as adults in later years for antibody titre for tetanus response; antibody response is not affected by previous PHA response (Reid et al., 2007). In all models, including null models, all explanatory variables found previously to influence response were included: *f*, sex, whether the individual's mother had been inoculated, number of days between inoculation and resampling and its quadratic term, and trial year (Reid et al., 2007). To assess TLR heterozygosity, models also included H_{TLR}, its quadratic term, and the interaction of *f* with each (model set 5A). To examine the effects of MHC allele diversity, models included Div_{MHC}, its quadratic term, and the interaction of *f* with each (model set 5C). To examine the effects of specific immune gene alleles, models included the presence of 15 specific TLR alleles (model set 5B) or the presence of 9 MHC supertypes (model set 5D).

Because nestlings and older individuals differ in the factors affecting their PHA response, individuals tested only as nestlings were excluded (Lukasch, Westerdahl, Strandh, Winkler, et al., 2017; Reid et al., 2003, 2007). PHA responses in two consecutive years were available for 76 and 86 individuals with MHC and TLR genotypes, respectively. In all models, including null models, explanatory variables included *f*, sex, and the trial year. To examine TLR heterozygosity, models also included H_{TLR}, its quadratic term, and the interaction of *f* with each (model set 6A). To examine MHC allele diversity, models also included Div_{MHC}, its quadratic term, and the interaction of *f* with each (model set 6C). To examine the effects of specific immune gene alleles, models were repeated with explanatory variables including presence of 13 specific TLR alleles (TLR15_6 and TLR15_7 were invariant in this sample and thus excluded; model set 6B) or the presence of 9 MHC supertypes (model 6D).

2.7 | Immune response assays and survival or longevity

We used Cox mixed effects models to test if immune response, *f*, and their linear and quadratic interaction terms influenced survival or longevity. We first examined longevity in adult individuals that had been involved in the tetanus immune response trial. Explanatory variables included: *f*, tetanus immune response, their interaction, the quadratic term for the tetanus response, and its interaction with *f*. Random effects in all models included brood identity, nested within natal year, based on a data set that included 86 individuals from 68 nests over 6 years.

We examined whether the PHA response predicts survival to adulthood and adult longevity. Previous work found that the PHA response in chicks was higher than in older birds (*t* test: $t = 17.47$, $p < .001$), and decreased as maternal but not chick *f* increased ($p = .002$ vs. 0.25, respectively); suggesting PHA response in chicks largely reflects natal environment (Reid et al., 2003). Explanatory variables investigating survival to adulthood included PHA response and its quadratic term, using data from 116 chicks from 57 nests hatched over 2 years. Some of adult longevity, terms included *f*, PHA response, their interaction, the quadratic term for PHA response, and its interaction with *f*. Although *f* predicts PHA response in older birds ($F = 71.5$, $p < .001$; Reid et al., 2007), both were included to explore their contributions to variance in adult longevity, using data from 118 individuals hatched from 88 nests over 11 years.

2.8 | Immune gene variability and inbreeding coefficient (f)

Inbreeding coefficient (*f*) is negatively related to heterozygosity estimated at neutral markers in song sparrows (Keller & Waller, 2002; Nietlisbach et al., 2017), though multilocus heterozygosity and *f* are only weakly correlated in other species (Slate et al., 2004). Heterozygosity at neutral markers has not

been linked to immune gene variability (Brambilla et al., 2018; Whittingham et al., 2018). We tested for relationships between *f* and immune gene heterozygosity (TLR) or diversity (MHC) by regressing H_{TLR} or Div_{MHC} on *f*. To facilitate comparisons with previous research, these terms were neither centred nor scaled for this analysis. *f* was estimated from the genetic pedigree using standard algorithms (Nietlisbach et al., 2017; Reid et al., 2014). This partially repeated an earlier analysis that used fewer datapoints (TLR and *f*; Nelson-Flower et al., 2018).

2.9 | Statistical analysis

Statistical analyses were performed in R version 4.2.0 (R Core Team, 2022). All statistical tests were two-tailed. Collinearity between explanatory variables was detected through calculation of variance inflation factors (VIF) (Zuur et al., 2009); all explanatory variables included in models had VIF scores of <3 indicating that collinearity was not problematic. We examined survival probabilities using the R package coxme version 2.2.16 (Therneau, 2015). For GLMs, we used R package glmmadmb version 0.8.3.3 (Skaug et al., 2014) to specify models with negative binomial error structures. For linear models, we used R package lme4 version 1.1.29 (Bates et al., 2015). We assessed model fit using Akaike's Information Criterion adjusted for small sample sizes (AICc; Burnham & Anderson, 2002). Tables are presented showing the full and null models as well as the models in the Δ2AICc model set after excluding "nested" models (models that were more complex versions of the top model) from the 2 ΔAICc model set (Arnold, 2010). When sample sizes were small, candidate models were limited in the number of terms they could contain (maximum number of terms was N/5). We employed model averaging whereby models within 2 AICc units of the best model were averaged using MuMIn version 1.43.6 (Bartoń, 2016); if averaging was employed, model estimates, standard errors, and 95% confidence intervals were calculated from "full" (as opposed to "conditional") models as suggested by Arnold (2010), representing a conservative approach. Means and standard errors presented in tables and figures were generated from reduced models identified using AICc comparison or multi-model averaging (reviewed in Grueber et al., 2011). Model estimates and standard errors of terms not retained in minimal or averaged models were calculated from global (full) models. Unless otherwise specified, all continuous explanatory variables were centred and scaled to reduce any influence of measurement scale on model results and to allow direct comparison of model coefficients; categorical variables involved in interactions were also centred for model comparisons and averaging (Grueber et al., 2011; Schielzeth, 2010). Due to the large number of explanatory variables in many of the reduced models, we calculated *q* values to correct *p*-values for the possibility of false discovery (Verhoeven et al., 2005). *q* values were calculated using the package FDRestimation version 1.0.1 (Murray & Blume, 2022). Figures of Cox model results were drawn using R package survminer version 0.9.4 (Kassambara & Kosinski, 2017) using estimates from Cox proportional hazards models.

3 | RESULTS

3.1 | Summary statistics of key variables

In total the data set included 380 song sparrows hatched between 1995 and 2015. Summary statistics of key variables used in analyses can be found in the Supporting Information in Table S2. Proportions of individuals carrying specific TLR alleles, MHC alleles, and MHC supertypes can be found in Table S3.

3.2 | Immune gene variability and survival or longevity

Survival to adulthood for 376 chicks decreased as *f* increased (coefficient ± SE: 0.405 ± 0.109, hazard ratio = 1.499, z-value = 3.73, $p < .001$, model set 1A, Table S4). In contrast, survival to adulthood was unrelated to any immune gene parameters, including H_{TLR} (−0.027 ± 0.111, hazard ratio = 0.973, $p = .810$, estimates from the full model in model set 1A, Table S4), specific TLR alleles (model set 1B, Table S4), Div_{MHC} (−0.102 ± 0.206, hazard ratio = 0.903, $p = .620$, estimates from the full model in model set 3A, Table S6), or specific MHC supertypes (model set 3B, Table S6). No evidence was found for a quadratic relationship of Div_{MHC} with survival to adulthood (0.315 ± 0.229, hazard ratio = 1.371, $p = .170$, estimates from the full model in model set 3A, Table S6).

H_{TLR} was unrelated to adult longevity (coefficient ± SE: −0.180 ± 0.193, hazard ratio = 0.835, $p = .350$, estimates from the full model in model set 2A, Table S5). Specific TLR alleles were also unrelated to adult longevity (model set 2B, Table S5). In contrast, the interaction between *f* and Div_{MHC} affected adult longevity (−1.449 ± 0.596, hazard ratio = 0.235, z-value = 3.16, $p = .002$, $q = 0.010$, model set 4A, Table S7, Figure 1); increases in MHC allele diversity decreased adult longevity, particularly for individuals with low *f*. No evidence was found for a quadratic relationship of Div_{MHC} with adult longevity (−0.016 ± 0.481, hazard ratio = 0.984, $p = .970$, estimates from the full model in model set 4A, Table S7). Adult longevity was lower in the presence of MHC supertype ST5 (0.848 ± 0.333, hazard ratio = 2.335, 95% CI: 0.195, 1.501, $p = .011$, $q = 0.028$, model set 4B, Table S7, Figure 2). An alignment of the amino acid resides making up the PBR of MHC alleles in ST5 is shown in Figure S1.

3.3 | Immune gene variability and immune response assays

Although H_{TLR} was unrelated to tetanus antibody titre (0.428 ± 0.321, estimate from the full model in model set 5A, Table S8), TLR3_2

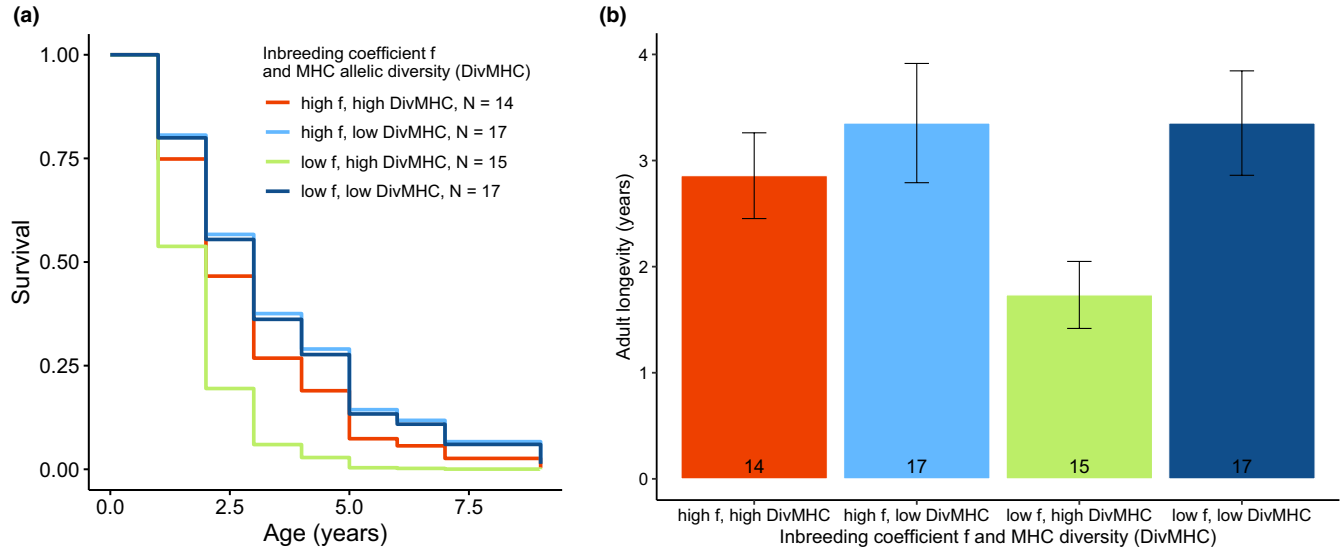

FIGURE 1 Effects of inbreeding coefficient *f* and MHC class II B exon 2 allele diversity (Div_{MHC}) on longevity of adults (individuals who survived to 1 year old). Both *f* and MHC allele diversity are categorized here using population means for illustrative purposes but both were continuous variables in our analyses. (a) Model output from a Cox proportional hazards model. (b) Raw data (mean ± SEM).

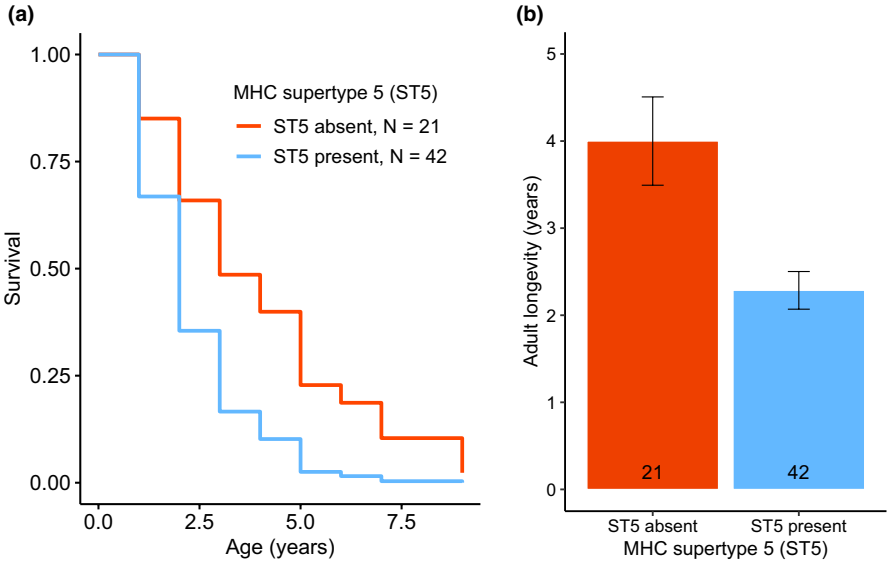

FIGURE 2 Effects of MHC class II B exon 2 supertype 5 (ST5) on longevity of adults (individuals who survived to 1 year old). (a) Model output from a Cox proportional hazards model. (b) Raw data (mean ± SEM).

was associated with reduced antibody titres (estimate ± SE: −1.242 ± 0.445, z-value = −2.79, $p = .005$, $q = 0.010$; model set 5B, Table S8, Figure 3). The tetanus antibody titre response was not influenced by Div_{MHC} (−0.360 ± 0.529, model set 5C, Table S9), the quadratic term for Div_{MHC} (−0.235 ± 0.486, model set 5C, Table S9), or by specific MHC supertypes (model set 5D, Table S9).

Phytohaemagglutinin (PHA) responses indicated that H_{TLR} (estimate ± SE: −0.025 ± 0.031, model set 6A, Table S10), specific TLR alleles (model set 6B, Table S10), and Div_{MHC} (0.009 ± 0.038, estimate from the full model in model set 6C, Table S11) had no effect on wing-web swelling. One specific MHC supertype did, however, affect PHA response: ST7 decreased PHA response (−0.069 ± 0.032, *t*-value = −2.13, $p = .037$, $q = 0.046$; model set 6D, Table S11, Figure 4).

3.4 | Immune response assays and longevity

Adult longevity was unrelated to the tetanus response (coefficient ± SE: 0.762 ± 0.501, hazard ratio = 2.143, z = 1.52, $p = .130$, estimate from the full model in model set 7A, Table S12). Similarly, survival to adulthood was unrelated to the PHA response for chicks (coefficient ± SE: −0.268 ± 0.189, hazard ratio = 0.765, z = −1.42, $p = .160$, estimate from the full model in model set 7B, Table S12).

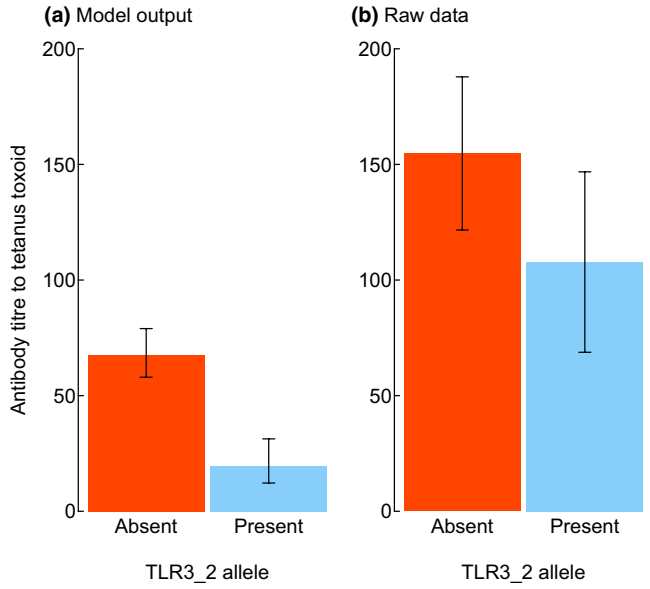

FIGURE 3 Effect of TLR3_2 allele on tetanus immune response (antibody titre to tetanus toxoid). Error bars indicate standard error. $N = 58$ individuals with TLR3_2 absent; $N = 8$ individuals with TLR3_2 present. (a) Model output of the minimal model, which includes other factors known to be important in antibody titre in this species (sex, *f*, days between inoculation and antibody titre measurement, maternal inoculation status, and year of experiment); (b) raw data.

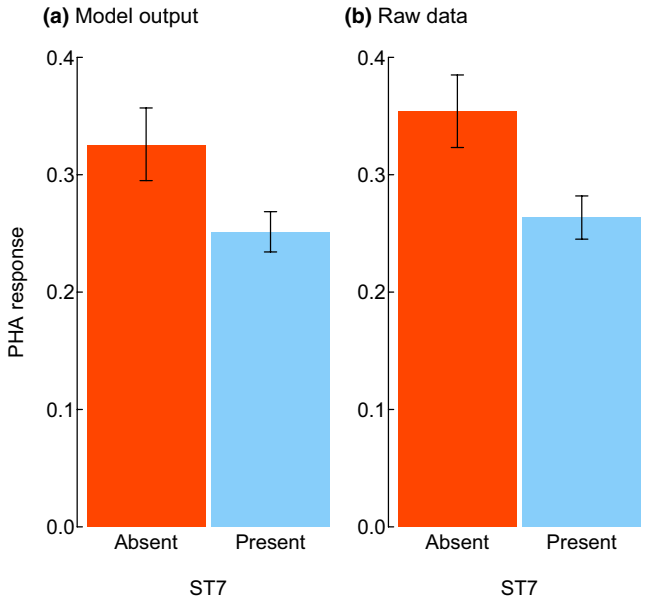

FIGURE 4 Effect of MHC class II B exon 2 supertype 7 (ST7) on PHA immune response. Error bars indicate standard error. $N = 22$ individuals with ST7 absent; $N = 54$ individuals with ST7 present. (a) Model output of the minimal model, which includes other factors known to be important in PHA response in this species (sex, *f*, and year of experiment); (b) raw data.

Adult longevity was also unrelated to the PHA response (coefficient ± SE: 0.277 ± 0.241, hazard ratio = 1.319, $z = 1.15$, $p = .250$, estimate from the full model in model set 7C, Table S12).

3.5 | Immune gene variability and *f*

f was unrelated to TLR heterozygosity (H_{TLR}; regression slope ± standard error = −0.140 ± 0.121, $t = -1.16$, $p = .247$); *f* was also unrelated to MHC class II B allele diversity (Div_{MHC}; 12.372 ± 11.893, $t = 1.04$, $p = .300$).

4 | DISCUSSION

Practical challenges in obtaining detailed data on individual fitness, immune genotype, and inbreeding in wild populations have to date severely hampered tests of the general hypothesis that immune genotype affects survival via the immune response (Møller & Saino, 2004). To address this research gap, we utilized free-living song sparrows assayed previously for humoral and innate immune responses, and measured MHC and TLR immune genotype and variation, juvenile survival to adulthood, and adult longevity to test if these factors were linked to immune response and/or inbreeding *f* in individual birds. Overall, juvenile survival to adulthood decreased with increasing *f*, but was unrelated to variation in immune genes as measured by our metrics. Adult longevity was affected by MHC in interaction with *f*, such that more outbred birds with increased MHC diversity experienced shorter lifespans; in addition, the presence of MHC supertype ST5 was associated with a decrease in adult longevity. Immune response was affected by specific immune genotypes: antibody response was decreased in the presence of the specific allele TLR3_2, and the PHA response was decreased in the presence of MHC supertype ST7. Surprisingly, immune response did not predict survival to adulthood or adult longevity, and there was no statistical association between immune genotype variability and *f*.

4.1 | Immune gene diversity or heterozygosity and survival to adulthood, adult longevity, and immune responses

Immune loci other than MHC have been suggested as useful candidate genes for heterozygosity-fitness correlations, including TLRs (Acevedo-Whitehouse & Cunningham, 2006). TLR gene heterozygosity at the extracellular binding domain did not affect survival or longevity in song sparrows, similar to prior studies of other species (Bateson et al., 2016; Grueber et al., 2013). In addition, though individuals that are more (or less) heterozygous at TLR alleles might have differing abilities to recognize PAMPs (thereby affecting immune responses), immune responsiveness in song sparrows was not associated with TLR heterozygosity. In contrast, increases in MHC class II allele diversity (Div_{MHC}) were associated with decreases in adult longevity in individuals, particularly in adults with low *f*. In general, adults with fewer MHC alleles (15 or fewer) lived on average

1 year longer than did those with more MHC alleles (more than 15). Similarly, in adult caribou, greater MHC class II functional diversity was associated with decreased survival (Gagnon et al., 2020). We discuss below that the MHC supertype ST5 was associated with a decrease in adult longevity. Individuals with ST5 were more likely to have a high MHC allele diversity (*t* test, $p < .001$); the presence or absence of ST5 may therefore drive this finding. Alternatively, one suggested cost of increased MHC allele diversity may be an increased risk of autoimmunity and/or a decrease in the repertoire of T cell receptors, leading to a decrease in functional immunity (reviewed in Radwan et al., 2020) as seen in bank voles (*Myodes glareolus*; Migalska et al., 2019). However, song sparrows on Mandarte Island are inbred (Keller, 1998) and innate immune genes have been shown to be less diverse than in mainland populations (Nelson-Flower et al., 2018), making this explanation unlikely.

4.2 | Effects of specific TLR alleles and MHC supertypes on survival to adulthood, adult longevity, and immune responses

Specific immune gene alleles or supertypes are often found to affect survival and immune responsiveness in wild bird populations (Bonneaud et al., 2005; Brouwer et al., 2010; Dunn et al., 2013; Lukasch, Westerdahl, Strandh, Knauer, et al., 2017; Lukasch, Westerdahl, Strandh, Winkler, et al., 2017; Sepil et al., 2013; Table 1). In adult song sparrows on Mandarte Island, MHC class II supertype ST5 was associated with decreased longevity; individuals that did not carry ST5 lived on average 1.5 years longer than those with ST5. We found no associations between specific TLR alleles and survival or longevity. This stands in contrast to findings in Seychelles warblers (*Acrocephalus sechellensis*) and Stewart Island robins (*Petroica australis rakiura*), in which specific TLR alleles affected survival (Davies et al., 2021; Grueber et al., 2013). As part of the innate immune system and a first line of defence, TLRs are generally highly conserved and, in song sparrows, experience purifying selection (Nelson-Flower et al., 2018). However, TLRs are under balancing selection in other species, indicating that further work is required to ascertain links between TLR variation and fitness (Minias & Vinkler, 2022).

The presence of a specific TLR allele (TLR3_2) was associated with decreases in the humoral immune response (tetanus toxoid antibody response) in song sparrows. TLR3 recognizes double-stranded RNA, which is indicative of some viral infections (Nie et al., 2018). However, the mechanism responsible for this result remains unclear; functional connections between TLR3 and humoral adaptive immunity are indirect. This result may represent type I error, despite our efforts to reduce the occurrence of such error. While research investigating the role of TLR alleles in immune responses is rare, prior results in Attwater's prairie-chicken found that a specific TLR1LB allele was associated with changes in the innate immune response (measured by lysozyme and haemolysis assays; Bateson et al., 2016). Future research investigating the role of specific TLR alleles in immune responses might be best served by development of specific immune challenges that target particular TLR loci: for example, the introduction of double-stranded RNA to assess the role of TLR3 genotypes in immune responses.

The ST5 supertype was common, found in 67% of the adult birds in our MHC data set, and may represent an example of negative frequency-dependent selection, discussed below. We also found that MHC class II supertype ST7 was associated with a small but significant decrease in PHA response in juvenile and adult song sparrows. Similarly, a specific MHC class II allele was associated with a decreased PHA response in juvenile sea lions (*Zalophus californianus*; (Montano-Frías et al., 2016)). PHA response was historically thought to represent cell-mediated immunity, and though MHC genes activate acquired, cell-mediated immunity including T cells (Janeway et al., 2001; Viney et al., 2005), the short elapsed time between injection of PHA and measurement of swelling probably precludes this mechanism. It is likely that the functional connection between the presence of MHC ST7 and a decrease in PHA response is indirect.

4.3 | Immune gene variability: Selection mechanisms at work

It can be difficult to identify the exact mechanism involved in selection for MHC polymorphism at a population level because the predictions made by hypotheses such as negative frequency-dependent selection and heterozygote advantage can overlap (Spurgin & Richardson, 2010). Other problems also arise: negative frequency-dependent selection implies changes in an allele's frequency over time, but many studies are accomplished over relatively short time periods and offer only a "snapshot" view of a population (reviewed in Radwan et al., 2020). In addition, the benefits of larger numbers of MHC alleles (heterozygote advantage) can be difficult to parse from the benefits of specific alleles that may be more common in heterozygotes (reviewed in Worley et al., 2010). Investigations of pathogen-mediated selection have been suggested to focus on identifying what mechanisms are at work rather than determining their relative importance in a system (Spurgin & Richardson, 2010).

Specific TLR alleles and MHC supertypes decreased longevity or immune responses in song sparrows, implying that negative frequency dependent selection could be at work. Negative frequency-dependent selection could be mediated by a particular immune gene allele or supertype (reviewed in Radwan et al., 2020). Under negative frequency-dependent selection, pathogens are susceptible to rare alleles, but over time pathogens evolve to resist these alleles as they become increasingly common (Takahata & Nei, 1990). Negative frequency-dependent selection is usually taken to mean that a rare allele is beneficial, rather than a common allele being detrimental. However, such impact of specific immune genotypes is not uncommon (Bateson et al., 2016; Dunn et al., 2013); for example, an MHC supertype decreased nestling survival in house sparrows (Lukasch, Westerdahl, Strandh, Knauer, et al., 2017), and a TLR3 allele decreased survival of adults in Seychelle's warblers (Davies et al., 2021). Specific MHC alleles have been shown to increase the

likelihood of pathogen infection in birds (Bonneaud et al., 2006; Worley et al., 2010).

Our results indicate no support in this population for the heterozygote advantage hypothesis, because individuals with larger numbers of MHC alleles did not experience greater fitness (survival/longevity) or any effects on immune responses. We also found no evidence for the optimality hypothesis, because there was no quadratic relationship between MHC allele diversity and survival to adulthood, adult longevity or immune responses.

4.4 | Immune response assays and longevity

We found no links between individual longevity and PHA or tetanus response, suggested previously to be valuable as predictors of fitness (though see alsoAdamo, 2004; Viney et al., 2005). However, observed PHA or antibody responses may not reflect aspects of the immune system most influential in fighting pathogens in particular individuals or sites, resulting in little or no relationship to fitness (Adamo, 2004; Viney et al., 2005). In addition, immune responses can be difficult to interpret in cases where the maximal response is not the optimal response with respect to fitness (Adamo, 2004; Graham et al., 2011; Viney et al., 2005). A different approach is to assay the specific immune mechanisms deployed to defend against common pathogens. For example, Soay sheep (*Ovis aries*) with high levels of antibodies to a common helminth were more likely to survive the subsequent winter (Nussey et al., 2014). Future work in song sparrows might therefore focus on specific common pathogens or parasites, MHC and TLR variation, and individual fitness.

4.5 | Immune gene variability and *f*

Prior work in our study population has revealed correlations between *f* and various fitness components (Keller, 1998; Reid et al., 2014; Taylor et al., 2010; Wolak et al., 2018), leading to speculation that *f* may reflect immune gene variability (Reid et al., 2007), given a negative correlation of *f* to genome-wide heterozygosity at neutral loci (Nietlisbach et al., 2017). However, we found no correlation between individual *f* and TLR heterozygosity at nonsynonymous SNPs, adding to mixed reports of correlations between *f*, genome-wide heterozygosity, and TLR heterozygosity (e.g., Hartmann et al., 2014; Bateson et al., 2016; Grueber et al., 2015). We also found that *f* was unrelated to MHC variability. Genome-wide heterozygosity and MHC variation were also found to be unrelated in wild ibex (*Capra ibex*; Brambilla et al., 2018), caribou (*Rangifer tarandus*; Gagnon et al., 2020), and common yellowthroat (Whittingham et al., 2018). MHC diversity can be maintained despite inbreeding and small population size (Aguilar et al., 2004; Grueber et al., 2017; Knafler et al., 2017; Richardson & Westerdahl, 2003), indicating that strong selection in response to pathogens can override the losses of genetic diversity that occur via inbreeding and genetic drift (Radwan et al., 2020).

While juvenile survival to adulthood declines with increasing *f* in song sparrows (Keller, 1998; Nietlisbach et al., 2017; Reid et al., 2014; Taylor et al., 2010; Wolak et al., 2018, this study), we found no correlation between juvenile survival and immune genotype, implying that reduced survival in highly inbred juveniles is unrelated to individual variation in the TLR or MHC genes considered here. In contrast, we found that adult longevity was unaffected by *f* on its own (also found previously by Reid et al., 2014), but strongly influenced by MHC allele diversity in interaction with *f*. Overall, immune genotype and diversity appear to influence survival and longevity separately from the expected effects of inbreeding *f* on individual immune response.

4.6 | Methodological limitations

The research presented here draws together multiple lines of investigation; some methodological limitations are evident, though we tried to mitigate their impacts. First, the immune response assays have limited specific functional relevance to some of the immune genes we investigated. For example, we examined TLR3 and TLR4, which bind double-stranded RNA and lipopolysaccharide from gram-negative bacteria, respectively; an assay introducing these antigens to individual birds could correlate more directly with the function of these TLR genes. Nevertheless, the PHA response and antibody titre response have been used as immunological parameters in field studies for many years and are likely to encompass some indirect associations between immune function and genotype. Second, this research encompasses many statistical analyses which, in some cases, have limited sample sizes, thus increasing the risk of type I error. We therefore limited the number of statistical terms in candidate models, removed "nested" models from top models sets, presented "full" (rather than "conditional") model averages, and calculated *q* values to correct false discovery rates. All of these measures increase the stringency of our statistical analyses, but the possibility remains that some of our results could be the result of type I error. Finally, many of the individuals were chosen for TLR and MHC genotyping because they were assayed immunologically as adults (Reid et al., 2003, 2007). By definition these individuals survived to adulthood, but genotyping only these would add a degree of bias, because most birds in this study population die before attaining adulthood. We therefore also genotyped a broodmate for each of these individuals regardless of the broodmate's survival. We chose this conservative approach over genotyping a randomly chosen individual as less likely to generate spurious results. However, this may have increased the likelihood of type II error in detecting differences in survival due to immune genotype.

4.7 | Conclusions

This research investigates immune gene diversity, immune function, and fitness in a wild population, while disentangling the effects of heterozygosity or diversity at candidate genes from those of genome-wide heterozygosity (indicated by *f*). Most studies of wild populations are hindered by practical limits on precisely

estimating individual fitness, immune function, and *f*, but we were able to use a long-term data set, an extensive collection of blood samples, and previously collected immune response data to overcome these challenges. In general, we found that specific immune genotypes were associated with decreases in immune responses and adult longevity, suggested a role for negative frequency-dependent selection in this population. We also found that high MHC allele diversity in outbred birds contributed to shorter adult longevity. Immune responses were not a proxy for fitness as measured by survival or adult longevity. Finally, immune gene diversity or heterozygosity did not reflect inbreeding coefficient *f*, suggesting that selection may maintain variation at functional loci even when populations are small.

AUTHOR CONTRIBUTIONS

Martha J. Nelson-Flower conceived the analyses, performed the TLR genotyping, carried out the statistical analyses and wrote the manuscript. Leanne A. Grieves performed the MHC genotyping with Savo Lazic and Elizabeth A. MacDougall-Shackleton. Jane M. Reid performed the immune response experiments. Ryan R. Germain provided data extraction and collation from the song sparrow database. Sabrina S. Taylor contributed reagents for TLR genotyping. Peter Arcese coordinated collection of blood samples and survival data on Mandarte Island. All authors contributed to editing drafts of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data can be accessed in Dryad at https://doi.org/10.5061/dryad.0gb5mkm5q (Nelson-Flower et al., 2022). Novel MHC alleles have been deposited in GenBank with accession numbers OQ617125-OQ617168.

BENEFIT-SHARING STATEMENT

Benefits generated: Benefits from this research accrue from the sharing of our data and results on public databases as described above.

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REFERENCES

Acevedo-Whitehouse, K., & Cunningham, A. (2006). Is MHC enough for understanding wildlife immunogenetics? *Trends in Ecology & Evolution*, *21*, 433–438. https://doi.org/10.1016/j.tree.2006.05.010

Adamo, S. A. (2004). How should behavioural ecologists interpret measurements of immunity? *Animal Behaviour*, *68*, 1443–1449. https://doi.org/10.1016/j.anbehav.2004.05.005

Aguilar, A., Roemer, G., Debenham, S., Binns, M., Garcelon, D., & Wayne, R. K. (2004). High MHC diversity maintained by balancing selection in an otherwise genetically monomorphic mammal. *Proceedings of the National Academy of Sciences*, *101*, 3490–3494. https://doi.org/10.1073/pnas.0306582101

Alcaide, M., & Edwards, S. V. (2011). Molecular evolution of the toll-like receptor multigene family in birds. *Molecular Biology and Evolution*, *28*, 1703–1715.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Arnold, T. W. (2010). Uninformative parameters and model selection using Akaike's information criterion. *Journal of Wildlife Management*, *74*, 1175–1178.

Bartoń, K. (2016). MuMIn: multi-model inference.

Bates, D., Machler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*, 1–48. https://doi.org/10.18637/jss.v067.i01

Bateson, Z. W., Hammerly, S. C., Johnson, J. A., Morrow, M. E., Whittingham, L. A., & Dunn, P. O. (2016). Specific alleles at immune genes, rather than genome-wide heterozygosity, are related to immunity and survival in the critically endangered Attwater's prairie-chicken. *Molecular Ecology*, *25*, 4730–4744.

Beutler, B. (2004). Innate immunity: An overview. *Molecular Immunology*, *40*, 845–859. https://doi.org/10.1016/j.molimm.2003.10.005

Birkhead, T. R., Fletcher, F., & Pellatt, E. J. (1999). Nestling diet, secondary sexual traits and fitness in the zebra finch. *Proceedings of the Royal Society of London—Series B: Biological Sciences*, *266*, 385–390. https://doi.org/10.1098/rspb.1999.0649

Bonneaud, C., Pérez-Tris, J., Federici, P., Chastel, O., & Sorci, G. (2006). Major histocompatibility alleles associated with local resistance to malaria in a passerine. *Evolution*, *60*(2), 383–389.

Bonneaud, C., Richard, M., Faivre, B., Westerdahl, H., & Sorci, G. (2005). An Mhc class I allele associated to the expression of T-dependent immune response in the house sparrow. *Immunogenetics*, *57*, 782–789. https://doi.org/10.1007/s00251-005-0046-5

Bonneaud, C., Sinsheimer, J. S., Richard, M., Chastel, O., & Sorci, G. (2009). *Mhc* polymorphisms fail to explain the heritability of phytohaemagglutinin-induced skin swelling in a wild passerine. *Biology Letters*, *5*, 784–787. https://doi.org/10.1098/rsbl.2009.0435

Brambilla, A., Keller, L., Bassano, B., & Grossen, C. (2018). Heterozygosity-fitness correlation at the major histocompatibility complex despite low variation in alpine ibex (*Capra ibex*). *Evolutionary Applications*, *11*, 631–644. https://doi.org/10.1111/eva.12575

Brouwer, L., Barr, I., van de Pol, M., Burke, T., Komdeur, J., & Richardson, D. S. (2010). MHC-dependent survival in a wild population: Evidence for hidden genetic benefits gained through extra-pair fertilizations. *Molecular Ecology*, *19*, 3444–3455. https://doi.org/10.1111/j.1365-294X.2010.04750.x

Burnham, K. P., & Anderson, D. R. (2002). *Model selection and multimodel inference*. Springer.

Charbonnel, N., Bryja, J., Galan, M., Deter, J., Tollenaere, C., Chaval, Y., Morand, S., & Cosson, J.-F. (2010). Negative relationships between cellular immune response, Mhc class II heterozygosity and secondary sexual trait in the montane water vole. *Evolutionary Applications*, *3*, 279–290. https://doi.org/10.1111/j.1752-4571.2009.00108.x

Cutrera, A. P., Zenuto, R. R., & Lacey, E. A. (2011). MHC variation, multiple simultaneous infections and physiological condition in the subterranean rodent Ctenomys talarum. *Infection, Genetics and Evolution*, *11*, 1023–1036. https://doi.org/10.1016/j.meegid.2011.03.016

Davies, C. S., Taylor, M. I., Hammers, M., Burke, T., Komdeur, J., Dugdale, H. L., & Richardson, D. S. (2021). Contemporary evolution of the innate immune receptor gene *TLR3* in an isolated vertebrate population. *Molecular Ecology*, *30*, 2528–2542. https://doi.org/10.1111/mec.15914

Dickel, L., Arcese, P., Nietlisbach, P., Keller, L. F., Jensen, H., & Reid, J. M. (2021). Are immigrants outbred and unrelated? Testing standard assumptions in a wild metapopulation. *Molecular Ecology*, *30*, 5674–5686. https://doi.org/10.1111/mec.16173

Doherty, P. C., & Zinkernagel, R. M. (1975). Enhanced immunological surveillance in mice heterozygous at the H-2 gene complex. *Nature*, *256*, 50–52.

Downing, T., Lloyd, A. T., O'Farrelly, C., & Bradley, D. G. (2010). The differential evolutionary dynamics of avian cytokine and TLR gene classes. *Journal of Immunology*, *184*, 6993–7000. https://doi.org/10.4049/jimmunol.0903092

Doytchinova, I. A., & Flower, D. R. (2005). In Silico identification of Supertypes for class II MHCs. *Journal of Immunology*, *174*, 7085–7095. https://doi.org/10.4049/jimmunol.174.11.7085

Dunn, P. O., Bollmer, J. L., Freeman-Gallant, C. R., & Whittingham, L. A. (2013). MHC variation is related to a sexually selected ornament, survival, and parasite resistance in common yellowthroats. *Evolution*, *67*, 679–687. https://doi.org/10.1111/j.1558-5646.2012.01799.x

Edwards, S. V., Gasper, J., & March, M. (1998). Genomics and polymorphism of Agph-DAB1, an Mhc class II B gene in red-winged blackbirds (Agelaius phoeniceus). *Molecular Biology and Evolution*, *15*, 236–250.

Gagnon, M., Yannic, G., Boyer, F., & Côté, S. D. (2020). Adult survival in migratory caribou is negatively associated with MHC functional diversity. *Heredity*, *125*, 290–303. https://doi.org/10.1038/s41437-020-0347-3

Gaigher, A., Burri, R., San-Jose, L. M., Roulin, A., & Fumagalli, L. (2019). Lack of statistical power as a major limitation in understanding MHC-mediated immunocompetence in wild vertebrate populations. *Molecular Ecology*, *28*, 5115–5132. https://doi.org/10.1111/mec.15276

Germain, R. R., Wolak, M. E., Arcese, P., Losdat, S., & Reid, J. M. (2016). Direct and indirect genetic and fine-scale location effects on breeding date in song sparrows. *The Journal of Animal Ecology*, *85*, 1613–1624. https://doi.org/10.1111/1365-2656.12575

Gloor, G. B., Hummelen, R., Macklaim, J. M., Dickson, R. J., Fernandes, A. D., MacPhee, R., & Reid, G. (2010). Microbiome profiling by Illumina sequencing of combinatorial sequence-tagged PCR products. *PLoS One*, *5*, e15406. https://doi.org/10.1371/journal.pone.0015406

Gonzalez, G., Sorci, G., Moller, A. P., Ninni, P., Haussy, C., & De Lope, F. (1999). Immunocompetence and condition-dependent sexual advertisement in male house sparrows (Passer domesticus). *The Journal of Animal Ecology*, *68*, 1225–1234. https://doi.org/10.1046/j.1365-2656.1999.00364.x

Graham, A. L., Shuker, D. M., Pollitt, L. C., Auld, S. K. J. R., Wilson, A. J., & Little, T. J. (2011). Fitness consequences of immune responses: Strengthening the empirical framework for ecoimmunology. *Functional Ecology*, *25*, 5–17. https://doi.org/10.1111/j.1365-2435.2010.01777.x

Grieves, L. A., Gloor, G. B., Bernards, M. A., & MacDougall-Shackleton, E. A. (2019). Songbirds show odour-based discrimination of similarity and diversity at the major histocompatibility complex. *Animal Behaviour*, *158*, 131–138. https://doi.org/10.1016/j.anbehav.2019.10.005

Grueber, C. E., Nakagawa, S., Laws, R. J., & Jamieson, I. G. (2011). Multimodel inference in ecology and evolution: Challenges and solutions: Multimodel inference. *Journal of Evolutionary Biology*, *24*, 699–711. https://doi.org/10.1111/j.1420-9101.2010.02210.x

Grueber, C. E., Knafler, G. J., King, T. M., Senior, A. M., Grosser, S., Robertson, B., Weston, K. A., Brekke, P., Harris, C. L. W., & Jamieson, I. G. (2015). Toll-like receptor diversity in 10 threatened bird species: Relationship with microsatellite heterozygosity. *Conservation Genetics*, *16*(3), 595–611. https://doi.org/10.1007/s10592-014-0685-x

Grueber, C. E., Sutton, J. T., Heber, S., Briskie, J. V., Jamieson, I. G., & Robertson, B. C. (2017). Reciprocal translocation of small numbers of inbred individuals rescues immunogenetic diversity. *Molecular Ecology*, *26*, 2660–2673. https://doi.org/10.1111/mec.14063

Grueber, C. E., Wallis, G. P., & Jamieson, I. G. (2013). Genetic drift outweighs natural selection at toll-like receptor (TLR) immunity loci in a re-introduced population of a threatened species. *Molecular Ecology*, *22*, 4470–4482. https://doi.org/10.1111/mec.12404

Grueber, C. E., Wallis, G. P., & Jamieson, I. G. (2014). Episodic positive selection in the evolution of avian toll-like receptor innate immunity genes. *PLoS One*, *9*, e89632. https://doi.org/10.1371/journal.pone.0089632

Hartmann, S. A., Schaefer, H. M., & Segelbacher, G. (2014). Genetic depletion at adaptive but not neutral loci in an endangered bird species. *Molecular Ecology*, *23*, 5712–5725. https://doi.org/10.1111/mec.12975

Hasselquist, D., Marsh, J. A., Sherman, P. W., & Wingfield, J. C. (1999). Is avian humoral immunocompetence suppressed by testosterone? *Behavioral Ecology and Sociobiology*, *45*, 167–175. https://doi.org/10.1007/s002650050550

Janeway, C. A., Travers, P., Walport, M., & Shlomik, M. J. (2001). *Immunobiology: The immune system in health and disease*. Garland Science.

Jombart, T., & Ahmed, I. (2011). Adegenet 1.3-1: New tools for the analysis of genome-wide SNP data.

Jombart, T., & Collins, C. (2015). A Tutorial for Discriminant Analysis of Principal Components (DAPC) Using Adegenet 2.0.0.

Kamiya, T., O'Dwyer, K., Westerdahl, H., Senior, A., & Nakagawa, S. (2014). A quantitative review of MHC-based mating preference: The role of diversity and dissimilarity. *Molecular Ecology*, *23*, 5151–5163. https://doi.org/10.1111/mec.12934

Kassambara, A., & Kosinski, M. (2017). Survminer: Drawing survival curves using "ggplot2".

Keller, L. F. (1998). Inbreeding and its fitness effects in an insular population of song sparrows (Melospiza melodia). *Evolution*, *52*, 240–250. https://doi.org/10.1111/j.1558-5646.1998.tb05157.x

Keller, L. F., & Arcese, P. (1998). No evidence for inbreeding avoidance in a natural population of song sparrows (Melospiza melodia). *The American Naturalist*, *152*, 380–392. https://doi.org/10.1086/286176

Keller, L. F., & Waller, D. M. (2002). Inbreeding effects in wild populations. *Trends in Ecology & Evolution*, *17*, 230–241. https://doi.org/10.1016/S0169-5347(02)02489-8

Klein, J., & Figueroa, F. E. (1986). Evolution of the major histocompatibility complex. *Critical Reviews in Immunology*, *6*(4), 295–386.

Knafler, G., Grueber, C., Sutton, J., & Jamieson, I. (2017). Differential patterns of diversity at microsatellite, MHC, and TLR loci in bottlenecked south Island saddleback populations. *New Zealand Journal of Ecology*, *41*, 98–106. https://doi.org/10.20417/nzjecol.41.8

Librado, P., & Rozas, J. (2009). DNAsp v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, *25*, 1451–1452.

Liston, A., Humblet-Baron, S., Duffy, D., & Goris, A. (2021). Human immune diversity: From evolution to modernity. *Nature Immunology*, *22*(12), 1479–1489. https://doi.org/10.1038/s41590-021-01058-1

Lukasch, B., Westerdahl, H., Strandh, M., Knauer, F., Winkler, H., Moodley, Y., & Hoi, H. (2017). Major histocompatibility complex genes partly explain early survival in house sparrows. *Scientific Reports*, *7*, 6571. https://doi.org/10.1038/s41598-017-06631-z

Lukasch, B., Westerdahl, H., Strandh, M., Winkler, H., Moodley, Y., Knauer, F., & Hoi, H. (2017). Genes of the major histocompatibility complex highlight interactions of the innate and adaptive immune system. *PeerJ*, *5*, e3679. https://doi.org/10.7717/peerj.3679

Marr, A. B., Arcese, P., Hochachka, W. M., Reid, J. M., & Keller, L. F. (2006). Interactive effects of environmental stress and inbreeding on reproductive traits in a wild bird population. *The Journal of Animal Ecology*, *75*, 1406–1415. https://doi.org/10.1111/j.1365-2656.2006.01165.x

Martin, L. B., II, Han, P., Lewittes, J., Kuhlman, J. R., Klasing, K. C., & Wikelski, M. (2006). Phytohemagglutinin-induced skin swelling in birds: Histological support for a classic immunoecological technique. *Functional Ecology*, *20*(2), 290–299. https://doi.org/10.1111/j.1365-2435.2006.01094.x

Migalska, M., Sebastian, A., & Radwan, J. (2019). Major histocompatibility complex class I diversity limits the repertoire of T cell receptors. *Proceedings of the National Academy of Sciences*, *116*(11), 5021–5026. https://doi.org/10.1073/pnas.1807864116

Minias, P., Pikus, E., Whittingham, L. A., & Dunn, P. O. (2018). A global analysis of selection at THE avian MHC. *Evolution*, *72*, 1278–1293. https://doi.org/10.1111/evo.13490

Minias, P., & Vinkler, M. (2022). Selection balancing at innate immune genes: Adaptive polymorphism maintenance in toll-like receptors. *Molecular Biology and Evolution*, *39*, msac102. https://doi.org/10.1093/molbev/msac102

Møller, A. P., & Saino, N. (2004). Immune response and survival. *Oikos*, *104*, 299–304. https://doi.org/10.1111/j.0030-1299.2004.12844.x

Montano-Frías, J. E., Vera-Massieu, C., Álvarez-Martínez, R., Flores-Morán, A., & Acevedo-Whitehouse, K. (2016). MHC class II transcription is associated with inflammatory responses in a wild marine mammal. *Infection, Genetics and Evolution*, *42*, 77–82. https://doi.org/10.1016/j.meegid.2016.04.022

Murray, M., & Blume, J. (2022). FDRestimation: Estimate, plot, and summarize false discovery rates.

Nelson-Flower, M. J., Germain, R. R., MacDougall-Shackleton, E. A., Taylor, S. S., & Arcese, P. (2018). Purifying selection in the toll-like receptors of song sparrows Melospiza melodia. *The Journal of Heredity*, *109*, 501–509. https://doi.org/10.1093/jhered/esy027

[dataset]Nelson-Flower, M. J., Grieves, L. A., Reid, J. M., Germain, R. R., Lazic, S., Taylor, S. S., MacDougall-Shackleton, E. A., & Arcese, P. (2022). Immune genotypes, immune responses, and survival in a wild bird population. In *Data for immune genotypes, immune responses, and survival in a wild bird population*; Dryad; DOI to be assigned upon Dryad submission.

Netea, M. G., Wijmenga, C., & O'Neill, L. A. J. (2012). Genetic variation in toll-like receptors and disease susceptibility. *Nature Immunology*, *13*, 535–542. https://doi.org/10.1038/ni.2284

Nie, L., Cai, S.-Y., Shao, J.-Z., & Chen, J. (2018). Toll-like receptors, associated biological roles, and signaling networks in non-mammals. *Frontiers in Immunology*, *9*, 1523. https://doi.org/10.3389/fimmu.2018.01523

Nietlisbach, P., Camenisch, G., Bucher, T., Slate, J., Keller, L. F., & Postma, E. (2015). A microsatellite-based linkage map for song sparrows (Melospiza melodia). *Molecular Ecology Resources*, *15*, 1486–1496. https://doi.org/10.1111/1755-0998.12414

Nietlisbach, P., Keller, L. F., Camenisch, G., Arcese, P., Reid, J. M., & Postma, E. (2017). Pedigree-based inbreeding coefficient explains more variation in fitness than heterozygosity at 160 microsatellites in a wild bird population. *Proceedings of the Royal Society B: Biological Sciences*, *284*, 20162763.

Norris, K., & Evans, M. R. (2000). Ecological immunology: Life history trade-offs and immune defense in birds. *Behavioral Ecology*, *11*, 19–26. https://doi.org/10.1093/beheco/11.1.19

Nowak, M. A., Tarczy-Hornoch, K., & Austyn, J. M. (1992). The optimal number of major histocompatibility complex molecules in an individual. *Proceedings of the National Academy of Sciences*, *89*(22), 10896–10899. https://doi.org/10.1073/pnas.89.22.10896

Nussey, D. H., Watt, K. A., Clark, A., Pilkington, J. G., Pemberton, J. M., Graham, A. L., & McNeilly, T. N. (2014). Multivariate immune defences and fitness in the wild: Complex but ecologically important associations among plasma antibodies, health and survival. *Proceedings of the Royal Society B: Biological Sciences*, *281*, 20132931. https://doi.org/10.1098/rspb.2013.2931

O'Connor, E. A., Westerdahl, H., Burri, R., & Edwards, S. V. (2019). Avian MHC evolution in the era of genomics: Phase 1.0. *Cell*, *8*, 1152. https://doi.org/10.3390/cells8101152

Owen-Ashley, N. T., Hasselquist, D., & Wingfield, J. C. (2004). Androgens and the Immunocompetence handicap hypothesis: Unraveling direct and indirect pathways of immunosuppression in song sparrows. *The American Naturalist*, *164*, 490–505. https://doi.org/10.1086/423714

R Core Team. (2022). R: A language and environment for statistical computing.

Råberg, L., & Stjernman, M. (2003). Natural selection on immune responsiveness in blue tits Parus caeruleus. *Evolution*, *57*(7), 1670. https://doi.org/10.1554/02-417

Radwan, J., Babik, W., Kaufman, J., Lenz, T. L., & Winternitz, J. (2020). Advances in the evolutionary understanding of MHC polymorphism. *Trends in Genetics*, *36*, 298–311. https://doi.org/10.1016/j.tig.2020.01.008

Radwan, J., Zagalska-Neubauer, M., Cichoń, M., Sendecka, J., Kulma, K., Gustafsson, L., & Babik, W. (2012). MHC diversity, malaria and lifetime reproductive success in collared flycatchers. *Molecular Ecology*, *21*(10), 2469–2479. https://doi.org/10.1111/j.1365-294X.2012.05547.x

Reid, J. M., Arcese, P., & Keller, L. F. (2003). Inbreeding depresses immune response in song sparrows (Melospiza melodia): Direct and intergenerational effects. *Proceedings of the Royal Society B: Biological Sciences*, *270*, 2151–2157. https://doi.org/10.1098/rspb.2003.2480

Reid, J. M., Arcese, P., Keller, L. F., Elliott, K. H., Sampson, L., & Hasselquist, D. (2007). Inbreeding effects on immune response in free-living song sparrows (Melospiza melodia). *Proceedings of the Royal Society B: Biological Sciences*, *274*, 697–706. https://doi.org/10.1098/rspb.2006.0092

Reid, J. M., Arcese, P., Nietlisbach, P., Wolak, M. E., Muff, S., Dickel, L., & Keller, L. F. (2021). Immigration counter-acts local micro-evolution of a major fitness component: Migration-selection balance in free-living song sparrows. *Evolution Letters*, *5*, 48–60. https://doi.org/10.1002/evl3.214

Reid, J. M., Keller, L. F., Marr, A. B., Nietlisbach, P., Sardell, R. J., & Arcese, P. (2014). Pedigree error due to extra-pair reproduction substantially biases estimates of inbreeding depression. *Evolution*, *68*, 802–815. https://doi.org/10.1111/evo.12305

Rekdal, S. L., Anmarkrud, J. A., Lifjeld, J. T., & Johnsen, A. (2021). Elevated phytohaemagglutinin-induced skin-swelling response at an intermediate number of MHC class II alleles in bluethroat nestlings. *Journal of Avian Biology*, *52*, jav.02734. https://doi.org/10.1111/jav.02734

Richardson, D. S., & Westerdahl, H. (2003). MHC diversity in two Acrocephalus species: The outbred great reed warbler and the inbred Seychelles warbler. *Molecular Ecology*, *12*, 3523–3529. https://doi.org/10.1046/j.1365-294X.2003.02005.x

Rodríguez, A., Broggi, J., Alcaide, M., Negro, J. J., & Figuerola, J. (2014). Determinants and short-term physiological consequences of PHA immune response in lesser kestrel nestlings: PHA immune response

on lesser kestrel nestlings. *Journal of Experimental Zoology. Part A, Ecological Genetics and Physiology*, *321*, 376–386. https://doi.org/10.1002/jez.1868

Saino, N., Bolzern, A. M., & Moller, A. P. (1997). Immunocompetence, ornamentation, and viability of male barn swallows (Hirundo rustica). *Proceedings of the National Academy of Sciences*, *94*, 549–552. https://doi.org/10.1073/pnas.94.2.549

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. *Journal of Medicinal Chemistry*, *41*, 2481–2491. https://doi.org/10.1021/jm9700575

Sardell, R. J., Keller, L. F., Arcese, P., Bucher, T., & Reid, J. M. (2010). Comprehensive paternity assignment: Genotype, spatial location and social status in song sparrows, Melospiza melodia. *Molecular Ecology*, *19*, 4352–4364. https://doi.org/10.1111/j.1365-294X.2010.04805.x

Schielzeth, H. (2010). Simple means to improve the interpretability of regression coefficients. *Methods in Ecology and Evolution*, *1*, 103–113. https://doi.org/10.1111/j.2041-210X.2010.00012.x

Schwensow, N., Fietz, J., Dausmann, K. H., & Sommer, S. (2007). Neutral versus adaptive genetic variation in parasite resistance: Importance of major histocompatibility complex supertypes in a free-ranging primate. *Heredity*, *99*(3), 265–277. https://doi.org/10.1038/sj.hdy.6800993

Sepil, I., Lachish, S., & Sheldon, B. C. (2013). *Mhc*-linked survival and lifetime reproductive success in a wild population of great tits. *Molecular Ecology*, *22*, 384–396. https://doi.org/10.1111/mec.12123

Sepil, I., Moghadam, H. K., Huchard, E., & Sheldon, B. C. (2012). Characterization and 454 pyrosequencing of major histocompatibility complex class I genes in the great tit reveal complexity in a passerine system. *BMC Evolutionary Biology*, *12*, 68. https://doi.org/10.1186/1471-2148-12-68

Skaug, H., Fournier, D., Nielsen, A., Magnusson, A., & Bolker, B. (2014). Generalized linear mixed models using AD model builder.

Slade, J. W. G., Sarquis-Adamson, Y., Gloor, G. B., Lachance, M.-A., & MacDougall-Shackleton, E. A. (2016). Population differences at MHC do not explain enhanced resistance of song sparrows to local parasites. *Journal of Heredity*, *108*, esw082–esw134. https://doi.org/10.1093/jhered/esw082

Slate, J., David, P., Dodds, K. G., Veenvliet, B. A., Glass, B. C., Broad, T. E., & McEwan, J. C. (2004). Understanding the relationship between the inbreeding coefficient and multilocus heterozygosity: Theoretical expectations and empirical data. *Heredity*, *93*, 255–265. https://doi.org/10.1038/sj.hdy.6800485

Smith, J. N. M., Keller, L. F., Marr, A. B., & Arcese, P. (2006). *Conservation and biology of small populations: The song sparrows of Mandarte Island*. Oxford University Press.

Spielman, D., Brook, B. W., Briscoe, D. A., & Frankham, R. (2004). Does inbreeding and loss of genetic diversity decrease disease resistance? *Conservation Genetics*, *5*, 439–448. https://doi.org/10.1023/B:COGE.0000041030.76598.cd

Spurgin, L. G., & Richardson, D. S. (2010). How pathogens drive genetic diversity: MHC, mechanisms and misunderstandings. *Proceedings of the Royal Society B: Biological Sciences*, *277*(1684), 979–988. https://doi.org/10.1098/rspb.2009.2084

Strandin, T., Babayan, S. A., & Forbes, K. M. (2018). Reviewing the effects of food provisioning on wildlife immunity. *Philosophical Transactions of the Royal Society B*, *373*, 20170088. https://doi.org/10.1098/rstb.2017.0088

Takahata, N., & Nei, M. (1990). Allelic genealogy under overdominant and frequency-dependent selection and polymorphism of major histocompatibility complex loci. *Genetics*, *124*, 967–978.

Taylor, S. S., Sardell, R. J., Reid, J. M., Bucher, T., Taylor, N. G., Arcese, P., & Keller, L. F. (2010). Inbreeding coefficient and heterozygosity-fitness correlations in unhatched and hatched song sparrow nestmates. *Molecular Ecology*, *19*, 4454–4461. https://doi.org/10.1111/j.1365-294X.2010.04824.x

Therneau, T.M. (2015). Coxme: Mixed effects cox models.

Verhoeven, K. J. F., Simonsen, K. L., & McIntyre, L. M. (2005). Implementing false discovery rate control: Increasing your power. *Oikos*, *108*, 643–647. https://doi.org/10.1111/j.0030-1299.2005.13727.x

Viney, M. E., Riley, E. M., & Buchanan, K. L. (2005). Optimal immune responses: immunocompetence revisited. *Trends in Ecology & Evolution*, *20*, 665–669. https://doi.org/10.1016/j.tree.2005.10.003

Vinkler, M., Svobodová, J., Gabrielová, B., Bainová, H., & Bryjová, A. (2014). Cytokine expression in phytohaemagglutinin-induced skin inflammation in a galliform bird. *Journal of Avian Biology*, *45*, 43–50. https://doi.org/10.1111/j.1600-048X.2011.05860.x

Wegner, K. M. (2003). Parasite selection for immunogenetic optimality. *Science*, *301*, 1343. https://doi.org/10.1126/science.1088293

Whittingham, L. A., Dunn, P. O., Freeman-Gallant, C. R., Taff, C. C., & Johnson, J. A. (2018). Major histocompatibility complex variation and blood parasites in resident and migratory populations of the common yellowthroat. *Journal of Evolutionary Biology*, *31*, 1544–1557. https://doi.org/10.1111/jeb.13349

Wilson, A. G., & Arcese, P. (2008). Influential factors for natal dispersal in an avian Island metapopulation. *Journal of Avian Biology*, *39*, 341–347.

Wilson, S., Norris, D. R., Wilson, A. G., & Arcese, P. (2007). Breeding experience and population density affect the ability of a songbird to respond to future climate variation. *Proceedings of the Royal Society B: Biological Sciences*, *274*, 2539–2545. https://doi.org/10.1098/rspb.2007.0643

Wolak, M. E., Arcese, P., Keller, L. F., Nietlisbach, P., & Reid, J. M. (2018). Sex-specific additive genetic variances and correlations for fitness in a song sparrow (*Melospiza melodia*) population subject to natural immigration and inbreeding. *Evolution*, *72*, 2057–2075. https://doi.org/10.1111/evo.13575

Worley, K., Collet, J., Spurgin, L. G., Cornwallis, C., Pizzari, T., & Richardson, D. S. (2010). MHC heterozygosity and survival in red junglefowl: MHC and survival in junglefowl. *Molecular Ecology*, *19*, 3064–3075. https://doi.org/10.1111/j.1365-294X.2010.04724.x

Zagalska-Neubauer, M., Babik, W., Stuglik, M., Gustafsson, L., Cichoń, M., & Radwan, J. (2010). 454 sequencing reveals extreme complexity of the class II major histocompatibility complex in the collared flycatcher. *BMC Evolutionary Biology*, *10*, 395. https://doi.org/10.1186/1471-2148-10-395

Zuur, A., Ieno, E., Walker, N., Saveliev, A., & Smith, G. (2009). *Mixed effects models and extensions in ecology with R*. Springer.

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