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Physiological condition reflects polymorphism at the toll-like receptors in a colonial waterbird

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ABSTRACT

Toll-like receptors (TLRs) are a crucial component of vertebrate innate immune response. Despite their importance, associations of TLR diversity with fitness-related traits have rarely been examined in wild animal populations. Here, we tested for associations of TLR polymorphism with physiological condition in a colonial waterbird, the Black-headed Gull (*Chroicocephalus ridibundus*). Physiological condition and polymorphism at 4 TLR loci were assessed in 60 gulls from a breeding colony in northern Poland. We found that blood hemoglobin and plasma albumin concentrations were positively associated with TLR diversity across all genotyped loci. Plasma concentrations of albumin and triglycerides were also associated with the presence of specific TLR variants and locus-specific diversity. All significant associations between physiological condition and TLRs were primarily apparent at the level of nucleotide, rather than amino acid allelic variants. Although the exact molecular mechanisms responsible for these associations require further investigation, our study provides strong correlational support for links between TLR diversity and physiological condition in a wild avian population, and it adds to the growing, but still modest, body of evidence for the fitness-related consequences of individual TLR repertoire in wild birds.

Keywords: Black-headed Gull, physiological condition, synonymous mutations, toll-like receptors

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LAY SUMMARY

- Toll-like receptors (TLRs) initiate innate immune response by recognition of conserved pathogen-associated molecular patterns.
- We tested for associations between TLR diversity and fitness-related traits (physiological condition) in the Black-headed Gull.
- Physiological condition was associated with both TLR diversity and presence of specific TLR variants, but these associations were mostly prevalent at the nucleotide, rather than amino acid, level.
- Our results raise the question on the adaptive significance of synonymous variation at TLR genes.

La condición fisiológica refleja polimorfismo en los receptores de tipo Toll en un ave acuática colonial

RESUMEN

Los receptores tipo Toll (TLR, por sus siglas en inglés) son un componente crucial de la respuesta inmune innata de los vertebrados. A pesar de su importancia, las asociaciones de la diversidad de TLR con rasgos relacionados con la aptitud biológica rara vez han sido examinadas en poblaciones de animales silvestres. Aquí, evaluamos las asociaciones del polimorfismo TLR con la condición fisiológica en un ave acuática colonial, la gaviota *Chroicocephalus ridibundus*. Se evaluó la condición fisiológica y el polimorfismo en 4 loci TLR en 60 gaviotas de una colonia reproductora en el norte de Polonia. Encontramos que las concentraciones de hemoglobina en sangre y albúmina plasmática se asociaron positivamente con la diversidad de TLR en todos los loci tipificados genéticamente. Las concentraciones plasmáticas de albúmina y triglicéridos también se asociaron con la presencia de variantes específicas de TLR y la diversidad específica de cada locus. Todas las asociaciones significativas entre la condición fisiológica y los TLR fueron evidentes principalmente a nivel de nucleótidos, en lugar de variantes alélicas de aminoácidos. Aunque los mecanismos moleculares exactos responsables de estas asociaciones requieren más investigación, nuestro estudio proporciona un fuerte apoyo correlacional para los

vínculos entre la diversidad de TLR y la condición fisiológica en una población de aves silvestres, y se suma al creciente, pero aún modesto, cuerpo de evidencia sobre las consecuencias relacionadas con la aptitud biológica del repertorio individual de TLR en las aves silvestres.

Palabras clave: Chroicocephalus ridibundus, condición fisiológica, mutaciones sinónimo, receptores de tipo Toll

INTRODUCTION

Toll-like receptors (TLRs) are central components of the innate immune system, which recognize specific pathogen-associated molecular patterns (PAMPs) and initiate a signaling cascade resulting in the stimulation of innate and adaptive immune responses (Janeway and Medzhitov 2002, Andreakos et al. 2004, Akira et al. 2006, Vijay 2018). The general TLR structure consists of an N-terminal ectodomain with 16–28 tandem leucine-rich repeats (LRRs) and 1–2 cysteine-rich regions, a transmembrane domain, and a highly conserved cytoplasmic Toll/IL-1 receptor (TIR) domain (Brownlie and Allan 2011). The LRR region is directly responsible for ligand binding and PAMP identification (Brodsky and Medzhitov 2007, Matsushima et al. 2007). The LRRs show strong variation between different TLR loci, which is crucial for the recognition of a broad spectrum of pathogens: fungi, bacteria, viruses, parasites, and protozoans, such as malaria, leishmaniasis, toxoplasmosis, and trypanosomiasis (Fujita et al. 2003, Gazzinelli et al. 2004, Akira et al. 2006). Also, polymorphisms in TLR genes may shift balance between pro- and anti-inflammatory cytokines, modulating the risk of infection. It has been shown that the ability of an individual to respond properly to TLR ligands may be impaired by single-nucleotide polymorphisms (SNPs) within TLR genes, resulting in an altered susceptibility to disease (Schröder and Schumann 2005, Vijay 2018). Although nonsynonymous SNPs within exonic regions are primarily responsible for associations between TLR alleles and disease resistance, similar associations have also been reported for mutations in introns, 5′ un-translated regions, and promoters (possibly affecting gene expression) (Mukherjee et al. 2019), as well as for synonymous (silent) exonic SNPs (e.g., Junjie et al. 2012, Taniguchi et al. 2013).

According to the heterozygote advantage (overdominance) hypothesis, gene polymorphism can contribute to higher fitness of an individual, as heterozygous genotype will have higher relative fitness than either the dominant or recessive homozygous genotype (Gemmell and Slate 2006, Hedrick 2012). There is strong evidence for the mechanism of heterozygote advantage at the highly diverse pathogen-recognition genes of the adaptive immune system, the major histocompatibility complex (MHC) (Hughes and Nei 1988, 1989, Niskanen et al. 2014). Higher number of alleles expressed within an individual across duplicated MHC loci allows more antigens to be bound and presented to T cells, thus conferring resistance against a broader spectrum of pathogens. In fact, heterozygote advantage is thought to be one of key mechanisms of pathogen-driven balancing selection, which generates and maintains extraordinary allelic variation of MHC in natural vertebrate populations (Spurgin and Richardson 2010), reaching thousands of alleles in some species (e.g., Biedrzycka et al. 2017). Although TLRs are generally much more conserved than the MHC and have majority of residues subject to purifying selection (Downing et al. 2010, Alcaide and Edwards 2011, Grueber et al. 2014), higher intra-individual TLR diversity is also expected to increase the spectrum of antigens recognized and it has been suggested that heterozygote advantage mechanism may operate at TLRs (Schröder et al. 2005). Also, recent comparative analyses of avian TLRs indicate that birds exhibit relatively high levels of intraspecific TLR variation with a number of residues being under diversifying rather than purifying selection (Świderská et al. 2018, Velová et al. 2018), which may enhance associations of TLR diversity with disease resistance and fitness.

While there is a considerable body of literature on the genetic basis of resistance in humans, there is little information on how variation in TLRs influences disease susceptibility in wild animal populations (Vinkler and Albrecht 2009, Tschirren et al. 2013, Gavan et al. 2015). So far, it was demonstrated that allelic variation at TLRs was associated with susceptibility to parasites in wild mammal populations, for example, in wood mouse (*Apodemus sylvaticus*) (Jackson et al. 2009), bank vole (*Myodes glareolus*) (Tschirren et al. 2013), and water vole (*Arvicola amphibius*) (Gavan et al. 2015). Generally, only a few studies have explored the effects of innate immune genes on fitness components in wild animal populations despite the fact that genetic variation at innate immune receptors can have a large impact on host resistance (Tschirren et al. 2013). Consequently, our knowledge on the associations between TLR polymorphism and fitness traits in wild birds is yet limited. Here, we expected that through its effects on diseases resistance, polymorphism and allelic composition of TLRs should indirectly affect physiological performance of birds. The immune system is dedicated to maintain organismal health; therefore, it is reasonable to expect that immunocompetence could have a detectable impact on physiological body condition and traits of individual quality (Costantini and Møller 2009, Hasselquist and Nilsson 2012). Although such associations are to be expected, the effects of TLR polymorphism on health and fitness may be generally small, as indicated by an extensive research on domestic animals. For example, an analysis of

110 sites across 7 TLR genes identified several SNPs that may potentially elicit relatively small effects on uterine health in dairy cattle (Pinedo et al. 2013), whereas even broader analysis across 10 bovine TLRs revealed only 6 SNPs potentially eliciting small effects on susceptibility to *Mycobacterium avium* spp. *paratuberculosis* infection (Fisher et al. 2011).

The aim of this study was to test for associations of TLR polymorphism with physiological condition in a colonial waterbird, the Black-headed Gull (*Chroicocephalus ridibundus*). For this purpose, we assessed polymorphism of 4 TLR loci and physiological condition in 60 Black-headed Gulls breeding in central and peripheral zones of a colony in northern Poland. We focused on TLRs that recognize a broad range of antigens, including triacyl lipopetides and other ligands from mycobacteria, Gram-negative bacteria and hematozoans (TLR1 and TLR4), double-stranded RNA from viruses (TLR3), and bacterial flagellin (TLR5). To control for the level of neutral heterozygosity, we also genotyped a set of 10 microsatellite loci for each individual. We hypothesized that individuals with higher allelic diversity in TLRs are expected to have better physiological condition, as measured with total blood hemoglobin concentration and plasma concentrations of albumin and triglycerides. All these measures are commonly used as robust condition indices in wild birds (Ferrer 1993, Jenni-Eiermann and Jenni 1998, Minias 2015). Blood hemoglobin concentration reflects diet quality (Pryke et al. 2011) and general condition (Minias 2015), but it has also been shown to decrease in response to various parasitic infections (Khan et al. 2006, Słomczyński et al. 2006, Mazur et al. 2007, Mandal et al. 2008, Krams et al. 2013). Plasma albumin and triglyceride concentrations can also be considered as reliable indices of nutritional status (Coles 1997, Jenni-Eiermann and Jenni 1998), and low albumin concentration may also be indicative for acute disease and chronic infection or inflammation (Hõrak et al. 2002). We predicted that associations of different physiological measures with infection status and immune function should facilitate occurrence of similar relationships between physiology and TLR diversity.

METHODS

General Field Procedures

The study was performed in the Black-headed Gull colony near Kusowo village (53.2473°N, 18.1327°E), northern Poland, in 2016. Gulls nested on a lake island situated in an agricultural landscape. The size of the colony was estimated at 800 breeding pairs. Central and peripheral zones of the colony were separated with a natural barrier of trees and shrubs, mainly Willow *Salix* sp. (Indykiewicz et al. 2019). Nesting habitat did not show conspicuous differences between the 2 zones and gulls nested primarily in herbaceous plants (fat hen [*Chenopodium album*] and gypsywort [*Lycopus europaeus*]) and grasses, which provided cover for the broods. Nesting densities were estimated at 2.23 pairs 10 m^{-2} in peripheries and 5.30 pairs 10 m^{-2} in the colony center. Adult Black-headed Gulls (n = 60) were captured across the entire colony between April 13 and April 30, and sample sizes were the same for the peripheral and central zones of the colony (30 birds per zone). All the birds were captured using spring traps (Ecotone, Sopot, Polska), which did not cause any losses in broods. Each bird was ringed and ~20 μL of blood from the ulnar vein was collected onto Flinders Technology Associates (FTA) Classic Cards (Whatman, Maidstone, UK) for DNA extraction. Following the recommendation by Gutiérrez-Corchero et al. (2002), each card was dried and kept at room temperature until analysis. We also took ~5 μL of blood for the purpose of whole-blood hemoglobin concentration measurements and ~50 μL of blood to measure the concentration of 2 basic plasma metabolites (albumin and triglycerides).

Physiological Condition

Physiological condition of gulls was assessed with whole-blood hemoglobin concentration and plasma concentrations of 2 basic metabolites, triglycerides and albumin. Hemoglobin concentration is a general measure of blood oxygen-carrying capacity in vertebrates and it has been shown to correlate with a wide spectrum of fitness-related traits in birds (reviewed in Minias 2015). Similarly, plasma metabolites reflect various aspects of physiological condition and characterize the feeding state of birds, such as food intake, diet quality, fat reserves, and size-adjusted body mass (Totzke et al. 1999). Total triglyceride levels reliably indicate nutrient status and they may vary in relation to environmental conditions and stress (Jenni-Eiermann and Jenni 1998, Jenni-Eiermann et al. 2002, Artacho et al. 2007, Ibañez et al. 2015, Albano et al. 2016). A decrease in serum albumin concentration accompanies almost all diseases, and low albumin levels are considered a prominent symptom of malnutrition and chronic infection or inflammation (Kawai 1973, Coles 1997, Hõrak et al. 2002).

In order to measure blood hemoglobin concentration, 5 μL of blood of each bird was collected into a disposable HemoCue microcuvette. The samples were analyzed in a portable HemoCue Hb 201+ photometer (HemoCue, Ängeholm, Sweden), which uses the azide-methemoglobin method to measure hemoglobin concentration. The photometer was internally calibrated and is considered to be suitable for measurements of avian blood (Harter et al. 2015). It was reported to yield high repeatability of hemoglobin measurements in birds (Barve et al. 2016). The absorbance, directly proportional to hemoglobin concentration, was measured within 5 min from blood sampling.

TABLE 1. Descriptive statistics for 3 measures of physiological condition in the Black-headed Gull.

Trait	Mean	SE	Min	Max	Skewness
Hemoglobin concentration (g L^{-1})	163.55	1.47	137.00	185.00	0.04
Plasma albumin concentration (g L^{-1})	15.45	0.70	6.50	32.80	0.92
Plasma triglyceride concentration (mg dL^{-1})	166.65	12.30	17.0	592.00	1.83

Blood for plasma metabolite measurements (50 µL) was collected into heparinized capillary tubes. Samples were centrifuged at 6,000 rpm for 5 min to separate plasma from blood cells and kept at −20°C until analysis. The measurements were conducted with a spectrophotometer (BTS-330, BioSystems Reagents & Instruments, Barcelona, Spain) using commercial kits. Bromocresol green and glycerol phosphate oxidase/peroxidase methods were used to measure albumin and triglyceride concentrations, respectively. Absorbance of each sample was measured in a flow cuvette against a blank reagent at the following wave lengths: 630 nm (albumin) and 500 nm (triglycerids). The measurements were calibrated against standards provided with reagents: bovine serum albumin 51.6 g L^{-1} (albumin) and glycerol equivalent of 200 mmol L^{-1} triolein (triglycerides). Our previous research on other avian species showed high repeatability of both measurements (Minias et al. 2015).

Descriptive statistics for all 3 physiological measures are presented in Table 1. Distribution of plasma triglyceride concentration strongly was strongly right-skewed (Table 1) and, thus, log-transformed prior to analyses. Distributions of other measurements (hemoglobin and plasma albumin) were reasonably close to normal (−1 < skewness < 1; Table 1) and, thus, left untransformed.

DNA Extraction and Microsatellite Genotyping
Nuclear DNA was obtained from blood stored on FTA cards using Bio-Trace DNA Purification Kit (EURx, Gdansk, Poland). A piece of dried blood sample (~2 mm^2) was removed from each card with a sterile cutter and used for DNA extraction, which followed a manufacturer's protocol (EURx, Gdansk, Poland). To assess neutral heterozygosity of each bird, we used 10 microsatellite loci, since even a relatively small panel of microsatellite markers has a strong informative power on genome-wide heterozygosity (comparable to hundreds of SNPs; Forstmeier et al. 2012). A similar panel of 10 (or fewer) microsatellite loci has been commonly used to estimate individual heterozygosity in a wide range of avian species from different phylogenetic lineages (e.g., Seddon et al. 2004, Jouventin et al. 2007, Gillingham et al. 2013), including gulls (Mulard et al. 2009). The markers were originally developed for the Kittiwake (*Rissa tridactyla*) (K6, K16, K31, and K32; Tirard et al. 2002) and the Red-billed Gull (*Larus novaehollandiae scopulinus*) (RBG13, RBG18, RBG20, RBG27, RBG28, and RBG29; Given et al. 2002; Table 2) but were previously shown to successfully cross-amplify in the Black-headed Gull (Indykiewicz et al. 2018). The protocol used for polymerase chain reaction (PCR) amplifications was described previously (Indykiewicz et al. 2018). Fragment size analysis was conducted with a capillary sequencer ABI 3730xl, and allele sizes were scored against GeneScan TM 600 LIZ Standard using Geneious v10.0.5 (Biomatters Ltd., Auckland, New Zealand) software. Frequency of null alleles at each locus was low (<0.055) and after controlling for the false discovery rate (FDR) (Benjamini and Hochberg 1995) we found no evidence for significant pairwise linkage disequilibrium between loci, as assessed with CERVUS v3.0.3 (Kalinowski et al. 2007). Although one locus (K31) showed a minor but significant deviation from the Hardy–Weinberg equilibrium (FDR-corrected P-value: $q = 0.015$), as assessed with the exact test (1,000,000 Markov chain length and 100,000 dememorization) in ARLEQUIN v1.3.5.2 (Excoffier and Lischer 2010), we decided to retain all the loci in the analyses. The number of alleles retrieved from each locus ranged from 7 to 25, while the observed and expected heterozygosities were estimated at 0.47–0.97 and 0.43–0.93 per locus, respectively. Neutral multilocus heterozygosity (MLH) was calculated for each individual as the proportion of heterozygote loci among all loci genotyped.

TLR Sequencing
To genotype TLRs in the Black-headed Gull, we used conservative primers developed by Alcaide and Edwards (2011). So far, these primers have been widely used to genotype and infer TLR diversity in wild bird populations (Grueber et al. 2012, Hartmann et al. 2014, Gonzalez-Quevedo et al. 2015).

All PCR amplifications were conducted in a final volume of 20 µL containing 10 µL of DreamTaq PCR Master Mix (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) and 0.2 µM of each primer. A 1-µL DNA extract from blood samples was added to each reaction. Annealing temperatures and PCR conditions followed the protocol of Alcaide and Edwards (2011). Each PCR amplification was checked on 2% agarose gels. We obtained successful amplifications for 4 out of the 10 TLR loci reported in birds: TLR1LB, TLR3, TLR4, and TLR5. PCR products were sequenced with Sanger sequencing technology in both forward and reverse directions. All alignments were

TABLE 2. Characterization of 10 polymorphic microsatellite loci in the Black-headed Gull (N = 60 individuals). N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; qHWE, statistical significance of deviations from the Hardy–Weinberg equilibrium (P-value adjusted for the FDR). Primer sequences, annealing temperatures, and PCR conditions followed the protocol developed by Given et al. (2002) [1] and by Tirard et al. (2002) [2].

Locus	N_A	H_O	H_E	qHWE	Reference
RBG13	9	0.65	0.64	0.61	1
RBG18	7	0.47	0.43	0.83	1
RBG20	18	0.90	0.91	1.00	1
RBG27	25	0.97	0.91	0.91	1
RBG28	7	0.82	0.75	0.99	1
RBG29	8	0.85	0.78	1.00	1
K6	11	0.80	0.82	0.22	2
K16	16	0.82	0.92	0.60	2
K32	16	0.90	0.86	0.96	2
K31	17	0.83	0.93	0.015	2

assembled, edited, and united in GENEIOUS v10.0.5 software. Final sizes of each alignment ranged from 831 to 1,203 base pairs (bp) (Table 3). Although we sequenced, on average, only 40% of the total coding region of each gene (1,959–3,180 bp according to Temperley et al. 2008), genotyping mostly focused on extracellular LRR motifs that are responsible for PAMP recognition.

Sequences from each locus were assigned to haplotypes using the PHASE algorithm (Stephens and Donnelly 2003) in DnaSP v6.10.03 (Rozas et al. 2017) using a burn-in of 1,000 iterations, 1,000 final iterations, and a thinning interval of 10. At each locus, we recorded homozygous genotypes (mean proportion of 29.2 ± 13.7% [mean ± SE] homozygotes per locus), which was expected to enhance reliable phasing of heterozygotes. All unique sequences were deposited in Genbank (accession numbers: MN995080–MN995167). TLR heterozygosity (MLH) was assessed at the level of nucleotide and amino acid sequences across all 4 loci for each captured individual. Because of low variation in TLR heterozygosity estimates at both levels (nucleotide and amino acid), we grouped these variables into 3 categories of low (0.25 and 0.5 heterozygosity of nucleotide variants, n = 18 individuals; 0 heterozygosity of amino acid variants, n = 9 individuals), intermediate (0.75 heterozygosity of nucleotide variants, n = 31 individuals; 0.25 heterozygosity of amino acid variants, n = 34 individuals), and high (1.0 heterozygosity of nucleotide variants, n = 11 individuals; 0.5 and 0.75 heterozygosity of amino acid variants, n = 17 individuals) heterozygosity. Following the approach of Hartmann et al. (2014), we also quantified SNP diversity at TLRs as the number of heterozygous SNPs (nucleotide level) and non-synonymous SNPs (amino acid level) across all 4 loci and separately for each locus. The numbers of SNPs were quantified both across all available TLR domains, as well as separately for ligand-binding (LRR) and all other (non-LRR) regions (including cytoplasmic Toll/interleukin 1 resistance domains and regions that had no specific function recognized).

Statistical Analyses

Associations of TLR polymorphism (heterozygosity and SNP diversity) with proxies of physiological condition (blood hemoglobin concentration and plasma concentrations of albumin and triglycerides) were

TABLE 3. Polymorphism of nucleotide and amino acid sequences at 4 TLR loci in the Black-headed Gull.

Sequence	Locus	Fragment size (bp)	Number of variants	Variant frequency (mean ± SE)	Observed heterozygosity (H_O)	Number of polymorphic sites	Number of SNPs (mean ± SE)
Nucleotide	TLR1LB	1,044	26	3.9 ± 0.9	0.85	19	2.58 ± 0.21
	TLR3	1,128	16	6.3 ± 2.6	0.73	11	1.92 ± 0.25
	TLR4	831	8	12.5 ± 9.8	0.32	7	0.37 ± 0.08
	TLR5	1,203	38	2.6 ± 0.6	0.93	14	2.55 ± 0.18
	All loci	4,206	88	4.6 ± 1.1	0.71	51	7.42 ± 0.35
Amino acid	TLR1LB	1,044	7	14.3 ± 12.8	0.18	5	0.20 ± 0.06
	TLR3	1,128	5	20.0 ± 17.1	0.20	4	0.13 ± 0.05
	TLR4	831	3	33.3 ± 30.4	0.05	2	0.05 ± 0.03
	TLR5	1,203	21	4.8 ± 1.8	0.73	7	1.50 ± 0.15
	All loci	4,206	36	11.1 ± 4.2	0.29	18	1.88 ± 0.17

analyzed with the general linear models. Each condition trait was entered as a response variable in a separate model. TLR heterozygosity was entered as a fixed factor (3-category variable), whereas TLR diversity was entered as a covariate. Separate models were run for each TLR polymorphism estimate at the nucleotide and amino acid levels. Associations with SNP diversity were tested both across all loci and separately for each locus. We also ran separate models for SNP diversity calculated across the entire genotyped sequence (total number of SNPs), as well as separately for ligand-binding (LRR) and other (non-LRR) regions. Finally, we tested for the effect of specific TLR variants on physiological condition of gulls. The occurrence of each TLR variant was entered as a binary fixed factor in the model, but variants with <10% or >90% frequency were excluded from the analysis. To reduce the inflated type I error rate resulting from multiple predictors, we corrected all P values from these models for the FDR (q values). In each analysis, we controlled for microsatellite heterozygosity and capture date (covariate), as well as for the effects of sex and breeding zone (fixed factor). The effect of breeding zone (center/periphery) was included, since gulls nesting in different parts of the colony were previously reported to exhibit differences in individual quality, including physiological traits (Indykiewicz et al. 2019). All models were run in the *lme4* package (Bates et al. 2014) developed for the R statistical environment (R Core Team 2013), whereas the *car* R package (Fox and Weisberg 2018) was used to infer Wald χ^2 statistics and P values for all independent variables. All values are reported as means ± SE.

RESULTS

TLR Polymorphism
The number of nucleotide sequence variants varied from 8 to 38 per TLR locus in our study population of the Black-headed Gull, and in total, we retrieved 88 variants (Table 3). Most of the nucleotide sequence variants (88.6%) had a low frequency of <10% and only 10 variants had a higher frequency in the population (1–3 variants per locus). The frequency of the most common variant at each locus ranged from 18.3% to 80.8%, and the mean frequency was 4.6 ± 1.1% across all loci (2.6%–12.5% per locus). The observed heterozygosity at each locus was 0.32–0.93 (Table 3). The number of polymorphic sites per locus ranged between 7 and 19 (0.84–1.82 per 100 bp per locus), whereas the number of heterozygous SNPs per individual across all loci ranged from 1 to 14 (mean = 7.42 ± 0.35) (Table 3). Most of the SNPs were recorded within the ligand-binding (LRR) domains (5.63 ± 0.32 vs. 1.72 ± 0.16 SNPs per locus for LRR and non-LRR regions, respectively). As expected, both TLR diversity measures (heterozygosity and total SNP numbers across all loci) were positively correlated

(Pearson product–moment coefficient: $r = 0.59$, $n = 60$, $P < 0.001$).

In total, we recorded 52 different nucleotide substitutions (at 51 polymorphic sites) across all TLR loci, but only 36.5% of them were non-synonymous and changed amino acid sequence (2–7 non-synonymous substitutions per locus). Consequently, amino acid polymorphism of TLR loci was much reduced when compared with the polymorphism at the level of nucleotide sequence. In total, we recorded 36 amino acid variants in our study population (3–21 variants per locus), and the mean variant frequency was 11.1 ± 4.2% across all loci (Table 3). Single high-frequency amino acid variants (88.3%–94.3%) were retrieved from TLR1LB, TLR3, and TLR4 loci, while the most common variant at TLR5 had much lower frequency (40.0%). The mean number of non-synonymous SNPs per individual ranged from 0 to 5 across all loci (mean = 1.88 ± 0.17) and the observed heterozygosity ranged from 0.05 to 0.73 (Table 3). Neither nucleotide nor amino acid TLR heterozygosity correlated with neutral heterozygosity across microsatellite loci (Pearson product–moment coefficient: $r = 0.04$, $n = 60$, $P = 0.76$ for nucleotide heterozygosity, $r = -0.20$, $n = 60$, $P = 0.12$ for amino acid heterozygosity).

TLR Polymorphism and Physiological Condition
We found a significant association between heterozygosity of TLR nucleotide sequence variants and blood hemoglobin concentration ($W = 7.61$, $P = 0.022$; Supplementary Material Table S1), as gulls with low TLR heterozygosity had lower blood hemoglobin concentrations than individuals with high ($\beta = 9.24 \pm 4.16$, $P = 0.031$) and intermediate ($\beta = 8.32 \pm 3.27$, $P = 0.014$) TLR heterozygosity (Figure 1). TLR heterozygosity was not significantly associated with plasma albumin ($W = 0.58$, $P = 0.75$; Supplementary Material Table S1) and triglycerides ($W = 0.61$, $P = 0.74$; Supplementary Material Table S1). However, plasma albumin concentration was positively associated with TLR diversity, as measured with the number of heterozygous SNPs across all loci ($\beta = 0.57 \pm 0.25$, $W = 5.12$, $P = 0.024$; Figure 2, Supplementary Material Table S2). There was no relationship between TLR diversity and other measures of physiological condition (hemoglobin concentration: $W = 0.32$, $P = 0.57$; plasma triglycerides concentration: $W = 0.14$, $P = 0.71$, Supplementary Material Table S2). When we separated SNPs from ligand-binding and other (non-LRR) regions, we found that the positive association between plasma albumin concentration and TLR diversity (across all loci) was primarily driven by polymorphisms within the LRR ($\beta = 0.77 \pm 0.27$, $W = 8.11$, $P = 0.004$; Supplementary Material Table S3) rather than non-LRR ($W = 0.58$, $P = 0.45$; Supplementary Material Table S3) domains. At the same time, we found a weak negative association between plasma triglyceride
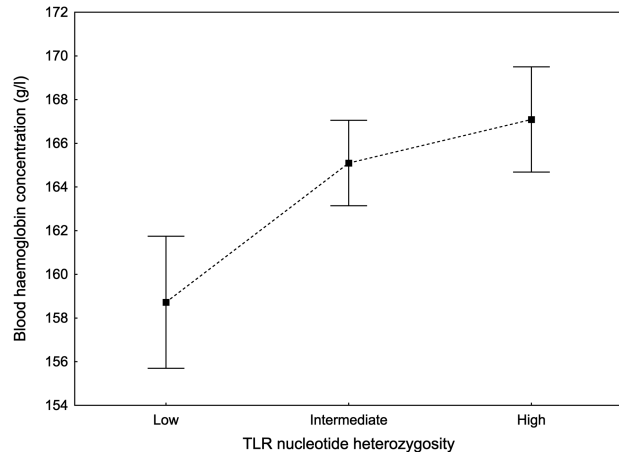
FIGURE 1. Association of blood hemoglobin concentration with TLR nucleotide heterozygosity, grouped into 3 categories: low (≤0.5 heterozygosity of nucleotide variants), intermediate (0.75 heterozygosity of nucleotide variants), and high (1.0 heterozygosity of nucleotide variants). Means ± SE are shown.

concentrations and the number of SNPs within the non-LRR regions (β = −0.05 ± 0.03, W = 3.87, P = 0.049; Supplementary Material Table S3).

The analysis of locus-specific nucleotide diversity measures provided support for the positive associations between plasma albumin concentration and the number of SNPs at TLR1LB locus (β = 0.86 ± 0.43, W = 4.05, P = 0.044; Supplementary Material Table S4), as well as between plasma triglycerides concentration and the number of SNPs at TLR3 locus (β = 0.04 ± 0.02, W = 4.29, P = 0.038; Supplementary Material Table S4). Both these associations remained significant when restricting SNPs to LRR domains (albumin: β = 2.67 ± 0.63, W = 17.75, P < 0.0003; triglycerides: β = 0.04 ± 0.02, W = 4.21, P = 0.04; Supplementary Material Table S5).

We also found associations between the presence of specific nucleotide sequence variants of TLRs and physiological condition of gulls. Individuals with TLR1LB*25 (n = 13) variant had higher plasma albumin concentration (β = 5.01 ± 1.70, W = 8.64, q = 0.043; Figure 3, Supplementary Material Table S6), whereas birds with TLR3*1 (n = 17) and TLR3*11 (n = 19) variants had significantly lower plasma triglyceride concentrations when compared with birds that lacked these variants (TLR3*1: β = −0.22 ± 0.07, W = 9.96, q = 0.021; TLR3*11: β = −0.22 ± 0.08, W = 7.93, q = 0.032; Figure 4, Supplementary Material Table S6). We found no association between blood hemoglobin concentration and the presence of specific nucleotide sequence variants of TLRs (all q > 0.05, Supplementary Material Table S6).

We found little evidence for associations between amino acid polymorphism TLR loci and condition of Black-headed Gulls. Neither heterozygosity nor diversity (measured as the number of non-synonymous SNPs across all loci) of TLR amino acid variants correlated
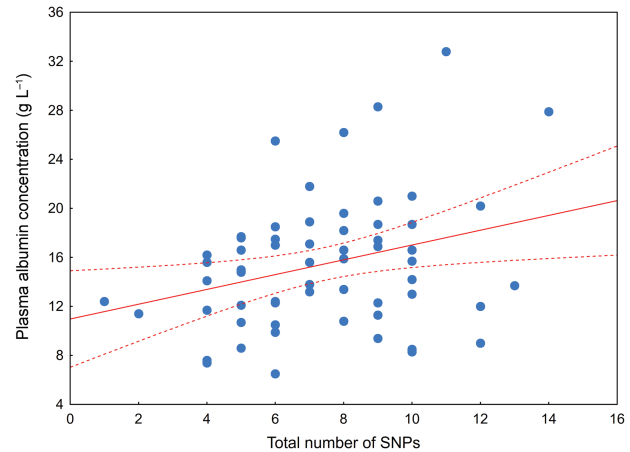

FIGURE 2. Relationship between plasma albumin concentration and TLR diversity, as measured with the total number of heterozygous SNPs across all loci.
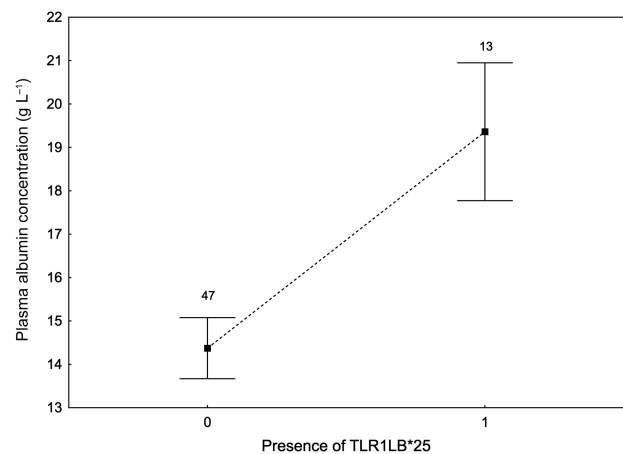
FIGURE 3. Association of plasma albumin concentration with the presence of TLR1LB*25 nucleotide sequence variant. Means ± SE are shown and sample sizes are reported for each group.

significantly with any of the condition indices (all P > 0.05; Supplementary Material Tables S1 and S2). Separate analyses of LRR and non-LRR SNPs across all loci provided support for the negative association between plasma albumin concentration and the number of non-synonymous SNPs within non-LRR regions (β = −3.48 ± 1.70, W = 4.16, P = 0.041; Supplementary Material Table S3). However, locus-specific analyses indicated that this association was primarily driven by the polymorphism at a single (TLR1LB) locus (β = −3.36 ± 1.52, W = 4.86, P = 0.028; Supplementary Material Table S4), while relationships of plasma albumin concentrations with amino acid polymorphisms at other loci were nonsignificant (all P > 0.05; Supplementary Material Table S4). We found no evidence for associations of hemoglobin and plasma triglyceride concentrations with locus-specific measures of amino acid diversity (all P > 0.05; Supplementary Material Table S4). Similarly, no
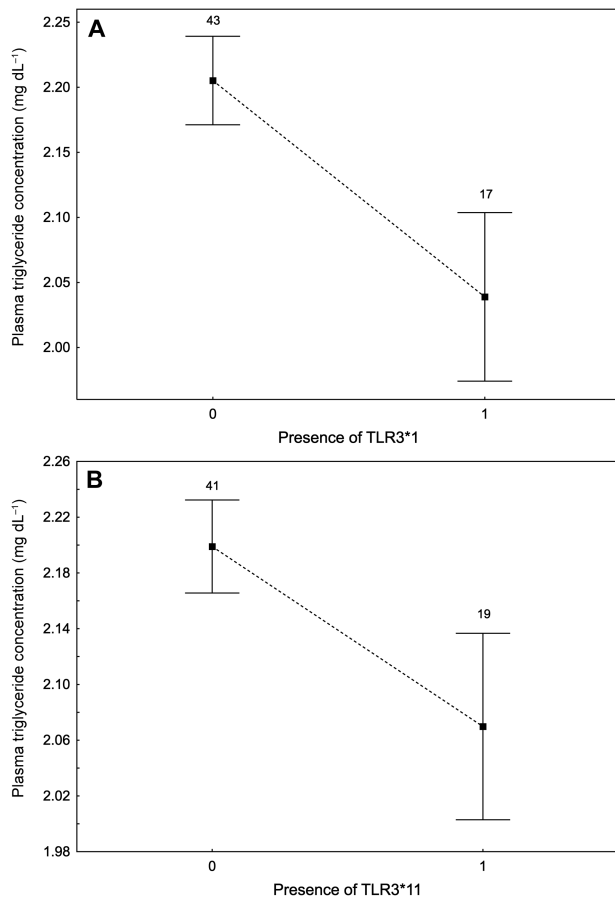
FIGURE 4. Associations of plasma triglyceride concentration with the presence of TLR3*1 (A) and TLR3*11 (B) nucleotide sequence variants. Means ± SE are shown and sample sizes are reported for each group.

significant associations were found between the occurrence of specific amino acid TLR variants and condition of gulls from our study population.

DISCUSSION

Our study provided support for an association between physiological condition and allelic diversity of TLRs genes in a wild population of Black-headed Gull. We have shown that blood hemoglobin concentration was positively associated with TLR heterozygosity at the nucleotide level, whereas plasma albumin concentration showed a positive correlation with the number of heterozygous SNPs across all genotyped TLR loci. Locus-specific analyses provided evidence for significant associations of physiological condition (plasma albumin and triglycerides concentration) with nucleotide diversity at particular TLR loci, and these associations were primarily due to SNPs present within the ligand-binding LRR regions. At the same time, we found associations between the presence of specific TLR

nucleotide variants and different aspects of physiological condition. Surprisingly, most of the significant associations between physiological condition measures and TLR polymorphism were apparent only at the level of nucleotide, rather than amino acid allelic variants.

Blood hemoglobin and plasma metabolite concentrations are well-known and useful indicators of general condition of birds (Jenni-Eiermann and Jenni 1998, Minias 2015). Low albumin concentration may reflect acute diseases and chronic infection or inflammation (Hõrak et al. 2002). A decrease of plasma albumin content during inflammation is usually accompanied by a simultaneous increase of gamma-globulin concentration that includes most of the known antibodies involved in immune response to protozoan, bacterial, and viral infections (Kawai 1973, Ots and Hõrak 1998). Blood hemoglobin concentration is negatively correlated with infestation rates of not only parasitic hematozoans (Sergent et al. 2004, Mandal et al. 2008, Krams et al. 2013), but also arthropods (Słomczyński et al. 2006), gastrointestinal helminths (Mazur et al. 2007), and ticks (Norte et al. 2013). For example, infection by *Plasmodium* spp. leads to a marked loss of red blood cells and causes anemia, whereas heavy parasitemia may often cause abnormal breakdown of red blood cells (Sherman 1979, Mohandas and An 2012, White 2018). Also, one of the general causes of anemia during bird hemosporidioses is the active removal of infected red blood cells from the blood circulation (Sherman 1979, Permin and Juhl 2002). This study focused on 4 TLR genes, 2 of which (TLR1 and TLR4) have been associated with occurrence of *Plasmodium* hemoparasites and clinical symptoms of malaria in humans (Leoratti et al. 2008, Basu et al. 2010, Manning et al. 2016), providing some possible explanation for correlations between TLR diversity and blood hemoglobin concentrations. In fact, our analyses indicated that higher TLR polymorphism (heterozygosity and SNP diversity) was associated with better physiological condition of gulls, as measured with blood hemoglobin and plasma albumin concentrations. Although this hypothesis is speculative and requires further testing, we suggest that these associations could possibly reflect a heterozygote advantage mechanism, where higher diversity of TLR genes allows to recognize a broader spectrum of pathogens and parasites, also enhancing physiological health and condition of birds. Polymorphism of genes from the TLR response pathway has been identified, so far, as clinically relevant in determining susceptibility to certain infectious diseases, inflammation, and allergic diseases, and has been found to play a role in tumorigenesis (Schröder and Schumann 2005, Turvey and Hawn 2006, Medvedev 2013). There is also some evidence for the mechanism of heterozygote advantage operating at TLR genes, for example, a heterozygous SNP within

the human TLR2 provided resistance to the late stage of Lyme disease caused by *Borrelia burgdorferi* (Schröder et al. 2005).

We also found that physiological condition was associated with the presence of specific TLR1LB and TLR3 variants. The extracellular TLR1B receptor is instrumental in recognition of bacterial triacyl lipoproteins (Takeda et al. 2002, Brownlie and Allan 2011), initiating immune responses against many common avian pathogens, such as mycoplasmas, *M. avium*, *Pseudomonas aeruginosa*, *Salmonella* spp., and *Chlamydia* spp. (Tötemeyer et al. 2005, Bas et al. 2008, Bhide et al. 2009). Therefore, plasma albumin concentration (which drops as a result of inflammatory processes) could reflect higher immunocompetence level provided by TLR1LB*25 allelic variant. We also observed a negative correlation between plasma triglycerides (which are directly linked to the nutritional condition) and the presence of TLR3*1 and TLR3*11 allelic variants. The TLR3 is an intracellular receptor identifying double-stranded viral DNA (Brownlie and Allan 2011) found in poxviruses, herpesviruses, and adenoviruses (Modrow et al. 2013). It is possible that these TLR3 variants confer impaired immune response against some of these viruses, which may have detrimental consequences for the organism. In fact, adenoviruses are known to cause serious gastrointestinal symptoms and can be responsible for extensive mortality in gulls (Bodewes et al. 2013, Karamendin et al. 2021). Nonetheless, we explicitly acknowledge that this complex interface between TLR allelic composition, pathogens, and physiological status was not tested here.

In our study, we measured TLR polymorphism of sequence variants at both nucleotide and amino acid levels. We showed that amino acid polymorphism of TLR loci was much reduced when compared with the polymorphism at the level of nucleotide sequence. This pattern was due to the presence of numerous synonymous SNPs, which is not surprising while taking the conservative nature of TLRs into consideration (Alcaide and Edwards 2011). Interestingly, synonymous (silent) mutations were primarily responsible for all significant associations between TLRs and physiological condition that were revealed in our study, and we failed to find any relationships at the level of amino acid sequence variants. For long, it has been assumed that synonymous SNPs are functionally inconsequential, as they do not lead to a change in primary polypeptide sequence. Nevertheless, there is rapidly accumulating evidence that synonymous mutations in immune genes may be implicated in determination of disease resistance in humans (Junjie et al. 2012, Beima-Sofie et al. 2013, Cho et al. 2013, Taniguchi et al. 2013). For example, 2 synonymous SNPs in the exon of TLR2 were associated with the risk of hepatocellular carcinoma HCC, which is the most common type of primary liver cancer (Junjie et al. 2012). A significant association was found between a synonymous mutation in the TLR9

exon and HIV-1 acquisition risk, although this could reflect a linkage disequilibrium with an intronic SNP that affected transcriptional activity (Beima-Sofie et al. 2013). Also, synonymous exonic polymorphisms in the TLR2 gene were associated with susceptibility to congenital cytomegalovirus infection, but it has not been resolved whether this was due to their own effect or whether they are linked with other polymorphisms directly responsible for resistance (Taniguchi et al. 2013). Finally, the majority of SNPs across 12 genes of the innate immune system that were associated with TLR-induced cytokine production (mostly signal transducer and activator of transcription [STAT], interferon regulatory factor [IRF], and spleen tyrosine kinase [SYK] families) were either synonymous or located in noncoding regions (Cho et al. 2013).

There are a broad range of mechanisms that can explain the role of synonymous mutation in disease resistance through modifications of protein level and conformation (Sauna and Kimchi-Sarfaty 2011). The amount of active and correctly folded protein is influenced by transcription, translation, or turnover of mRNAs and proteins, but the regulation at the translation level is thought to dominate (Schwanhäusser et al. 2011). Synonymous mutations are often implicated in the translation processes through the mechanisms of splicing disruption (via the effect on the function of exonic splicer enhancers), mRNA degradation and reduced efficiency of translation initiation (via less stable mRNA secondary structure), changes in local translation elongation rates (via changes in relative synonymous codon usage), regulation of co-transcriptional protein folding (via the effect on tertiary protein structure during synthesis), and proteostasis (via the effect of a misfolded protein on other protein tertiary structure and proteotoxicity) (Sauna and Kimchi-Sarfaty 2011). Although our results provided support for robust associations between physiological condition and nucleotide (rather than amino acid) polymorphism at TLR genes in the Black-headed Gull, we did not examine molecular mechanisms that could be responsible for these relationships and we may only speculate whether they reflect direct effects or not. In fact, we cannot rule out alternative indirect explanations for the pattern that we observed. First, synonymous SNPs may be in linkage disequilibrium with polymorphisms located in regions not targeted by our primers (e.g., within introns or gene expression regulative regions), and this scenario has already been evoked to explain associations between synonymous SNPs in TLRs with disease resistance in humans (Beima-Sofie et al. 2013). It is also possible that the variability observed in TLRs reflects the variability at non-TLR loci or genome-wide heterozygosity. Although we controlled for neutral heterozygosity at microsatellite loci in the analyses, our panel of microsatellite markers was relatively small, and its power to produce reliable estimates of genome-wide heterozygosity could be limited.

Furthermore, our indices of physiological condition may be considered as health-related traits, but they are not directly linked with susceptibility to any particular diseases, which does not allow us to draw any conclusions as to the nature of their associations with TLR diversity. Finally, although we corrected for the FDR where possible, it is possible that our statistical analyses could still suffer from type I error due to the large number of models tested. Taking all these limitations into account, the mechanisms responsible for the links between TLR polymorphism and physiological condition in the Black-headed Gull certainly require a more rigorous examination in the future.

So far, there is surprisingly little information on how variation in TLRs affects fitness and disease susceptibility in wild animals. For example, it was shown that polymorphisms at TLR2 were associated with *Borrelia afzelii* infection status in wild bank voles (Tschirren et al. 2013), whereas infection intensity with *Heligmosomoides polygyrus* nematode and *Polyplax serrata* louse was negatively correlated with TLR-based innate immune responsiveness in wood mouse (Jackson et al. 2009). TLR4 genotype was also found to be associated with gamasid mite, flea (*Megabothris walkeri*), and sheep tick larva (*Ixodes ricinus*) burdens in the isolated population of water voles (Gavan et al. 2015). Although our knowledge on the associations between TLR allelic variation and pathogen resistance in wild mammals is limited, it seems to provide a clear support for the important role of TLR genes in determination of disease susceptibility. At the same time, similar information for wild birds is virtually lacking. The only study of this kind that we are aware of revealed significant associations of allele frequencies and heterozygosity at 3 TLR loci (TLR1A, TLR2B, and TLR7) with *Haemoproteus* infection status in wild Bananaquit (*Coereba flaveola*) (Antonides et al. 2019). Information on the associations of TLR polymorphism with other fitness-related traits in wild birds is also fragmentary and inconclusive. For example, post-release survival of captive-bred endangered Attwater's Prairie-Chicken (*Tympanuchus cupido attwateri*) was related to the presence of specific TLR1B alleles (Bateson et al. 2016). In contrast, a study on Song Sparrows (*Melospiza melodia*) found no evidence for associations between TLR heterozygosity and survival (Nelson-Flower et al. 2018), whereas a negative relationship was found between TLR diversity (number of SNPs across 6 TLR loci) and survival in a bottlenecked population of Pale-headed Brushfinch (*Atlapetes pallidiceps*) (Hartmann et al. 2014). Considering how little we know about TLR-fitness associations in birds, we believe that our study provides a valuable contribution to our understanding of the role of immunogenetic variation in wild animals.

Conclusions

Our study provided correlational evidence for associations of TLR diversity with physiological condition in the Black-headed Gull. Surprisingly, we found that these associations were primarily apparent at the nucleotide rather than at the amino acid level. While the exact mechanisms for this kind of associations certainly merit further research, we believe that our study adds to the growing, but still modest, body of evidence for the fitness-related consequences of individual TLR repertoire in wild birds.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Ornithology* online.

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