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All sequence data generated in this study were deposited in GenBank under accession numbers MN394222 - MN394376 (*TLR4*), MN313018 - MN313169 (*TLR5*), MN312870 - MN313017 (*TLR7*), and MN566362 - MN566421 (mitochondrial control region, HVR1).

Abstract

Over evolutionary time, pathogen challenge shapes the immune phenotype of the host to better respond to an incipient threat. The extent and direction of this selection pressure depends on the local pathogen composition, which is in turn determined by biotic and abiotic features of the environment. However, little is known about adaptation to local pathogen threats in wild animals. The Gentoo penguin (*Pygoscelis papua*) is a species complex that lends itself to the study of immune adaptation because of its circumpolar distribution over a large latitudinal range, with little or no admixture between different clades. In this study, we examine the diversity in a key family of innate immune genes - the Toll-like receptors (TLRs) - across the range of the Gentoo. The three TLRs that we investigated present varying levels of diversity, with *TLR4* and *TLR5* greatly exceeding the diversity of *TLR7*. We present evidence of positive selection in *TLR4* and *TLR5*, which points to pathogen-driven adaptation to the local pathogen milieu. Finally, we demonstrate that two positively selected co-segregating sites in *TLR5* are sufficient to alter the responsiveness of the receptor to its bacterial ligand, flagellin. Taken together, these results suggest that Gentoo penguins have experienced distinct pathogen-driven selection pressures in different environments, which may be important given the role of the Gentoo as a sentinel species in some of the world's most rapidly changing environments.

Introduction

All organisms are challenged by pathogens in their surrounding environments, but it is clear that the pathogen pressure can vary by location. Similarly to free-living metazoans, a latitudinal species richness gradient has been identified in several parasitic and pathogenic taxa, which may be driven by temperature and other abiotic and biotic factors (Rohde and Heap 1998; Guernier, et al. 2004; Dionne, et al. 2007). Given this gradient in pathogen pressure, it follows that natural selection on the host will favour distinct immune phenotypes in different environments, as suggested by MHC II genetic diversity patterns in Humboldt penguins associated with higher pathogen diversity in lower latitudes (Sallaberry-Pincheira, et al. 2015; Sallaberry-Pincheira, et al. 2016). In our study, we sought to test the hypothesis that pathogen-driven selection can drive distinct patterns of host immune system genotype and phenotype, using the Gentoo penguin (*Pygoscelis papua* ssp.) as a model species complex.

The Gentoo penguin complex (Vianna, et al. 2017; Clucas, et al. 2018) is ideally suited for investigating pathogen-driven selection on the immune system. Firstly, it has a circumpolar range, spanning the largest latitudinal range of any penguin species, between 46-66° S, with breeding

colonies in most of the Southern Ocean's sub-Antarctic islands, as well as the islands off Tierra del Fuego in South America, South Georgia, the Scotia Arc, and the Western Antarctic Peninsula (Stonehouse 1970). Secondly, population monitoring of the species has shown it to be growing at the southern end of its range (Lynch, et al. 2012), with highly fluctuating changes over time in colonies in the South Atlantic and Indian Oceans (Lescroel and Bost 2006; Trathan, et al. 2007). Thirdly, the Gentoo penguin is a highly philopatric seabird known to remain close to its breeding colonies year round (Trivelpiece, et al. 1987; Wilson, et al. 1998; Clausen and Putz 2003; Hinke, et al. 2017), limiting gene flow across breeding regions (Levy, et al. 2016; Vianna, et al. 2017; Clucas, et al. 2018).

Furthermore, across its range, Gentoo penguins overlap with (and occasionally co-occur in mixed colonies with) King (*Aptenodytes patagonicus*), Magellanic (*Spheniscus magellanicus*), Macaroni (*Eudyptes chrysolophus*), and Southern Rockhopper penguins (*Eudyptes chrysocome*) in sub-Antarctic colonies, as well as congeneric Adélie (*P. adeliae*) and Chinstrap penguins (*P. antarcticus*) in its Antarctic range. Gentoo penguin colonies are also frequented by a number of flying birds with vast ranges, including albatrosses and petrels (Order: Procellariiformes), as well as predator-scavengers like skuas (genus *Stercorarius*) and sheathbills (genus *Chionis*) that could introduce and/or spread novel avian pathogens. Levels of human interaction also vary across the range, from permanent settlements with livestock present near colonies in the Falkland/Malvinas Islands, to seasonal or year-round scientific research stations in Sub-Antarctic and Antarctic colonies, and an increasing presence of Antarctic tourism. Differences in sympatric interactions with other species across the range of the Gentoo is likely to result in different pathogen challenges and therefore different selective pressures.

To investigate genetic diversity across the immune system, many immunogenetic studies on penguins have focused on the major histocompatibility complex (MHC; Tsuda, et al. 2001; Bollmer, et al. 2007; Knafler, et al. 2012; Sallaberry-Pincheira, et al. 2016). Increasingly, however, the Toll-like receptors (TLRs) are recognised as important monogenic determinants of disease resistance phenotypes, and are therefore important operands for natural selection (Grueber, et al. 2014). Toll-like receptors are the best-studied family of pattern-recognition receptors in the vertebrate innate immune system, representing the front line of detection of pathogen challenge (Kawai and Akira 2006). TLRs respond to highly conserved microbe-associated molecular patterns (MAMPs) that are structurally conserved in large groups of pathogens. Upon binding of a MAMP, TLRs undergo dimerization and initiate an intracellular signalling cascade that culminates in the production of anti-pathogen effector molecules (Akira, et al. 2001; Botos, et al. 2011).

Vertebrates have six major families of TLRs which are typically conserved across evolutionary time to retain specificity for a particular MAMP or family of MAMPs. In most avian species, there are ten

recognized Toll-like receptors (Roach, et al. 2005; Boyd, et al. 2007; Brownlie and Allan 2011). Of these, TLR4 and TLR5 respond to the bacterial agonists lipopolysaccharide and flagellin, respectively, while TLR7 responds to single-stranded RNA of viruses in the endosomal compartment (Chow, et al. 1999; Gewirtz, et al. 2001; Lund, et al. 2004).

To investigate TLR diversity across the range of the Gentoo penguin complex, we sequenced the full coding sequences of *TLR4*, *TLR5* and *TLR7*, as opposed to targeted portions of certain exons as in previous studies (Dalton, et al. 2016a). These three genes represent bacterial- and viral-sensing Toll-like receptors that are present in almost all vertebrates. Samples (n = 155) were obtained from a broad geographic range across the range of the species (**Figure 1**), representing the largest geospatial scale of any immunogenetics study outside of humans. We describe patterns of diversity in TLRs that have a clear spatial component, and provide evidence that some of the diversity in TLR4 and TLR5 is driven by positive selection between different locations. We also demonstrate that two of the positively selected residues in TLR5 yield a phenotypic difference in the response of the receptor to flagellin, providing further evidence that Gentoo penguins have experienced differential pathogen-driven selection pressures in different environments.

Results

Amplification of TLR genes in the Gentoo penguin

Through successful amplification by PCR or whole-genome sequencing, we were able to confirm that Gentoo penguins have clear homologs of *TLR4*, *TLR5*, and *TLR7*, finding no evidence of gene loss or pseudogenization, as has been reported in other avian lineages for *TLR5* (Velová, et al. 2018) or in African penguins for *TLR7* (Dalton, et al. 2016a).

The length of the *P. papua TLR4* coding sequence matches the longest reported length (2550 bp/849 aa) in other bird species. For *TLR5*, there is a start codon that yields an open reading frame in line with the length of previously published *TLR5* sequences (2589 bp/862 aa; Velová, et al 2018), but the ORF continues upstream of the putative start codon, yielding a complete ORF that is 2643 bp/880 aa, which is 54 bp longer than other reported avian *TLR5* sequences. Both the longer and shorter ORF respond to flagellin in our *in vitro* system (data not shown), suggesting both could be functional *in vivo*. The length of the *TLR7* coding exon, at 3126 bp/1042 aa falls within the reported range of avian coding sequence lengths.

Diversity Indices and Population Differentiation

Mitochondrial Hypervariable Region (HVR1)

All colonies with more than two sampled individuals presented high levels of mitochondrial hypervariable region 1 haplotype diversity (H_d = 0.60-1.00; **Figure 2** and **Supplementary Table S1**)

as obtained in DnaSP 6.12.10 (Rozas, et al. 2017). Differentiation between colonies at this locus was significant and in line with previous data for this species (see **Supplementary Table S2D**), with four clearly differentiated clades obtained through population-level analyses in Arlequin v3.5.1.3 (Excoffier and Lischer 2010): (1) a southern clade consisting of colonies South of the Polar Front in South Georgia, the South Orkneys, the South Shetlands, and Western Antarctic Peninsula; (2) a South American/Falklands/Malvinas clade, (3) a Kerguelen clade and (4) a North Indian Ocean clade (Marion and Crozet Islands).

Our BEAST 2 phylogenetic analysis of HVR1 showed support for a division within the Gentoo penguin complex occurring approximately 3.36 Mya (1.72-4.88 Mya), when the North Indian Ocean clade diverges from all others. The Kerguelen lineage diverges from the Atlantic lineages 2.36 Mya (1.14-3.51 Mya), and the populations North and South of the Polar Front within the Atlantic Ocean appear to diverge 1.19 Mya (0.51-1.75 Mya; **Figure 3)**. Within each clade, no clear site-specific mtDNA structure was noted.

TLR4

For *TLR4*, 13 polymorphic sites were identified, with a total of 21 distinct phased haplotypes coding for 9 unique protein variants (**Supplementary Table S1**). Indian Ocean colonies (CR, MAR, COU, and MO) presented the highest levels of haplotype diversity (H_d = 0.66-0.90), while the South American colony in Tierra del Fuego (MT) and the Falkland/Malvinas Islands (CB and BR) had very low diversity (H_d = 0.00-0.05; **Figure 2**). Among Gentoo penguin colonies south of the Polar Front, diversity was highest in the central colonies of SIG, COP, SP, and BO (H_d = 0.51-0.63), decreasing sharply in BI in South Georgia (H_d = 0.00), as well as southward down the Western Antarctic Peninsula (GGV and JP; H_d = 0.08-0.26).

Comparing pairwise genetic distances (F_{ST} and Φ_{ST}) for in Arlequin *TLR4* (**Figure 5** and **Supplementary Table S2A**), the Indian Ocean populations of Crozet and Marion differed significantly ($F_{ST} > 0.3$, $p < 0.01$) or near-significantly (corrected $p \sim 0.01$) from all other colonies in the Atlantic. Crozet and Marion did not differ significantly from each other (F_{ST} = 0.001, p = 0.40), while some haplotypes were shared between CR/MAR and the Kerguelen Island colonies of COU and MO, yielding some non-significant pairings among the four Indian Ocean colonies. In the Atlantic, one haplotype, also present in the Indian Ocean in lower frequencies, dominated across all the colonies (**Figure 4**). The central sites in the Scotia Arc (COP/SP on King George Island, Signy Island, and BO on the northern end of the Western Antarctic Peninsula) exhibited greater diversity than other Atlantic sites and contained private alleles. The overall pattern of significance for F_{ST} and Φ_{ST} comparisons can be seen in **Figure 5**. Not surprisingly, colonies within the same island group that are in close proximity to each other (COU/MO, CB/BR, COP/SP) did not differ significantly, despite variations in sample sizes.

Hierarchical population structure was detected for *TLR4* across Gentoo penguin colonies using Arlequin (AMOVA, global $F_{ST} = 0.32$, $p < 0.0001$). The proportion of variation resulting from differences among groups was 24.01% ($F_{CT} = 0.24$, $p = 0.003$) when colonies were placed into four groups, coinciding with the four mtDNA clades: (1) Marion/Crozet archipelagos; (2) Kerguelen Is.; (3) Falkland/Malvinas and Tierra del Fuego; and (4) south of the Polar Front in the Scotia Arc and Antarctic Peninsula. However, F_{CT} increased to 28.19% ($p = 0.007$) when Bird Island was grouped separately from other Southern Gentoo penguin colonies, a pattern also suggested in genomic-level analyses (Clucas, et al. 2018).

TLR5

TLR5 was the most diverse TLR locus analysed. Twenty polymorphic sites were identified, with a total of 46 distinct phased haplotypes coding for 32 unique protein variants (**Supplementary Table S1**). Five Gentoo penguin colonies north of the Polar Front (COU/MO in Kerguelen, CB/BR in Falklands/Malvinas and MT in Tierra del Fuego) exhibited the highest diversity measures (6-17 haplotypes, $H_d = 0.77$-0.92, **Figure 2**), despite differences in sample size. This is unexpected given that Martillo Island is the smallest known population of this species ($N_c = 12$ breeding pairs, Ghys, et al. 2008), yet still maintained high diversity (5 unique protein variants in a sample of n = 5) at this locus. Interestingly, Crozet and Marion Island colonies exhibited substantially lower genetic variation at this locus with only two haplotypes ($H_d = 0.13$-0.44), though these were shared with the Kerguelen colonies ($H_d = 0.86$-0.87; **Figure 4**). Southern colonies exhibited moderate diversity ($H_d = 0.51$-0.65), with the exception of SP in the South Shetland Islands and the Western Antarctic Peninsula at the edge of the range, with only two or three haplotypes ($H_d = 0.12$-0.38). Strikingly, Atlantic colonies south of the Polar Front were dominated by one haplotype found less frequently (10-28%) in the northern Atlantic colonies and completely absent from all Indian Ocean colonies. The second-most prevalent haplotype in southern colonies was completely absent from all northern colonies.

Pairwise F_{ST} and Φ_{ST} values obtained in Arlequin revealed significant clustering by clade in terms of genetic distance at the *TLR5* locus (**Figure 5** and **Supplementary Table S2B**). Within three of the four clades mentioned above, there were no significant differences (CR/MAR, COU/MO, and CB/BR/MT). The only significantly differentiated within-clade pairs lay in Gentoo penguin colonies south of the Polar Front, where Jougla Point differed significantly from COP (highest diversity among Southern Gentoo penguins) and BI (at the opposite geographic edge of this clade's range). All other colonies differed significantly from all colonies outside their clade (F_{ST} range 0.1-0.88, $p < 0.01$), with CR/COU being near-significant ($F_{ST} = 0.13$, $p = 0.02$). This was reflected in AMOVA results, where a four-clade grouping presented a proportion of variation resulting from differences among groups of 32.70% ($F_{CT} = 0.327$, $p < 0.0001$), and isolating Jougla Point increased the F_{CT} to 56.56% ($p < 0.0001$).

TLR7

TLR7 was the least diverse TLR locus, with 9 polymorphic sites, 10 phased haplotypes, and 8 unique protein variants present across the study colonies (**Figure 2** and **Supplementary Table S1**). One haplotype was predominant in all colonies (frequency of 70-100%) and 7 of the 10 haplotypes were private alleles, only found in single colonies (**Figure 4**). In the Indian Ocean, Kerguelen colonies (COU/MO) had relatively more diversity (H_d = 0.34-0.50) than the Crozet and Marion Island colonies (H_d = 0.00), which had only one haplotype. In the Atlantic, South Georgia (BI) had only one haplotype (H_d = 0.00), while all northern Atlantic colonies (CB/BR/MT) and SP in the South Shetland Islands presented two haplotypes (H_d = 0.06-0.47). Other southern colonies contained 3-4 haplotypes (H_d = 0.38-0.60), while the southernmost colony at Jougla Point (JP) on the Western Antarctic Peninsula exhibited the most unique haplotypes (H = 5; H_d = 0.38) and contained two private alleles. Unsurprisingly, only 1 of 91 pairwise comparisons between colonies were significant in terms of F_{ST} and 3/91 for Φ_{ST} ($p < 0.01$), with no pattern to this differentiation (**Figure 5** and **Supplementary Table S2C**). Different AMOVA groupings yielded an among-group variation (F_{CT}) no higher than 2.85% (four-clade grouping), further highlighting the lack of structure in this locus.

Effect of Population Size on Diversity

Census population size was not significantly correlated to TLR haplotype diversity (See **Supplementary Figure S1** and **Supplementary Tables S3 and S4**: *TLR4* p = 0.067; *TLR5* p = 0.75; *TLR7* p = 0.64).

Isolation by Distance

Significant isolation by distance, using shortest distances by sea between colonies in a Mantel's test, was detected in both *TLR4* (r = 0.515, p = 0.001) and *TLR5* (r = 0.593, p = 0.001; **Supplementary Table S5A**). mtDNA HVR1 was less strongly correlated to isolation by distance (r = 0.312), though marginally significant (p = 0.015). On the other hand, the lack of diversity and structure in the *TLR7* locus yielded no significant correlation across the range of Gentoo penguin colonies sampled.

Analysis of Positive Selection

We investigated the *P. papua TLR4*, *TLR5* and *TLR7* genes for evidence of positive selection, which could be an indicator of adaptation to local pathogen environments across the natural range of the species. Neutrality tests (Tajima's *D* and Fu's *Fs*) did not yield observable patterns of significant deviation from neutrality across the full length of these genes (**Supplementary Table S1**). Using a codon-specific approach, the site models in the *codeml* package of programs in PAML v4.9 (Yang 1997; Yang 2007) were employed to test for signatures of positive selection in *P. papua* TLR loci.

Non-synonymous sites observed and analysed are graphically depicted in their relative positions on the proteins in **Figure 6**.

As expected, the majority of codons (*TLR4*, 98.8%; *TLR5*, 98.4%) were predicted to be under purifying selection with the ratio of non-synonymous to synonymous substitutions (dN/dS) being < 1. Interestingly, 1.2% (*TLR4*) or 1.6% (*TLR5*) of codons in the alignment were found to be positively selected using M2a, and similar frequencies, 1.2% (*TLR4*) or 1.7% (*TLR5*), were found using M8. For *TLR7*, 99.9% of sites were found to be under purifying selection, while the remaining 0.1% were predicted to be under neutral selection. We investigated whether models that permit positive selection were a significantly better fit to the multiple alignments than models where $dN/dS \not> 1$ by performing likelihood ratio tests between pairs of models. For *TLR4* and *TLR5*, all model comparisons (M1a *vs.* M2a, M7 *vs.* M8, and M8a *vs.* M8) significantly favoured the positive selection model compared to the neutral model (*TLR4*, $p \leq 0.017$; *TLR5*, $p \leq 7.2\times10^{-23}$ for all comparisons), indicating that *P. papua* *TLR4* and *TLR5* have likely undergone positive selection. In contrast, for *TLR7*, the data were not a significantly better fit to the positive selection model compared to the neutral model ($p = 1$) so the null hypothesis of codons being negatively- and neutrally-selected was not rejected.

For *TLR4* and *TLR5*, we then used the Bayes Empirical Bayes (BEB) algorithm to infer the posterior probability that a particular codon has experienced positive selection. For *TLR4*, three codons were predicted to have undergone positive selection at posterior probability of >0.90 under model M2a (**Supplementary Tables S6A-C**). All but one of the *TLR4* polymorphic residues (12, 82, 236, 316 and 445) were located in the extracellular (LRR) domain, of which two were positively selected (12 and 236). The final positively selected site (659) was located in the transmembrane domain (**Figure 6**). Of the three selected sites, one (12V/A) is a relatively non-conservative change (see **Supplementary Table S7** for amino acid distance metrics). One site in TLR4 (12) has previously been found to be under positive selection in birds (Velová, et al. 2018), while the remaining two (236 and 659) are novel selected sites in penguins (**Supplementary Table S8**).

For TLR5, there were 13 amino acid variants, of which nine were predicted to have undergone positive selection at posterior probability of >0.90 in both M2a and M8 (**Supplementary Table S6D-F**). Of these, four were located in the extracellular domain (10, 285, 442 and 535), one was in the transmembrane domain (667), and four were located in the TIR (intracellular) domain (698, 747, 788 and 845; **Figure 6**). Two sites (442 and 698) have previously been reported as being positively selected in other birds (Grueber, et al. 2014; Velová, et al. 2018), while the remaining seven sites are novel selected sites in penguins. Interestingly, one site in the TLR5 extracellular domain (285) is adjacent to two residues known to be important for flagellin binding in Interface B (Yoon, et al. 2012; Song, et al. 2017), and so could be important for ligand preference (**Supplementary Table S8**; **Supplementary Figure S2**). Four positively selected sites in TLR5 were non-conservative changes

(10C/Y, 442A/V, 667S/F and 788S/C). It is interesting to note that the non-conservative TLR5 667F/S polymorphism is located in the transmembrane domain, a region that is typically constrained by the physiochemical requirement to embed in the cell membrane. Furthermore, the homology-based methods of amino acid substitution consequence prediction SIFT (Ng and Henikoff, 2003) and PolyPhen-2 (Adzhubei, et al. 2013) both predict the S667F change to be of high functional consequence (SIFT score = 0.01; PolyPhen-2 score = 0.998; **Supplementary Table S9**). Transmembrane integrity is predicted to remain intact, despite the non-conservative polymorphism, and the Phobius tool (Käll, et al. 2004) predicts the transmembrane domain is unchanged by the polymorphism. While the vast majority (57/64, 89% of available sequences) of avian TLR5 have a serine in this position in the transmembrane domain, only one other bird - the Northern Fulmar (*Fulmarus glacialis*) - has a phenylalanine in this position (**Supplementary Figure S3**), indicating there likely to have been a functional consequence to the S667F change in the Gentoo penguin.

Functional Analysis of Selected TLR5 Residues

While *in silico* methods can be useful indicators of protein residues under selection, functional study is the only means to isolate the selected phenotype and its relevance. In order to assess whether key selected sites identified in the positive selection analyses have functional consequences, we developed an *in vitro* assay using transient expression of TLRs in a reporter cell line.

Since extracellular domain polymorphisms in TLRs are likely to give rise to preferences in ligand type (Faber, et al. 2018), we focused on TMD/TIR domain polymorphisms that are likely to give rise to differential signalling in response to the same agonist. Given that five *TLR5* polymorphisms were located in the TMD/TIR domain, we tested the two polymorphisms with the highest posterior probability of selection from the PAML analysis – residues 667 (TMD) and 845 (TIR). Polymorphisms at these positions segregate well with geographical location: birds from Crozet and Marion were all homozygous for the 667S/845I haplotype, while 87.3% (n = 71) of birds from colonies South of the Polar Front were homozygous for the derived haplotype, 667F/845V (**Figure 7**). In the Kerguelen Islands, the ancestral 667S/845I haplotype predominated, but variants at both positions were present at lower frequencies. South American/Falklands/Malvinas colonies were the most diverse at the positions of interest, where 46.7% (n = 14) of birds were heterozygous at one or both of the sites. Overall, however, polymorphisms at these loci tended to co-segregate: 72.4% (n = 110) of birds were homozygote for either the ancestral (667S/845I) or derived (667F/845V) haplotypes.

Given the strong tendency for the alleles at these positions to co-segregate at the extremes of the range of *P. papua*, and the fact that these were the TIR/TMD polymorphisms with the highest likelihood of positive selection, the functional consequences of altering both residues together were investigated. FLAG-tagged constructs of both of the TLR5 TIR/TMD variants with the same LRR domain were

transiently expressed in *TLR5*$^{-/-}$ HEK-Blue™ Null1 NF-κB reporter cells and protein expression levels were normalised using an anti-FLAG ELISA. Cells expressing either constructs were then treated with *Salmonella enterica* serovar Typhimurium-derived flagellin, or PBS control, and the NF- κB response was measured. Cells expressing either construct responded to flagellin, demonstrating that the *P. papua* TIR domain interacts efficiently with human adapter molecules. Interestingly, there was a marked enhancement (~1.5 fold, $p = 0.04$) of the flagellin response in the variant that was predominantly found in the Southern Gentoo compared to the variant found in the Indian Ocean clades, suggesting that the derived haplotype 667F/845V has enhanced signalling capability compared to the ancestral genotype 667S/845I (**Figure 7**). These data provide further evidence that *P. papua TLR5* has undergone positive selection for different immune capabilities during the expansion of the species below the Polar Front towards the Antarctic Peninsula.

Discussion

All vertebrates are subject to challenge by a plethora of pathogens that can exert strong selective pressures on host populations. The innate immune system, and in particular the Toll-like receptors, is responsible for both recognizing, and responding to, a pathogen threat by inducing inflammation and priming the adaptive immune response. To investigate TLR adaptation in Gentoo penguins, we sequenced the entire coding regions of *TLR4, TLR5* and *TLR7*, which recognize products from bacterial and viral pathogens. Multiple individuals were sequenced from colonies at the extremes of the species' range (~8000 km between the most distant colonies), providing an extensive geospatial component to our analysis. We found spatially-associated patterns of diversity in the TLRs, although greater diversity was observed in *TLR4* and *TLR5* compared to *TLR7*. Furthermore, clear evidence of positive selection in both *TLR4* and *TLR5* was identified, which was further reinforced by the demonstration that two of the TLR5 TMD/TIR domain polymorphisms are sufficient to alter the magnitude of responsiveness to flagellin. To our knowledge, no other TLR study outside of humans has supported predictions of positive selection with confirmation of functional polymorphism.

Patterns of Diversification and Selection

Most studies of TLR genetic diversity in wild populations have investigated small, bottlenecked, and/or endangered avian populations (Grueber, et al. 2012; Grueber, et al. 2013; Hartmann, et al. 2014; González-Quevedo, et al. 2015; Dalton, et al. 2016a; Dalton, et al. 2016b). In these populations, drift, rather than selection, is suspected to have been the main driver of sampled diversity due to recent bottlenecks or pronounced founder effects. Several studies have documented TLR diversity in domesticated animals such as pigs (Darfour-Oduro, et al. 2016), cows (Novak, et al. 2019), and chickens (Świderská, et al. 2018), but large-scale studies on whole-gene TLR variation in a wild population are lacking. Although study design can dramatically affect the diversity detected in different studies it is noteworthy that with Gentoo penguins *TLR5* exhibited higher diversity than

TLR4 whereas with domestic chicken breeds (n=110, 25 breeds; Świderská, et al. 2018) and grey partridge (n=10; Vinkler, et al. 2015) *TLR4* was more diverse than *TLR5* (**Supplementary Table S10**). The diversity of *TLR7* (compared with *TLR4* and/or *TLR5*) was relatively low in Gentoo penguins, domestic chicken breeds and grey partridges (**Supplementary Table S10**).

To provide an internal reference for TLR diversity, we sequenced the mitochondrial hypervariable region (HVR1) as a neutral marker in the same individuals. In line with previous studies, we found evidence of at least four deeply divergent clades in *P. papua* based on HVR1 sequence (Vianna, et al. 2017; Clucas, et al. 2018; Pertierra, et al. in review). These more recent analyses support a revision, first proposed by de Dinechin et al. (2012), of the previously-accepted two subspecies model that was based on morphological characteristics (Stonehouse 1970). These four clades are likely to have much greater divergence (millions of years) than what would be expected at the intraspecific level. We found evidence of differentiation according to this underlying population structure in *TLR4* and *TLR5* which further supports the argument for taxonomic revision of the species, with particular focus on the classification of colonies in South Georgia and the Indian Ocean. Conversely, *TLR7* was highly conserved across the species range and is clearly not subject to the same selection pressures as *TLR4* and *TLR5*. Overall, our study highlights that the genetic differentiation across Gentoo penguin clades is not just driven by drift, but by clear population-specific adaptations to the environment.

Diversity has been widely reported to vary between different families of TLRs, particularly comparing extracellular and intracellular TLRs. Some authors have reported that TLRs that respond to viral ligands are more likely to be under purifying selection, at least in mammals (Barreiro, et al. 2009; Wlasiuk and Nachman 2010; Wang, et al. 2016; Kloch, et al. 2018), although the pattern may not be consistent in birds, with *TLR3* displaying the greatest number of non-synonymous variants of the four TLRs tested in different chicken breeds (Świderská, et al. 2018), and *TLR7* diversity in the house finch (*Carpodacus mexicanus*) far exceeding that of *TLR4* and *TLR5* (Alcaide and Edwards 2011). Consistent with the pattern observed in mammals (and also the lesser kestrel, *Falco naumanni*; Alcaide and Edwards 2011), we observed overall nucleotide diversity measurements that were several times higher for the extracellular/bacterial TLRs *4* and *5* (*TLR4*: $6.1 \times 10^{-4} \pm 0.5 \times 10^{-4}$; *TLR5*: $14.8 \times 10^{-4} \pm 0.6 \times 10^{-4}$) compared to the intracellular/viral *TLR7* ($1.0 \times 10^{-4} \pm 0.1 \times 10^{-4}$), indicating strong purifying selection for maintenance of function in *TLR7*. In addition, we found no evidence of any codons under selection in *TLR7*, compared to three and nine sites in *TLR4* and *TLR5*, respectively, similar to the pattern of positively selected residues reported in several avian species (Alcaide and Edwards 2011).

Within TLR sequences, levels of variation are not uniformly distributed across the domains of the receptor. TLRs are type I integral membrane glycoproteins with highly conserved architecture across large phylogenetic distances (Botos, et al. 2011). Typical TLR structure comprises an N-terminal

extracellular (or intraluminal for intracellular TLRs) leucine-rich repeat (LRR) domain for ligand binding, a single transmembrane helix, and a C-terminal cytoplasmic signalling (Toll/interleukin-1 receptor, TIR) domain interacting with intracellular adapter proteins (Bell, et al. 2003). The leucine-rich repeat domain directly binds microbe-derived ligands in all known vertebrate TLRs, with the exception of TLR4 recognition of lipopolysaccharide via an accessory molecule, myeloid differentiation factor, MD-2 (Park, et al. 2009). Since the LRR domain represents the interface between host and pathogen, and pathogens exhibit variable MAMPs to evade detection (Andersen-Nissen, et al. 2005), there is often an excess of diversity in the LRR domain compared to the TIR domain (Świderská, et al. 2018; Velová, et al. 2018). In contrast, TIR domains interact with adapter proteins such as MyD88 (myeloid differentiation primary-response protein 88) which are shared between several TLRs, although a MyD88-independent pathway also facilitates TLR3 and TLR4 signalling (Akira and Takeda 2004). Unsurprisingly, TIR domains were found to be much more highly conserved than their corresponding extracellular domains in a study of 366 vertebrate TLRs from 96 species with the exception of *TLR10* (Mikami, et al. 2012). Within species, the same trend is evident: in a study of *TLR3*, *4*, *5* and *7* diversity across domestic chicken breeds, only three of the 46 non-synonymous polymorphisms (two in *chTLR3* and one in *chTLR7*) were located in the TIR domain (Świderská, et al. 2018).

In line with previous evidence for the asymmetric distribution of polymorphisms in TLR domains, we identified an excess of polymorphisms in the LRR domain compared to the TIR domain in two of the three TLRs studied (*TLR4*: 8 LRR vs. 0 TIR; *TLR7*: 6 LRR vs. 0 TIR). Interestingly, *P. papua TLR5* contained a greater number of TIR domain polymorphisms than would be expected from other species (11 LRR vs. 7 TIR), particularly given the LRR is over three-times the length of the TIR domain. Of the seven *TLR5* TIR domain polymorphisms, five were non-synonymous substitutions, suggesting that the TIR domain of *TLR5* has been under selection to modulate signalling intensity.

Somewhat surprisingly, we also identified non-synonymous polymorphic sites in the transmembrane domains of both *TLR4* (659 Ala/Thr) and *TLR5* (667 Ser/Phe). The transmembrane domain is an uncommon location for TLR polymorphisms, presumably because the region is highly constrained by chemical and functional requirements. As such, the effects of polymorphisms in this region are often large. For instance, the human *TLR1* 602S variant is associated with disrupted cell surface localization of the receptor but is protective against pathology associated with leprosy. It is also noteworthy that the Gentoo penguin TLR5 transmembrane polymorphic site (667) identified in this study is highly conserved elsewhere in avian phylogeny. Of the other birds with published TLR5 sequences, displayed in the alignment (**Supplementary Figure S3**), 57 (89%) have a serine (ancestral *P. papua* genotype) at this position in the transmembrane domain, and only one other bird – the Northern Fulmar (*Fulmarus glacialis*) – has a phenylalanine residue (derived *P. papua* genotype). The high conservation of serine at this position in the protein points to a widespread pressure for maintenance

of function across avian phylogeny, and provides more evidence of a positively selected residue with functional consequences.

Functional polymorphisms in *TLR5* support positive selection

We identified a number of positively selected codons in both *TLR4* and *TLR5*, making both of these receptors candidates for further functional investigation. Polymorphisms in TLR LRR domains have the potential to yield preferences for subtly different microbial ligands, such as LPS or flagellins from different bacterial species (Nahori, et al. 2005; Faber, et al. 2018). However, very limited data are available regarding which pathogens are present in the environments of each of the Gentoo clades, and therefore elucidation of any differences in ligand preference will require further study. We did, identify one positively selected site in TLR5 (285) that is adjacent to two important resides for flagellin binding in Interface B (Yoon, et al. 2012; Song, et al. 2017). This site would be a good candidate for functional investigation of changes in flagellin ligand preferences, but this would be difficult in the absence of known flagellin variants in candidate *P. papua* pathogens. Instead, we chose to investigate TIR and transmembrane domain polymorphisms for functional consequences because these can yield signalling intensity differences in response to the same agonist (Faber, et al. 2018). Given that the TIR domain of TLR4 did not show any non-synonymous polymorphisms, we focused on the TLR5 TIR/transmembrane domain, and in particular the two residues with the strongest signature of positive selection (667 and 845). Site 667 was likely to be of significant functional consequences because of its transmembrane location, non-conservative amino acid change (serine to phenylalanine) and both SIFT and PolyPhen-2 predicting the change to be of high importance.

The Gentoo penguin is reported to have undergone a circumpolar expansion, with ancestral populations in the Indian Ocean seeding northern populations that expanded into the Atlantic, and further expansions south of the Polar Front and to the West Antarctic Peninsula – the southernmost extreme of the range (de Dinechin, et al. 2012; Peña, et al. 2014). It is interesting to note that one of the ancestral Indian Ocean clades (Marion and Crozet archipelagos) is completely dominated by birds of the 667S/845I genotype, while the most derived clade of Gentoo penguins south of the Polar Front are almost entirely dominated by the 667F/845V genotype. These data may reflect an incipient selective sweep of the 667F/845V genotype in Southern Gentoo penguins.

An alternative explanation for the reduction in diversity at residues 667 and 845 could be genetic bottlenecking during the expansion of *P. papua* south of the Polar Front. However, evidence from neutral markers in this study and a previous study (Levy, et al. 2016) reveal that neutral variation is maintained in the Southern Gentoo penguin colonies at levels comparable with the northern Atlantic Gentoo penguin clade up to the southernmost extreme of the range. In *TLR5*, we also saw no correlation between census population size and haplotype diversity. These findings support the

hypothesis that a selective sweep, rather than a bottlenecking event, is responsible for the near-fixation of the *TLR5* 667F/845V haplotype in the Southern Gentoo penguin. Perhaps more importantly, the finding that the 667F/845V haplotype has enhanced signalling capability provides a functional basis for selection of this TLR5 haplotype.

Potential drivers of selection

Toll-like receptors, like other genes of the immune system, are subject to competing types of selection. Balancing selection works to maintain diversity at a population level in response to the diversity of pathogens in the environment, as was recently proposed in TLRs of the bank vole (*Myodes glareolus*; Kloch, et al. 2018). In contrast, purifying selection may predominate (to retain key functionality), which has been described in large-scale studies of human TLRs in different ethnic backgrounds (Mukherjee, et al. 2014). Finally, positive selection may promote the fixation of novel variants that confer a fitness advantage in the response to pathogens. In the present study, we found evidence of positive selection in *TLR4* and *TLR5*, which likely indicates that the pathogen composition differs substantially between distinct locations in the Gentoo penguin's range.

Spatial heterogeneity in the profile of pathogens that afflict Gentoo penguins would be a key driver for the patterns of selection identified in the TLR variants. Latitudinal species diversity gradients have been described for pathogens (and their hosts) (Rohde and Heap, 1998; Guégan, et al. 2008, Dionne, et al. 2007), which might suggest fewer pathogens in Antarctic species. However, a diverse range of pathogens are found in these environments (discussed below). Moreover, within the Gentoo range there are diverse biotic and abiotic characters that exhibit spatial variation (Trathan, et al. 2007, Barbosa, et al. 2009, Barbosa, et al. 2013, Lamont, et al. 2018; Chown, et al. 2015) and these factors will affect the transmission of pathogens. Indeed, the regionalized selection of TLR alleles in different sectors of the Gentoo range support the premise that different challenges are more prevalent or pathogenic in different populations.

The dense colonial conditions and ubiquitous guano (faeces) that characterise Gentoo penguin habitats provide ideal conditions for the transmission of a wide range of pathogens transmitted by direct contact or faeces. Furthermore, penguins as a group are known to be highly susceptible to a variety of infectious diseases, including, avian cholera (Jaeger, et al. 2018), avian pox (Kane, et al. 2012), avian malaria (Fix, et al. 1988; Grilo, et al. 2016) and aspergillosis (Flach, et al. 1990). A number of infection associated mass mortality events have been documented in both wild and captive penguin populations (Grimaldi, et al. 2015). However, little is known about the pathogens that exist in sub-Antarctic and Antarctic regions, their prevalence, or their fitness costs on penguin populations. Limited data are available on the prevalence of diseases in penguin populations (Clarke and Kerry 2000; Barbosa and Palacios 2009; Woods, et al. 2009; Grimaldi, et al. 2015), and most studies rely upon short notes, observations, and case reports closely tied to obvious signs of disease and mass

mortality in well-studied and highly visited penguin colonies. Studies that survey the environmental and host microbiomes to characterise pathogen presence in polar regions remain limited to sites near major polar research stations, have small sample sizes, and/or do not cover large spatial and temporal ranges (Zdanowski, et al. 2004; Dewar, et al. 2013; Fan, et al. 2013; Ma, et al. 2013; Dewar, et al. 2014).

The presence of Gram-negative bacteria exhibiting both lipopolysaccharides and flagella, including *Campylobacter*, *Escherichia*, *Salmonella* and others, has been demonstrated in Gentoo penguin colonies (Dimitrov, et al. 2009; Bonnedahl 2011; Barbosa, et al. 2013; González-Acuna, et al. 2013; García-Peña, et al. 2017). However, it is not known whether any of these (or other bacterial pathogens) vary across the Gentoo penguin's range, or may have played a role in the selection of Gentoo penguin *TLR4* or *TLR5* variants.

Studies of single-stranded RNA viruses (which would typically be recognised by TLR7) are similarly lacking in Gentoo penguins. Though single-stranded RNA viruses, including the causative agents of Newcastle disease virus and avian influenza, have occasionally been detected in *Pygoscelis* penguins through immunological assays and direct isolation (Morgan and Westbury 1988; Wallensten, et al. 2006; Neira, et al. 2017; Olivares, et al. 2019; Wille, et al. 2019), the fitness consequences of viral infection on penguin populations are unknown. We know of only one case report from Signy Island where evidence of a puffinosis outbreak (normally caused by Coronavirus) was described in Gentoo penguin chicks (Mac Donald and Conroy 1971). The viral drivers behind the strong purifying selection we observed in *TLR7* are unknown, but it could be that the ssRNA viruses that affect Gentoo penguins are less diverse across the species range than flagellated Gram-negative bacteria.

Sympatric interactions with a diversity of migratory flying birds and their parasites may be important contributors to pathogen diversity in Gentoo penguin colonies. Birds such as albatrosses, petrels, shearwaters, sheathbills, shags, gulls, terns, and skuas are often observed in close proximity to Gentoo penguin colonies and there are 46 species recognised in Antarctica alone (Lepage, et al. 2014). There is some evidence that ectoparasites and blood parasites are transmitted between co-occurring bird species (Barbosa et al. 2011; Levin, et al. 2013). It is plausible that cross-species transmission events are important in cross-colony transmission and structuring the profile of pathogens afflicting particular Gentoo penguins.

It remains unclear why the two functionally tested TMD/TIR residues in TLR5 would confer increased responsiveness to flagellin in Gentoo penguins south of the Polar Front. TLR signalling must be tightly controlled and aberrant TLR-induced inflammation can lead to immune pathology, toxic shock syndrome and death. It is unsurprising, therefore, that TLR polymorphisms have been described that confer reduced sensitivity to their agonist and a state of tolerance. For instance, the replacement of a highly conserved proline residue by a histidine at position 712 of TLR4 confers

endotoxin resistance in certain strains of mice (Qureshi, et al. 1999). It could be that the exposure to (or diversity of) pathogens is decreased for the Southern Gentoo penguin clade compared to other clades of Gentoo penguins, and thus individuals can tolerate enhanced signalling to a prevailing infection. Alternatively, the enhanced signalling could be a manifestation of the Southern Gentoo penguin adapting to a particular pathogen that is present in the West Antarctic Peninsula and absent elsewhere. The finding of adaptive changes in the Gentoo penguin immune system necessitates a much better understanding of the pathogen threats faced by Gentoo penguins in order for their significance to be realized.

Concluding remarks

This wide-ranging immunogenetic study of Toll-like receptors in wild Gentoo penguins reveals differential selection and adaptation to local pathogen pressure. While the drivers behind the observed patterns of diversity and selection remain unclear in the context of currently available data, it is clear that the Gentoo penguin has undergone adaptation to local pathogen assemblages across its range.

Infectious disease threats to penguins are likely to become ever more severe in the coming decades given the rapidly changing polar climate (Mayewski 2012; Lynch, et al. 2012). There is also evidence of reverse zoonosis of enteric bacteria being transmitted from humans to sea bird species in Antarctica (Cerda-Cuellar, et al. 2019), which could further increase transmission, especially in light of increasing tourist and scientific research program presence in Antarctica. Although the Gentoo penguin is not currently one of the 13 out of 18 penguin species with a conservation state of threatened or near-threatened, certain sub-Antarctic populations have experienced sharp declines (Crawford, et al. 2003; Lescroel and Bost 2006; Crawford, et al. 2009; Crawford, et al. 2014). Consequently, the vulnerability of pathogen-naïve populations of penguins should not be underestimated, nor should the importance of the Gentoo penguin as a sentinel species in the Southern Ocean (Carpenter-Kling, et al. 2019).

Our findings have important implications for the conservation of not just Gentoo penguins, but also many other vertebrate species, both in the wild and in captivity. Until now, most efforts to genetically delineate conservation units have relied mostly on neutral markers. The ever-increasing availability of genomic data allows targeted analysis of pathogen-recognition and other immune genes to assess whether different populations possess specific functional adaptations to their environments and should therefore be conserved separately. The approach used here, together with pathogen discovery and surveillance systems, could better define conservation units in species that occupy varied habitats and ecological niches in order to focus resources on potentially susceptible populations.

Methods

Sample Collection

This study used 155 blood samples from Gentoo penguins, previously obtained in the framework of other projects. Samples were collected between 1999-2017 at the 14 sites shown in **Figure 1** (details in **Supplementary Table S11**). To take blood, penguins were held with the flippers restrained and the head placed under the arm of the handler, or they were wrapped in cushioned material covering the head and preventing movement, to minimize stress during handling (Lemaho, et al. 1992). A second handler took up to 1 mL blood from the brachial, intertarsal or jugular vein using a 25G or 23G needle and 1 mL syringe or capillary, after cleaning the area with an alcohol swab. Total restraint time was generally two to three minutes. The animal was then released at the edge of the colony and observed to ensure it returned to its normal behavior. Blood was stored in 95% ethanol or Queen's Lysis Buffer at -20 °C for transport at room temperature and subsequent storage at -20 °C upon arrival. All blood samples were imported under the appropriate animal by-product import licenses.

Sampling was conducted under permits from each site's territorial government or governing agency. These permits for animal handling were issued following independent institutional ethical review of the sampling protocols, in accordance with Scientific Committee for Antarctic Research (SCAR) guidelines.

DNA extraction

DNA for samples from MO, CB, BI, COP, and JP was extracted from blood samples using QIAGEN DNeasy Blood and Tissue kits. The digestion step was modified to include 40 µL proteinase K and extended to 3 hours for blood samples. Details of the modifications made to the protocols for tissue samples are available in (Younger, Emmerson, et al. 2015; Younger, Clucas, et al. 2015). All these samples were treated with 1 µL Riboshredder (Epicentre) to reduce RNA contamination and DNA was visualized on a 1% agarose gel to confirm high molecular weight DNA was present. DNA concentration and purity was measured on a Qubit and Nanodrop (Thermofisher Scientific), respectively. These samples are stored at the University of Oxford for future analysis. DNA from all other sampling sites was isolated using a modified salt protocol (Aljanabi and Martinez 1997), with details in Vianna, et al. (2017), stored at the Pontificia Universidad Católica de Chile for future analysis.

TLR Genotyping

Primers for *TLR4*, *5*, and *7* coding regions were designed using the primer design feature based on Primer3 2.3.7 (Untergasser, et al. 2012) in Geneious v.11.0 (Biomatters, http://www.geneious.com). Reference coding sequences for primer design were derived from the congeneric Adélie penguin (*Pygoscelis adeliae*) reference genome (Genbank accession JMFP01000000) and unpublished Gentoo penguin genomic data. *TLR4* amplifications for samples from MO, CB, BI, COP, and JP, were conducted in 12 µL volumes (9 µL Qiagen *Taq* PCR Master Mix, 2 µL 10 µM forward and reverse primer mix, and 1 µL of template DNA diluted 1:100). *TLR5* and *TLR7* amplifications for these

samples were conducted in 25 µL volumes containing 5 µL 5X Phusion High Fidelity (HF) Buffer (New England Biolabs, UK), 0.5 µL 10 mM dNTPs, 1.25 µL of 10 µM forward primer, 1.25 µL of 10 µM reverse primer, 2 µL of template DNA diluted 1:100, 0.25 µL of Phusion Hot Start Flex DNA Polymerase (New England Biolabs, UK), and 14.75 µL nuclease-free water. One GC-rich region in *TLR7* required the use of Phusion GC buffer, rather than HF buffer for amplification. PCR products were visualized on a 1% agarose gel stained with SYBR Safe. The primers and PCR reaction conditions are fully detailed in the Supplementary Information (**Supplementary Table S12**). PCR products were sequenced using Macrogen Europe's EZ-Seq (*TLR4*) or Eco-Seq (*TLR5* and *TLR7*) services (http://www.macrogen.com, Netherlands) for purification and Sanger sequencing, using the same PCR primers for sequencing.

For all remaining sampling sites, which underwent whole genome sequencing, a total of 100 ng of genomic DNA was fragmented to an average of 350 base pairs to construct paired-end libraries using the Illumina TruSeq Nano kit with the included indexed adapter and barcode. A total of six PCR cycles were used for enrichment, purified with Sample Purification beads, quantified using a Qubit fluorometer and then sequenced to ~20x coverage with 150 paired-end reads using an Illumina HiSeq X platform at MedGenome (USA).

Sequences for each TLR coding region were assembled, edited, and aligned using Geneious v.11.0. For *TLR4*, which has multiple exons along the coding region, exon sequences were extracted and concatenated for further analysis. Heterozygous sites and single nucleotide polymorphisms (SNPs) were detected by visually examining chromatograms. In cases of doubt, resequencing was accomplished so that only high-quality reads from multiple sequencing runs were called as SNPs. All heterozygous sites also had homozygous individuals within the dataset, and each gene had at least one haplotype homozygous across the full length of the gene. All alleles were verified using a combination of multiple independent Sanger sequencing runs and where available, the whole genome sequencing data. The International Union of Pure and Applied Chemistry (IUPAC) code for degenerate nucleotides was used to label heterozygous positions.

mtDNA Genotyping

For mitochondrial DNA, the hypervariable region of the mitochondrial control region (HVR1), also known as Domain I, was amplified using the primers GPPAIR3F and GPPAIR3R (Clucas, et al. 2014) for samples from MO, CB, BI, COP, and JP. Amplifications were conducted in 25 µL volumes according to the manufacturer's instructions, using Phusion Hot Start Flex DNA Polymerase (New England Biolabs, UK) and 2 µL of template DNA diluted 1:100. Amplifications involved a two-step PCR, with an initial cycle of 98 °C for 30 seconds, 40 cycles of 98 °C for 10 seconds and 72 °C for 20 seconds, followed by a 10-minute extension at 72 °C. PCR products were visualized on a 1% agarose gel stained with SYBR Safe. PCR fragments were purified using an ethanol/sodium acetate

precipitation, and sequencing was performed using the Applied Biosystems BigDye Terminator v3.1 sequencing kit (Applied Biosystems) with the same PCR primers as sequencing primers.

Published mtDNA HVR1 sequences for the samples from BI (GenBank accessions KJ646314-KJ646330, n = 16) and COP (KJ646361-KJ646382, n = 21) were included in the analysis for the relevant individuals. mtDNA data was not available for the individuals from two Antarctic sites. Though other individuals from those sites have sequence data available, we only included data from individuals sequenced for both TLRs and HVR1.

Individual mtDNA fragments from all remaining sites (CR, MAR, COU, BR, MT, SIG, SP) were amplified using the primers tRNAGlu and AH530 (Roeder, et al. 2002). These reactions included 0.4 uM of each primer, 1.5 mM 1X of PCR reaction buffer, $MgCl_2$, 200 uM of each dNTP, and 1U of Taq Polymerase Platinum (Invitrogen) in a two-phase touchdown program (Korbie and Mattick 2008): (1) 10 minutes at 95 °C, and 11 cycles of 95 °C for 15 seconds; a touchdown with an annealing temperature of 60 °C–50 ºC for 30 seconds, with one cycle per 1 °C interval, and 72 °C for 45 seconds; (2) 35 amplification cycles at 95 °C for 15 seconds, 50 °C for 30 seconds, and 72 °C for 45 seconds; and a final extension period of 30 minutes at 72 °C. The purification of these PCR products and sequencing was carried out by Macrogen using an ABI PRISM 3730XL.

Only overlapping segments of the HVR1 sequences common to all samples were used by aligning and editing with Geneious v11.0. Consensus sequences for the resulting 273 bp region of interest were extracted for analysis.

Population-Level Analyses

Haplotypes were inferred for each of the diploid TLR loci using the PHASE algorithm (Stephens, et al. 2001) implemented in DnaSP 6.12.10 (Rozas, et al. 2017) with 10,000 iterations and 1,000 burn-in iterations. Phased haplotype data was used as input to determine standard genetic diversity measures of each population, including number of polymorphic sites, haplotypes, haplotype diversity, and nucleotide diversity, using DnaSP 6.12.10 and Arlequin v3.5.1.3 (Excoffier and Lischer 2010). Minimum spanning haplotype networks were constructed and visualized using PopART 1.7 (Bandelt, et al. 1999; Leigh and Bryant 2015) for each locus. DnaSP was also used to identify synonymous and non-synonymous polymorphic sites and frequencies. Arlequin was used to calculate Tajima's *D* (Tajima 1989), and Fu's *Fs* (Fu 1997). FSTAT v.2.9.3 (Goudet 1995) was used to calculate allelic richness, taking into account differences in sample size.

Because TLR nucleotide diversity has been observed to have a correlation to population size in some island bird populations (Gilroy, et al. 2017), we evaluated the relationship between Gentoo penguin census population sizes (N_c) and haplotype diversity (H_d) at each locus. Gentoo penguins are

philopatric, but also show evidence of admixture within island groups and adjacent coastlines (Levy, et al. 2016 and Vianna, et al. 2017). For this reason, the census population sizes selected for the analysis were numbers of breeding pairs from the most recent available surveys of each archipelago or region (in the case of the South Shetland Islands and Western Antarctic Peninsula). The N_c size and source survey reference is available in **Supplementary Table S3**. Spearman's rank correlations and *p* values were calculated for each diversity-size comparison.

For population differentiation comparisons, Arlequin was used to calculate pairwise F_{ST} distances based on haplotype frequencies (Weir and Cockerham 1984), and pairwise Φ_{ST}s for the TLR and mtDNA sequences (Excoffier, et al. 1992). FindModel (Posada and Crandall 1998) was used to find the best fit substitution model for use in Arlequin. Φ_{ST} calculations for the TLR loci were obtained using the Tamura and Nei substitution model (Tamura and Nei 1993), while mtDNA Φ_{ST} analysis was carried out using the Kimura 2-Parameter model (Kimura 1980) with a gamma of 0.27. Analysis of molecular variance (AMOVA) was used to compute hierarchical F-statistics, with 10,000 permutations, to evaluate likely patterns of genetic structure, seeking to identify the population grouping that maximized the among-group variation (F_{CT}) and minimized the variation among colonies within groups (F_{SC}) (Excoffier, et al. 1992). Significance of overall and pairwise genetic distances were computed using 1,000,000 permutations. We used the SGoF+ method (Carvajal-Rodriguez and de Una-Alvarez 2011) within the Myriads software (Carvajal-Rodriguez 2018) to correct for multiple tests.

To test for isolation-by-distance, shortest distances by sea (during summer ice extent), in km, were computed between each sampling location, using Google Earth v7.3.2.5776 (**Supplementary Table S5B**), which were then related to pairwise F_{ST} in a Mantel test, implemented in Arlequin.

Population Divergence and Phylogeography

Phylogenetic reconstruction and estimates of divergence time were carried out using BEAST 2.5.2 (Bouckaert, et al. 2019). The evolutionary model for mtDNA analysis was selected using jModelTest v. 2.1.10 (Darriba, et al. 2012), testing 88 candidate models and selecting the best fit using the Bayes Information Criterion (BIC). All 88 models were within the 100% confidence interval, with HKY+G selected for further divergence analyses (Hasegawa, et al. 1985). A total of 15 Adélie penguin (*P. adeliae*) and 15 Chinstrap penguin (*P. antarcticus*) mitochondrial HVR1 GenBank sequences (Clucas, et al. 2014), aligned and cropped to the equivalent size of the Gentoo sequences to avoid bias, were included in the analysis as outgroups.

The most recent common ancestor prior was set for the *Pygoscelis* genus at 7.6 Mya (Subramanian, et al. 2013), derived from the fossil calibration for *Pygoscelis grandis* (Walsh and Suarez 2006), with a normal distribution, and standard deviation (σ) of 1.3 Mya. A strict molecular clock with a starting

prior of 1.0 and a Yule speciation process for branching rates, with uniform priors for birth and clock rates of 1.0 was applied. Four independent runs of 30 million MCMC chains were performed, logging parameters every 3,000 steps. The four independent runs were then combined using LogCombiner v.2.5.2 and assessed for convergence within Tracer v.1.7.1 (Rambaut, et al. 2018). All parameters converged with ESS values greater than 6,000. A maximum clade credibility tree was then generated using TreeAnnotator v.2.5.2 (part of the BEAST software distribution) and visualized in FigTree v.1.4.3.

Selection Analyses

Phylogenetic inference was carried out on phased sequence data for each TLR locus using RAxML-NG using a GTR substitution matrix (Kozlov, et al. 2019). To detect selection, maximum likelihood analysis of ratios of non-synonymous to synonymous nucleotide substitutions (dN/dS; ω) was performed with the *codeml* package of programs in PAML v. 4.9 (Yang 1997; Yang 2007). Various models were fitted to the multiple alignments: M1a (neutral model; two site classes: $0 < \omega_0 < 1$ and $\omega_1 = 1$); M2a (positive selection; three site classes: $0 < \omega_0 < 1$, $\omega_1 = 1$ and $\omega_2 > 1$); M7 (neutral model; values of ω fit to a beta distribution where $\omega > 1$ disallowed); M8 (positive selection; similar to M7 but with an additional codon class of $\omega > 1$); M8a (neutral model; similar to M8 but with a fixed codon class at $\omega = 1$). Likelihood ratio tests were performed on pairs of models to assess whether models allowing positively selected codons gave a significantly better fit to the data than neutral models (model comparisons were M1a vs. M2a, M7 vs. M8, and M8a vs. M8). In situations where the null hypothesis of neutral codon evolution could be rejected ($p < 0.05$), the posterior probability of codons under selection in M2a and M8 were inferred using the Bayes Empirical Bayes algorithm (Yang, et al. 2005).

In Silico Prediction of Polymorphism Functional Consequences

Physiochemical distances between amino acid variants were assessed using distance matrices provided by several authors (Sneath, 1966; Epstein, 1967; Grantham, 1974; Miyata, 1979; Urbina, et al. 2006; **Supplementary Table S7**). Predicted functional consequences of amino acid substitutions were assessed using the homology-based tools SIFT (Ng and Henikoff, 2003) and PolyPhen-2 (Adzhubei, et al. 2013), using online servers (https://sift.bii.a-star.edu.sg/ and http://genetics.bwh.harvard.edu/pph2/; both accessed December 2019). Transmembrane domain positions were predicted using Phobius (Käll, et al. 2004; http://phobius.sbc.su.se/; accessed December 2019).

Functional Analysis of TLR5 Genotype Expression in CRISPR-Cas9 edited HEK-Blue Cells

In order to functionally assess positively selected *TLR5* polymorphisms *in vitro*, two full-length *TLR5* sequences were synthesised including the two signalling domain polymorphisms that had the

strongest signature of selection (GBlocks, IDT). Synthetic genes were cloned using the Gibson assembly method (Gibson, et al. 2009) into the p3XFLAG-CMV™-14 expression vector (Sigma) which incorporates a 3x-FLAG sequence on the C-terminus of the expressed construct. Insert-containing vector was purified using the ZymoPURE II plasmid Maxiprep with the optional endotoxin-removal step (Zymo). Both constructs were transiently expressed using TransIT®-2020 (Mirus Bio) in custom HEK-Blue™ Null1 cells (InvivoGen) that had undergone genome editing using the CRISPR-Cas9 technique to disrupt endogenous human *TLR5*. Cells expressing Gentoo *TLR5* constructs were challenged with *Salmonella* Typhimurium-derived flagellin (FLA-ST; InvivoGen) at 100 ng/mL and incubated for 24 h. Cell supernatants were harvested and NF-κB activity was assessed by measuring the absorbance at 405 nm on a FLUOstar® Omega microplate reader (BMG Labtech) following the addition of p-nitrophenyl phosphate substrate, according to the manufacturer's instructions (SIGMAFAST™, Sigma). Expression levels were monitored by subjecting cell lysates to a direct anti-FLAG ELISA. Cell lysates were harvested in ice-cold RIPA buffer (ThermoFisher) and proteins were immobilised on high-bind ELISA plates (VWR) using coating buffer (BioLegend) overnight at 4 °C. Wells were blocked using StartingBlock™ (PBS) blocking buffer (ThermoFisher) for 1 h and then incubated with mouse monoclonal anti-FLAG M2 antibody (Sigma, 1:1000) at 37 °C for 1 h, followed by incubation with goat anti-mouse IgG-HRP (ThermoFisher, 1:10000) for 1 h at 37 °C. Reactive protein amount was then assessed by the addition of 3,3',5,5'-tetramethylbenzidine substrate and measurement of absorbance at 650 nm. Expression data were then used to normalise signalling data. Statistical differences between means were determined by a two-tailed Student's t-test, and statistical significance was considered to be $p < 0.05$. Transfections were carried out in three independent wells per condition, and the experiment was conducted on at least three independent occasions.

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Author Contributions

HL and SRF conducted all analyses and wrote the paper, guided by TH and ALS who designed the study. HL, JAV, DN, GVC, MJP, CAB, RAP, SC, GDM, PP, FB, CLB, AAB, PT, ARR, and TH performed fieldwork and contributed samples. HL, GVC, and DN extracted all DNA. HL, SRF, and JKHS performed all PCR amplifications. JAV and DN prepared and provided whole genome sequence data. SRF and JKHS performed all cell culture, cloning, and expression assays. JAV, DN and LAFF provided analytical support with phylogenetic analyses. All authors discussed the study results and implications and contributed to editing the manuscript.

References

Adzhubei I, Jordan DM, Sunyaev SR. 2013. Predicting functional effect of human missense mutations using PolyPhen-2. Current Protocols in Human Genetics 76(1)7.20.1-7.20.41.

Akira S, Takeda K. 2004. Toll-like receptor signalling. Nature Reviews Immunology 4:499-511.

Akira S, Takeda K, Kaisho T. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nature Immunology 2:675-680.

Alcaide M, Edwards SV. 2011. Molecular Evolution of the Toll-Like Receptor Multigene Family in Birds. Molecular Biology and Evolution 28:1703-1715.

Aljanabi SM, Martinez I. 1997. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Research 25:4692-4693.

Andersen-Nissen E, Smith KD, Strobe KL, Barrett SLR, Cookson BT, Logan SM, Aderem A. 2005. Evasion of Toll-like receptor 5 by flagellated bacteria. Proceedings of the National Academy of Sciences of the United States of America 102:9247-9252.

Bandelt HJ, Forster P, Rohl A. 1999. Median-joining networks for inferring intraspecific phylogenies. Molecular Biology and Evolution 16:37-48.

Barbosa A, Benzal J, Vidal V, D'Amico V, Coria N, Diaz J, Motas M, Palacios MJ, Cuervo JJ, Ortiz J, et al. 2011. Seabird ticks (Ixodes uriae) distribution along the Antarctic Peninsula. Polar Biology 34:1621-1624.

Barbosa A, De Mas E, Benzal J, Diaz JI, Motas M, Jerez S, Pertierra L, Benayas J, Justel A, Lauzurica P, et al. 2013. Pollution and physiological variability in gentoo penguins at two rookeries with different levels of human visitation. Antarctic Science 25:329-338.

Barbosa A, Palacios MJ. 2009. Health of Antarctic birds: a review of their parasites, pathogens and diseases. Polar Biology 32:1095-1115.

Barreiro LB, Ben-Ali M, Quach H, Laval G, Patin E, Pickrell JK, Bouchier C, Tichit M, Neyrolles O, Gicquel B, et al. 2009. Evolutionary Dynamics of Human Toll-Like Receptors and Their Different Contributions to Host Defense. Plos Genetics 5.

Bell JK, Mullen GED, Leifer CA, Mazzoni A, Davies DR, Segal DM. 2003. Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends in Immunology 24:528-533.

Bollmer JL, Vargas FH, Parker PG. 2007. Low MHC variation in the endangered Galapagos penguin (Spheniscus mendiculus). Immunogenetics 59:593-602.

Bonnedahl J. 2011. Antibiotic Resistance in Enterobacteriaceae Isolated from Wild Birds. In. Uppsala University.

Botos I, Segal DM, Davies DR. 2011. The Structural Biology of Toll-like Receptors. Structure 19:447-459.

Bouckaert R, Vaughan TG, Barido-Sottani J, Duchene S, Fourment M, Gavryushkina A, Heled J, Jones G, Kuhnert D, De Maio N, et al. 2019. BEAST 2.5: An advanced software platform for Bayesian evolutionary analysis. Plos Computational Biology 15.

Boyd A, Philbin VJ, Smith AL. 2007. Conserved and distinct aspects of the avian Toll-like receptor (TLR) system: implications for transmission and control of bird-borne zoonoses. Biochemical Society Transactions 35:1504-1507.

Brownlie R, Allan B. 2011. Avian toll-like receptors. Cell and Tissue Research 343:121-130.
Carpenter-Kling T, Handley JM, Connan M, Crawford RJM, Makhado AB, Dyer BM, Froneman W, Lamont T, Wolfaardt AC, Landman M, et al. 2019. Gentoo penguins as sentinels of climate change at the sub-Antarctic Prince Edward Archipelago, Southern Ocean. Ecological Indicators 101:163-172.
Carvajal-Rodriguez A. 2018. Myriads: P-value-based multiple testing correction. Bioinformatics 34:1043-1045.
Carvajal-Rodriguez A, de Uña-Alvarez J. 2011. Assessing Significance in High-Throughput Experiments by Sequential Goodness of Fit and q-Value Estimation. PLoS One 6:e24700.
Cerda-Cuellar M, More E, Ayats T, Aguilera M, Munoz-Gonzalez S, Antilles N, Ryan PG, Gonzalez-Solis J. 2019. Do humans spread zoonotic enteric bacteria in Antarctic? Science of the Total Environment 654:190-196.
Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. Journal of Biological Chemistry 274.
Chown S, Clarke A, Fraser C, Cary SC, Moon KL, McGeoch MA. 2015. The changing form of Antarctic biodiversity. Nature 522(7557):431–438.
Clarke J, Kerry K. 2000. Diseases and parasites of penguins. Penguin Conservation 13:5-24.
Clausen A, Putz K. 2003. Winter diet and foraging range of gentoo penguins (Pygoscelis papua) from Kidney Cove, Falkland Islands. Polar Biology 26:32-40.
Clucas GV, Dunn MJ, Dyke G, Emslie SD, Levy H, Naveen R, Polito MJ, Pybus OG, Rogers AD, Hart T. 2014. A reversal of fortunes: climate change 'winners' and 'losers' in Antarctic Peninsula penguins. Scientific Reports 4.
Clucas GV, Younger JL, Kao D, Emmerson L, Southwell C, Wienecke B, Rogers AD, Bost C-A, Miller GD, Polito MJ, et al. 2018. Comparative population genomics reveals key barriers to dispersal in Southern Ocean penguins. Molecular Ecology 27:4680-4697.
Crawford RJM, Cooper J, Du Toit M, Greyling MD, Hanise B, Holness CL, Keith DG, Nel JL, Petersen SL, Spencer K, et al. 2003. Population and breeding of the gentoo penguin Pygoscelis papua at Marion Island, 1994/95-2002/03. African Journal of Marine Science 25:463-474.
Crawford RJM, Dyer BM, Upfold L, Makhado AB. 2014. Congruent, decreasing trends of gentoo penguins and Crozet shags at sub-Antarctic Marion Island suggest food limitation through common environmental forcing. African Journal of Marine Science 36:225-231.
Crawford RJM, Whittington PA, Upfold L, Ryan PG, Petersen SL, Dyer BM, Cooper J. 2009. Recent trends in numbers of four species of penguins at the Prince Edward Islands. African Journal of Marine Science 31:419-426.
Dalton DL, Vermaak E, Roelofse M, Kotze A. 2016a. Diversity in the Toll-Like Receptor Genes of the African Penguin (Spheniscus demersus). Plos One 11.
Dalton DL, Vermaak E, Smit-Robinson HA, Kotze A. 2016b. Lack of diversity at innate immunity Toll-like receptor genes in the Critically Endangered White-winged Flufftail (Sarothrura ayresi). Scientific Reports 6.
Darfour-Oduro KA, Megens H-J, Roca A, Groenen MAM, Schook LB. 2016. Evidence for adaptation of porcine Toll-like receptors. Immunogenetics 68:179-189.
Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nature Methods 9:772-772.
de Dinechin M, Dobson FS, Zehtindjiev P, Metcheva R, Couchoux C, Martin A, Quillfeldt P, Jouventin P. 2012. The biogeography of Gentoo Penguins (Pygoscelis papua). Canadian Journal of Zoology-Revue Canadienne De Zoologie 90:352-360.
Dewar ML, Arnould JPY, Dann P, Trathan P, Groscolas R, Smith S. 2013. Interspecific variations in the gastrointestinal microbiota in penguins. Microbiologyopen 2:195-204.
Dewar ML, Arnould JPY, Krause L, Dann P, Smith SC. 2014. Interspecific variations in the faecal microbiota of Procellariiform seabirds. Fems Microbiology Ecology 89:47-55.
Dimitrov K, Metcheva R, Kenarova A. 2009. Salmonella presence - an indicator of direct and indirect human impact on Gentoo in Antarctica. Biotechnology & Biotechnological Equipment 23:246-249.
Dionne M, Miller KM, Dodson JJ, Caron F, Bernatchez L. 2007. Clinal variation in MHC diversity with temperature: evidence for the role of host-pathogen interaction on local adaptation in Atlantic salmon. Evolution 61:2154-2164.

Epstein CJ. 1967. Non-randomness of ammo-acid changes in the evolution of homologous proteins. Nature 215(5099):355-359.
Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10:564-567.
Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes - application to human mitochondrial-DNA restriction data. Genetics 131:479-491.
Faber E, Tedin K, Speidel Y, Brinkmann MM, Josenhans C. 2018. Functional expression of TLR5 of different vertebrate species and diversification in intestinal pathogen recognition. Scientific Reports 8.
Fan J, Li L, Han J, Ming H, Li J, Na G, Chen J. 2013. Diversity and structure of bacterial communities in Fildes Peninsula, King George Island. Polar Biology 36:1385-1399.
Fix AS, Waterhouse C, Greiner EC, Stoskopf MK. 1988. Plasmodium relictum as a cause of avian malaria in wild-caught magellanic penguins (Spheniscus magellanicus). Journal of Wildlife Diseases 24:610-619.
Flach EJ, Stevenson MF, Henderson GM. 1990. Aspergillosis in Gentoo penguins (Pygoscelis papua) at Edinburgh Zoo, 1964 to 1988. Veterinary Record 126:81-85.
Freeman NM, Lovenduski NS, Gent PR. 2016. Temporal variability in the Antarctic Polar Front (2002-2014). Journal of Geophysical Research-Oceans 121:7263-7276.
Fu YX. 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. Genetics 147:915-925.
García-Peña FJ, Llorente MT, Serrano T, Ruano MJ, Belliure J, Benzal J, Herrera-Leon S, Vidal V, D'Amico V, Perez-Boto D, et al. 2017. Isolation of Campylobacter spp. from Three Species of Antarctic Penguins in Different Geographic Locations. Ecohealth 14:78-87.
Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL. 2001. Cutting edge: Bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. Journal of Immunology 167:1882-1885.
Ghys MI, Raya Rey A, Schiavini A. 2008. Population trend and breeding biology of Gentoo penguin in Martillo Island, Tierra Del Fuego, Argentina. Waterbirds 31(4):625-631.
Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, III, Smith HO. 2009. Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature Methods 6:343-U341.
Gilroy DL, van Oosterhout C, Komdeur J, Richardson, DS. 2017. Toll-like receptor variation in the bottlenecked population of the endangered Seychelles warbler. Anim Conserv 20:235-250.
González-Acuna D, Hernandez J, Moreno L, Herrmann B, Palma R, Latorre A, Medina-Vogel G, Kinsella MJ, Martin N, Araya K, et al. 2013. Health evaluation of wild gentoo penguins (Pygoscelis papua) in the Antarctic Peninsula. Polar Biology 36:1749-1760.
Grantham R. 1974. Amino acid difference formula to help explain protein evolution. Science 185(4154:862-864)
González-Quevedo C, Spurgin LG, Carlos Illera J, Richardson DS. 2015. Drift, not selection, shapes toll-like receptor variation among oceanic island populations. Molecular Ecology 24:5852-5863.
Goudet J. 1995. FSTAT (Version 1.2): A computer program to calculate F-statistics. Journal of Heredity 86:485-486.
Grilo ML, Vanstreels RET, Wallace R, Garcia-Parraga D, Braga EM, Chitty J, Catao-Dias JL, Madeira de Carvalho LM. 2016. Malaria in penguins - current perceptions. Avian Pathology 45:393-407.
Grimaldi WW, Seddon PJ, Lyver POB, Nakagawa S, Tompkins DM. 2015. Infectious diseases of Antarctic penguins: current status and future threats. Polar Biology 38:591-606.
Grueber CE, Wallis GP, Jamieson IG. 2014. Episodic Positive Selection in the Evolution of Avian Toll-Like Receptor Innate Immunity Genes. Plos One 9.
Grueber CE, Wallis GP, Jamieson IG. 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.
Grueber CE, Wallis GP, King TM, Jamieson IG. 2012. Variation at Innate Immunity Toll-Like Receptor Genes in a Bottlenecked Population of a New Zealand Robin. Plos One 7.

Guégan JF, Prugnolle F, Thomas F. 2007. Global spatial patterns of infectious diseases and human evolution. In: Stearns SC, Koella JC, editors. Evolution in health and disease. Oxford Scholarship Online. DOI: 10.1093/acprof:oso/9780199207466.001.0001 .
Guernier V, Hochberg ME, Guégan JF. 2004. Ecology drives the worldwide distribution of human diseases. PLoS Biol 2:e141.
Hartmann SA, Schaefer HM, Segelbacher G. 2014. Genetic depletion at adaptive but not neutral loci in an endangered bird species. Molecular Ecology 23:5712-5725.
Hasegawa M, Kishino H, Yano TA. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial-DNA. Journal of Molecular Evolution 22:160-174.
Hinke JT, Cossio AM, Goebel ME, Reiss CS, Trivelpiece WZ, Watters GM. 2017. Identifying Risk: Concurrent Overlap of the Antarctic Krill Fishery with Krill-Dependent Predators in the Scotia Sea. Plos One 12.
Jaeger A, Lebarbenchon C, Bourret V, Bastien M, Lagadec E, Thiebot J-B, Boulinier T, Delord K, Barbraud C, Marteau C, et al. 2018. Avian cholera outbreaks threaten seabird species on Amsterdam Island. Plos One 13.
Käll L, Krogh A, Sonnhammer ELL. 2004. A Combined Transmembrane Topology and Signal Peptide Prediction Method. Journal of Molecular Biolog, 338(5):1027-1036.
Kane OJ, Uhart MM, Rago V, Pereda AJ, Smith JR, Van Buren A, Clark JA, Boersma PD. 2012. Avian Pox in Magellanic Penguins (Spheniscus magellanicus). Journal of Wildlife Diseases 48:790-794.
Kawai T, Akira S. 2006. TLR signaling. Cell Death and Differentiation 13:816-825.
Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111-120.
Kloch A, Wenzel MA, Laetsch DR, Michalski O, Welc-Faleciak R, Piertney SB. 2018. Signatures of balancing selection in Toll-like receptor (TLRs) genes novel insights from a free-living rodent. Scientific Reports 8.
Knafler GJ, Clark JA, Boersma PD, Bouzat JL. 2012. MHC Diversity and Mate Choice in the Magellanic Penguin, Spheniscus magellanicus. Journal of Heredity 103:759-768.
Korbie DJ, Mattick JS. 2008. Touchdown PCR for increased specificity and sensitivity in PCR amplification. Nature Protocols 3:1452-1456.
Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A. 2019. RAxML-NG: A fast, scalable, and user-friendly tool for maximum likelihood phylogenetic inference. Bioinformatics (Oxford, England).
Leigh JW, Bryant D. 2015. POPART: full-feature software for haplotype network construction. Methods in Ecology and Evolution 6:1110-1116.
Lemaho Y, Karmann H, Briot D, Handrich Y, Robin JP, Mioskowski E, Cherel Y, Farni J. 1992. Stress in birds due to routine handling and a technique to avoid it. American Journal of Physiology 263:R775-R781.
Lepage D, Vaidya G, Guralnick R. 2014. Avibase - a database system for managing and organizing taxonomic concepts. Zookeys:117-135.
Lescroel A, Bost CA. 2006. Recent decrease in gentoo penguin populations at Iles Kerguelen. Antarctic Science 18:171-174.
Levin II, Zwiers P, Deem SL, Geest EA, Higashiguchi JM, Iezhova TA, Jimenez-Uzcategui G, Kim DH, Morton JP, Perlut NG, et al. 2013. Multiple Lineages of Avian Malaria Parasites (Plasmodium) in the Galapagos Islands and Evidence for Arrival via Migratory Birds. Conservation Biology 27:1366-1377.
Levy H, Clucas GV, Rogers AD, Leache AD, Ciborowski KL, Polito MJ, Lynch HJ, Dunn MJ, Hart T. 2016. Population structure and phylogeography of the Gentoo Penguin (Pygoscelis papua) across the Scotia Arc. Ecology and Evolution 6:1834-1853.
Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A, Flavell RA. 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proceedings of the National Academy of Sciences of the United States of America 101:5598-5603.
Lynch HJ, Naveen R, Trathan PN, Fagan WF. 2012. Spatially integrated assessment reveals widespread changes in penguin populations on the Antarctic Peninsula. Ecology 93:1367-1377.

Ma D, Zhu R, Ding W, Shen C, Chu H, Lin X. 2013. Ex-situ enzyme activity and bacterial community diversity through soil depth profiles in penguin and seal colonies on Vestfold Hills, East Antarctica. Polar Biology 36:1347-1361.
Mac Donald JW, Conroy JWH. 1971. Virus disease resembling puffinosis in the gentoo penguin (Pygoscelis papua) on signy island south orkney islands. British Antarctic Survey Bulletin:80-82.
Mayewski P. 2012. State of the Antarctic climate system. Excerpts from SASOCS (Mayewski et al. 2009). Anales del Instituto de la Patagonia 40:25-30.
Mikami T, Miyashita H, Takatsuka S, Kuroki Y, Matsushima N. 2012. Molecular evolution of vertebrate Toll-like receptors: Evolutionary rate difference between their leucine-rich repeats and their TIR domains. Gene 503:235-243.
Miyata T. 1979. Two types of amino acid substitution in protein evolution. Journal of Molecular Evolution 12(3):219-236
Moore JK, Abbott MR, Richman JG. 1999. Location and dynamics of the Antarctic Polar Front from satellite sea surface temperature data. Journal of Geophysical Research-Oceans 104:3059-3073.
Morgan IR, Westbury HA. 1988. Studies of viruses in penguins in the Vestfold Hills. Hydrobiologia 165:263-269.
Mukherjee S, Ganguli D, Majumder PP. 2014. Global Footprints of Purifying Selection on Toll-Like Receptor Genes Primarily Associated with Response to Bacterial Infections in Humans. Genome Biology and Evolution 6:551-558.
Nahori MA, Fournie-Amazouz E, Que-Gewirth NS, Balloy V, Chignard M, Raetz CRH, Saint Girons I, Werts C. 2005. Differential TLR recognition of leptospiral lipid A and lipopolysaccharide in muine and human cells. Journal of Immunology 175:6022-6031.
Neira V, Tapia R, Verdugo C, Barriga G, Mor S, Ng TFF, García V, Del Río J, Rodrigues P, Briceño C, et al. 2017. Novel Avulaviruses in Penguins, Antarctica. Emerg Infect Dis 23:1212-1214.
Ng PC, Henikoff S. 2003. SIFT: Predicting amino acid changes that affect protein function. Nucleic Acids Research 31(13):3812-3814.
Novak K, Bjelka M, Samake K, Valcikova T. 2019. Potential of TLR-gene diversity in Czech indigenous cattle for resistance breeding as revealed by hybrid sequencing. Archives Animal Breeding 62:477-490.
Olivares F, Tapia R, Gálvez C, Meza F, Barriga GP, Borras-Chavez R, Mena-Vasquez J, Medina RA, Neira V. 2019. Novel penguin Avian avulaviruses 17, 18 and 19 are widely distributed in the Antarctic Peninsula. Transbound Emerg Dis.
Park BS, Song DH, Kim HM, Choi B-S, Lee H, Lee J-O. 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature 458:1191-U1130.
Peña M F, Poulin E, Dantas GPM, González-Acuña D, Petry MV, Vianna JA. 2014. Have Historical Climate Changes Affected Gentoo Penguin (Pygoscelis papua) Populations in Antarctica? Plos One 9.
Pertierra L SN, Martínez P, Barbosa A, Raya Rey A, Pistorius P, Polanowski A, Bonadonna F, Le Bohec C, Bi K, et al. in review. Integrated phylogenomic and niche analyses reveal cryptic speciation in Gentoo penguins driven by local adaptation. In. Diversity and Distribution.
Posada D, Crandall KA. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics 14:817-818.
Qureshi ST, Lariviere L, Leveque G, Clermont S, Moore KJ, Gros P, Malo D. 1999. Endotoxin-tolerant mice have mutations in toll-like receptor 4 (Tlr4). Journal of Experimental Medicine 189.
Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. 2018. Posterior Summarization in Bayesian Phylogenetics Using Tracer 1.7. Systematic Biology 67:901-904.
Roach JC, Glusman G, Rowen L, Kaur A, Purcell MK, Smith KD, Hood LE, Aderem A. 2005. The evolution of vertebrate Toll-like receptors. Proc Natl Acad Sci U S A 102:9577-9582.
Roeder AD, Ritchie PA, Lambert DM. 2002. New DNA markers for penguins. Conservation Genetics 3:341-344.
Rohde K, Heap M. 1998. Latitudinal differences in species and community richness and in community structure of metazoan endo- and ectoparasites of marine teleost fish. Int J Parasitol 28:461-474.
Rozas J, Ferrer-Mata A, Sanchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sanchez-Gracia A. 2017. DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. Molecular Biology and Evolution 34:3299-3302.

Sallaberry-Pincheira N, González-Acuña D, Herrera-Tello Y, Dantas GP, Luna-Jorquera G, Frere E, Valdés-Velasquez A, Simeone A, Vianna JA. 2015. Molecular Epidemiology of Avian Malaria in Wild Breeding Colonies of Humboldt and Magellanic Penguins in South America. Ecohealth 12:267-277.

Sallaberry-Pincheira N, González-Acuña D, Padilla P, Dantas GPM, Luna-Jorquera G, Frere E, Valdés-Velásquez A, Vianna JA. 2016. Contrasting patterns of selection between MHC I and II across populations of Humboldt and Magellanic penguins. Ecol Evol 6:7498-7510.

Sneath PHA. 1966. Relations between chemical structure and biological activity in peptides. Journal of Theoretical Biology 12(2):157-195.

Song WS, Jeon YJ, Namgung B, Hong M, Yoon SI. 2017. A conserved TLR5 binding and activation hot spot on flagellin. Scientific Reports 7: 40878

Stephens M, Smith NJ, Donnelly P. 2001. A new statistical method for haplotype reconstruction from population data. American Journal of Human Genetics 68:978-989.

Stonehouse B. 1970. Geographic Variation in Gentoo Penguins Pygoscelis-Papua. Ibis 112:52-57.

Subramanian S, Beans-Picon G, Swaminathan SK, Millar CD, Lambert DM. 2013. Evidence for a recent origin of penguins. Biology Letters 9.

Świderská Z, Smidova A, Buchtova L, Bryjova A, Fabianova A, Munclinger P, Vinkler M. 2018. Avian Toll-like receptor allelic diversity far exceeds human polymorphism: an insight from domestic chicken breeds. Scientific Reports 8.

Tajima F. 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123:585-595.

Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Molecular Biology and Evolution 10:512-526.

Trathan PN, Forcada J, Murphy EJ. 2007. Environmental forcing and Southern Ocean marine predator populations: effects of climate change and variability. Philosophical Transactions of the Royal Society B-Biological Sciences 362:2351-2365.

Trivelpiece WZ, Trivelpiece SG, Volkman NJ. 1987. Ecological segregation of Adelie, Gentoo, and Chinstrap penguins at King George Island, Antarctica. Ecology 68:351-361.

Tsuda TT, Tsuda M, Naruse T, Kawata H, Ando A, Shiina T, Fukuda M, Kurita M, LeMaho I, Kulski JK, et al. 2001. Phylogenetic analysis of penguin (Spheniscidae) species based on sequence variation in MHC class II genes. Immunogenetics 53:712-716.

Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, Rozen SG. 2012. Primer3-new capabilities and interfaces. Nucleic Acids Research 40.

Urbina D, Tang B, Higgs PG. 2006. The response of amino acid frequencies to directional mutation pressure in mitochondrial genome sequences is related to the physical properties of the amino acids and to the structure of the genetic code. Journal of Molecular Evolution 62(3):340-361.

Velová H, Gutowska-Ding MW, Burt DW, Vinkler M. 2018. Toll-Like Receptor Evolution in Birds: Gene Duplication, Pseudogenization, and Diversifying Selection. Molecular Biology and Evolution 35:2170-2184.

Vianna JA, Noll D, Dantas GPM, Petry MV, Barbosa A, Gonzalez-Acuna D, Le Bohec C, Bonadonna F, Poulin E. 2017. Marked phylogeographic structure of Gentoo penguin reveals an ongoing diversification process along the Southern Ocean. Molecular Phylogenetics and Evolution 107:486-498.

Vinkler, M., Bainová, H., Bryjová, A. Tomášek, O, Albrecht, T, Bryja, J. 2015. Characterisation of Toll-like receptors 4, 5 and 7 and their genetic variation in the grey partridge. Genetica 143(1):101-112.

Wallensten A, Munster VJ, Osterhaus A, Waldenstrom J, Bonnedahl J, Broman T, Fouchier RAM, Olsen B. 2006. Mounting evidence for the presence of influenza A virus in the avifauna of the Antarctic region. Antarctic Science 18:353-356.

Walsh SA, Suarez ME. 2006. New penguin remains from the Pliocene of northern Chile. Historical Biology 18:119-130.

Wang J, Zhang Z, Liu J, Zhao J, Yin D. 2016. Ectodomain Architecture Affects Sequence and Functional Evolution of Vertebrate Toll-like Receptors. Scientific Reports 6.

Weir BS, Cockerham CC. 1984. Estimating F-Statistics for the Analysis of Population Structure. Evolution 38:1358-1370.

Wille M, Aban M, Wang J, Moore N, Shan S, Marshall J, González-Acuña D, Vijaykrishna D, Butler J, Hall RJ, et al. 2019. Antarctic Penguins as Reservoirs of Diversity for Avian Avulaviruses. J Virol 93.
Wilson RP, Alvarrez B, Latorre L, Adelung D, Culik B, Bannasch R. 1998. The movements of gentoo penguins Pygoscelis papua from Ardley Island, Antarctica. Polar Biology 19:407-413.
Wlasiuk G, Nachman MW. 2010. Adaptation and Constraint at Toll-Like Receptors in Primates. Molecular Biology and Evolution 27:2172-2186.
Woods R, Jones HI, Watts J, Miller GD, Shellam GR. 2009. Diseases of Antarctic seabirds. Health of Antarctic wildlife: a challenge for science and policy.:35-55.
Yang ZH. 2007. PAML 4: Phylogenetic analysis by maximum likelihood. Mol Biol Evol 24:1586-1591.
Yang ZH. 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. Computer Applications in the Biosciences 13:555-556.
Yang ZH, Wong WSW, Nielsen R. 2005. Bayes empirical Bayes inference of amino acid sites under positive selection. Mol Biol Evol 22:1107-1118.
Yoon SI, Kurnasov O, Natarajan V, Hong M, Gudkov AV, Osterman AL, Wilson IA. 2012. Structural basis of TLR5-flagellin recognition and signaling. Science 335(6070):859-864.
Younger J, Emmerson L, Southwell C, Lelliott P, Miller K. 2015. Proliferation of East Antarctic Adelie penguins in response to historical deglaciation. Bmc Evolutionary Biology 15.
Younger JL, Clucas GV, Kooyman G, Wienecke B, Rogers AD, Trathan PN, Hart T, Miller KJ. 2015. Too much of a good thing: sea ice extent may have forced emperor penguins into refugia during the last glacial maximum. Global Change Biology 21:2215-2226.
Zdanowski MK, Weglenski P, Golik P, Sasin JM, Borsuk P, Zmuda MJ, Stankovic A. 2004. Bacterial diversity in Adelie penguin, Pygoscelis adeliae, guano: molecular and morpho-physiological approaches. Fems Microbiology Ecology 50:163-173.

Figures

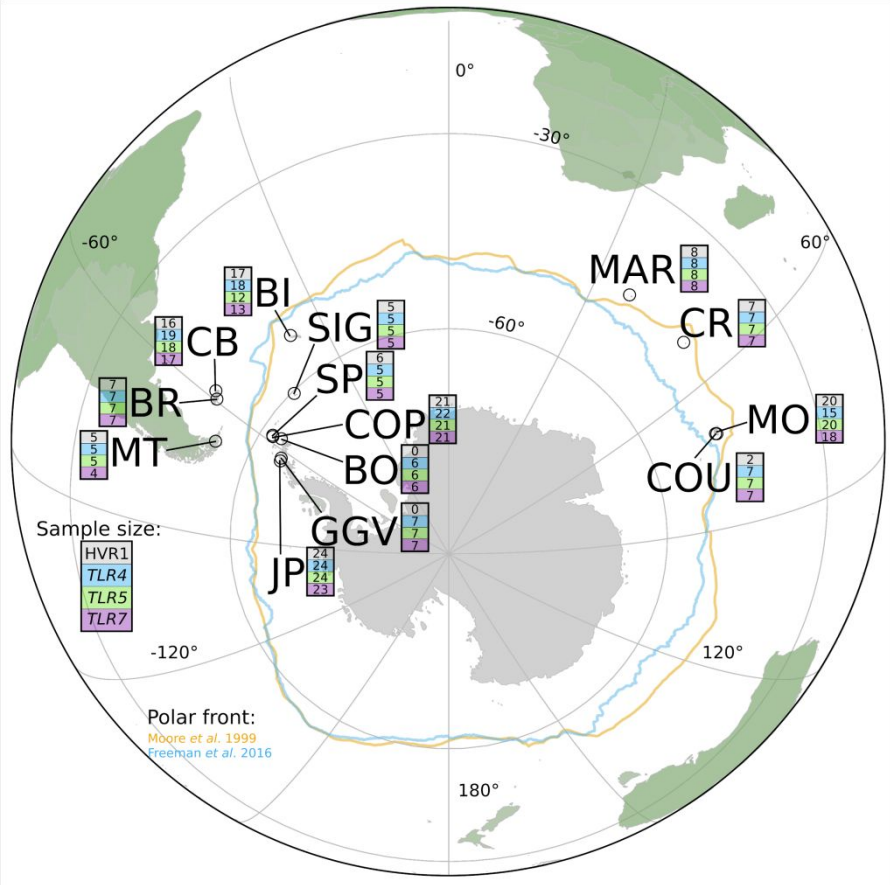


Figure 1. Locations and per-locus sample sizes for each sampled Gentoo penguin colony in the Southern Ocean and Antarctica. Solid colored lines represent the reported position of the Polar Front, based on the analyses of Freeman et al. (2016) and Moore et al. (1999). Depending on the analysis, the Kerguelen and Crozet Islands can lie just north or just south of the Polar Front (Moore, et al. 1999; Freeman, et al. 2016). (CR = Crozet Island; MAR = Marion Island; COU = Courbet Peninsula, Kerguelen; MO = Pointe du Morne, Kerguelen; CB = Cow Bay, Falkland/Malvinas Islands; BR = Bull Roads, Falkland/Malvinas Islands; BI = Bird Island, South Georgia; MT = Martillo Island, Tierra del Fuego; SIG = Signy Island, South Orkney Islands; COP = Copacabana (Admiralty Bay), King George Island, South Shetland Islands; SP = Stranger Point, King George Island, South Shetland Islands; BO = Bernardo O'Higgins Base, Western Antarctic Peninsula; GGV = Gabriel González Videla Base, Western Antarctic Peninsula; JP = Jougla Point, Western Antarctic Peninsula).

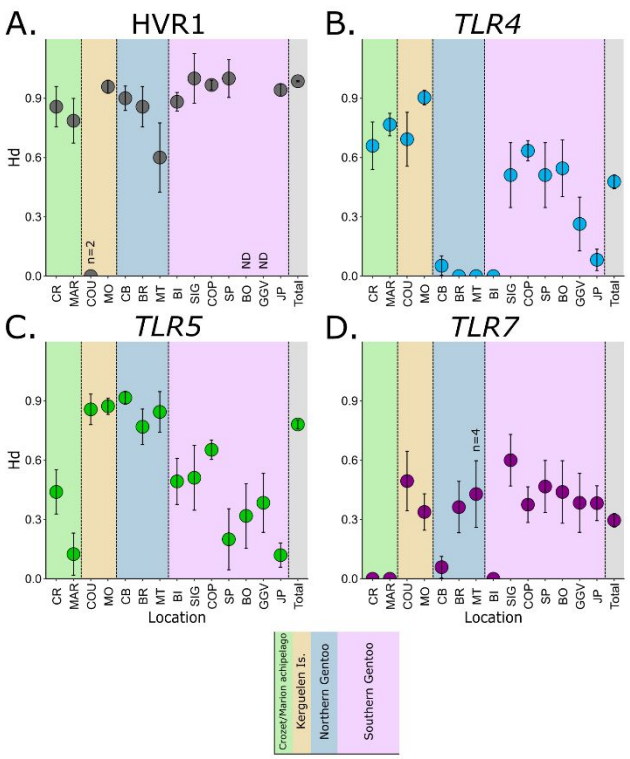


Figure 2. Haplotype diversity (Hd) for the hypervariable mitochondrial control region (HVR1; A) and three TLR loci (B, C, D), for all sampling sites for which data was available. All sites had n ≥ 5 unless otherwise indicated (ND = no data). Location abbreviations are the same as in previous figure.

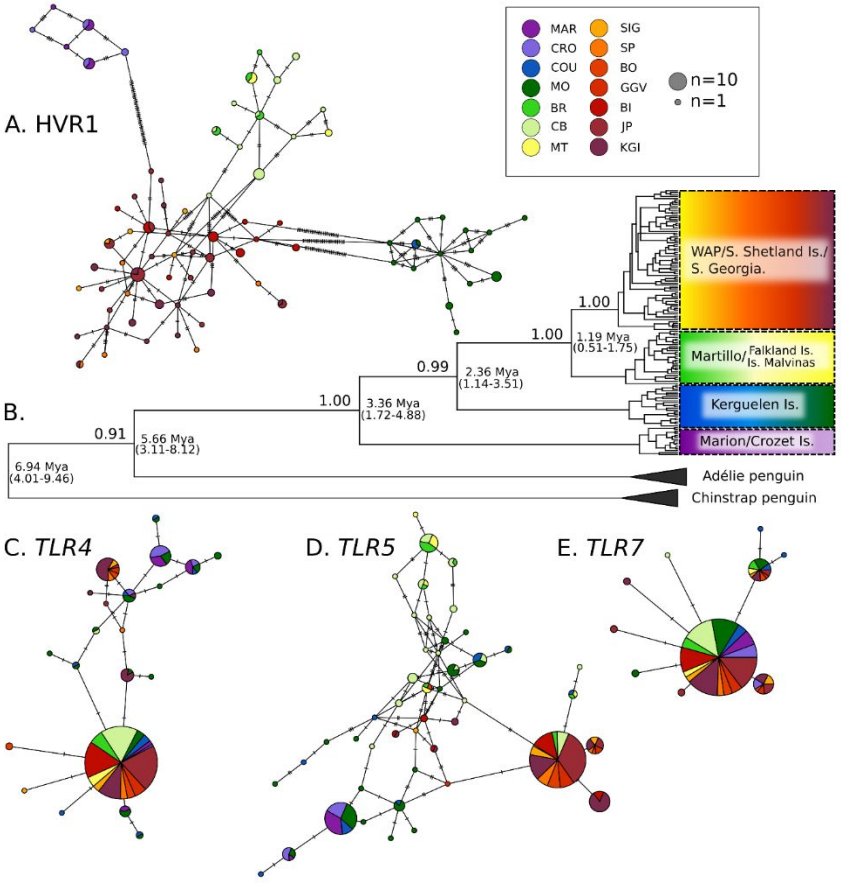
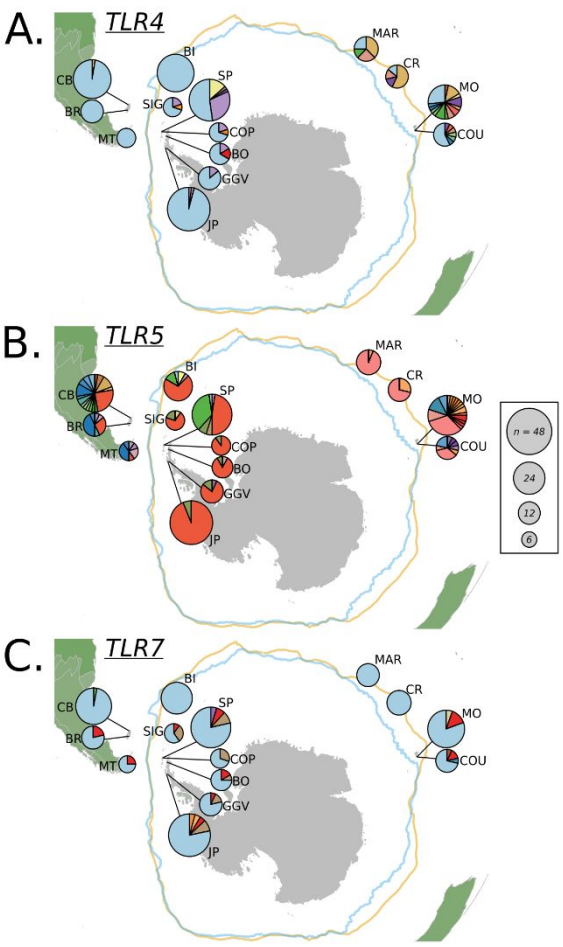


Figure 3. Minimum spanning haplotype networks for mtDNA HVR1 (A), TLR4 (C), TLR5 (D), and TLR7 (E) for Gentoo penguin colonies, along with mtDNA HVR1 maximum clade credibility tree (B), using congeneric penguin species as outgroups. Location abbreviations are the same as in previous figures. For minimum spanning haplotype networks, pie charts represent single haplotypes, while segment size refers to the contribution of individual sampled sites to the proportion of overall haplotype frequency. Size of pie charts reflects the number of individual birds with the observed haplotype. Dashes on connecting lines each denote one nucleotide change.



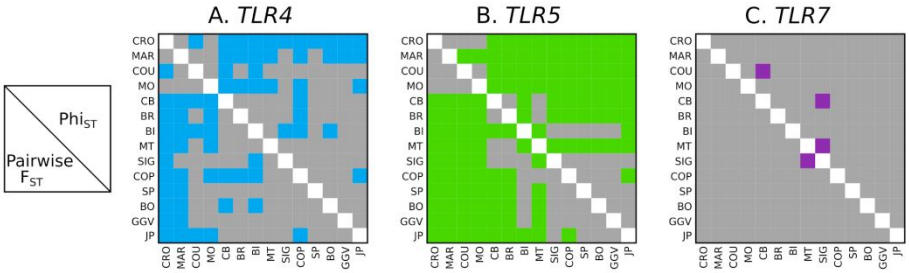


Figure 4. Haplotype diversity across Gentoo penguin sample populations for (A) *TLR4*, (B) *TLR5*, and (C) *TLR7*. For each locus, different colors represent unique haplotypes and each segment size reflects the proportion of birds in each location with that haplotype. Overall size of the pie chart reflects the number of birds sampled in each location. Location abbreviations are the same as in previous figures.

Figure 5. Visualization of pairings of Gentoo penguin breeding colonies, where pairwise F_{ST} values (below diagonal) or Φ_{ST} values (above diagonal) with significance $p < 0.01$ after correction for multiple tests using SGOF+ (Carvajal-Rodriguez and de Uña-Alvarez 2011) are shown in color for (A) *TLR4*, (B) *TLR5*, and (C) *TLR7*. Location abbreviations are the same as in previous figures.

Figure 6. Positions of polymorphic and positively selected sites in *P. papua* TLR4, 5 and 7. Schematic diagrams of TLRs in the extracellular (TLR4 and 5) and endosomal (TLR7) compartments show positions of amino acid variants resulting from non-synonymous nucleotide substitutions. Variant positions are marked with stars and are colored according to the likelihood of positive selection as determined by the M2a model in the *codeml* program in PAML.

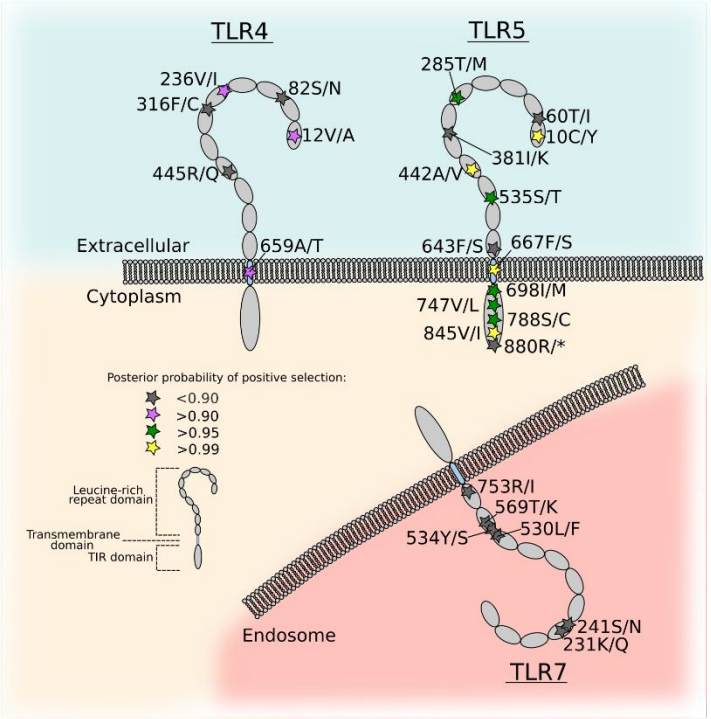


Figure 7. Distribution and functional differences of two selected amino acid residues in TLR5. (A) Mapped areas represent the four clades of *P. papua* as determined by HVR1 analysis. Pie charts represent the allele proportions at the two selected residues in TLR5, and chart area is proportional to the number of samples. (B) Bar chart displays NF-kB response of the two genotypes following stimulation with FLA-ST.