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MHC Reflects Fine-Scale Habitat Structure in White-Tailed Eagles, *Haliaeetus albicilla*

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Abstract

The major histocompatibility complex (MHC) genes code for key immune receptors responsible for recognition of intra- and extracellular pathogens (MHC class I and class II, respectively). It was hypothesized that MHC polymorphism can be maintained via fluctuating selection resulting from between-habitat variation in pathogen regimes. We examined associations between MHC class I and class II genes and habitat structure in an apex avian predator, the white-tailed eagle, *Haliaeetus albicilla*. We genotyped MHC class I and class II genes in ca. 150 white-tailed eagle chicks from nearly 100 nesting territories distributed across 3 distinct populations in Poland. Habitat structure was quantified at the level of foraging territories and directly at the nest sites. We found strong support for associations of habitat traits with diversity and allelic composition at the MHC class II. Forest area within territory and forest productivity were identified as the major habitat predictors of MHC class II polymorphism, whereas other habitat traits (distance to nearest open water, grassland, and water area within territory or understory presence) showed fewer associations with class II alleles. In contrast, there was little support for associations between MHC class I genes and habitat structure. All significant associations were apparent at the within-population level rather than between populations. Our results suggest that extracellular (rather than intracellular) pathogens may exert much stronger selective pressure on the white-tailed eagle. Associations of habitat structure with MHC class II may reflect fluctuating (balancing) selection, which maintains MHC diversity within populations.

Subject area: Molecular adaptation and selection
Key words: birds, fluctuating selection, habitat, major histocompatibility complex, population divergence, white-tailed eagle

Habitat is often recognized as one of the leading factors that shape genetic structure of natural meta-populations (Keyghobadi 2007). Genetic differentiation across landscapes may be driven by stochastic processes, such as genetic drift, and it should be affected by the rate of gene flow (migration rate) between subpopulations or habitat patches (Miles et al. 2019). Highly isolated populations in fragmented landscapes are expected to diverge at a higher rate than populations with fewer or no migration barriers and extensive gene flow. However, genetic divergence between populations or landscapes could also be enhanced by adaptive processes. Natural selection is

known to vary in space, and each local population should evolve traits that provide an advantage under the local environmental conditions (habitats), regardless of their adaptive value and fitness consequences in other habitats (Kawecki and Ebert 2004). This mechanism is recognized as the local adaptation and it assumes that resident genotypes in each population are expected to have a higher relative fitness in their local habitat than genotypes originating from other habitats (Kawecki and Ebert 2004). Local adaptation has often been evoked to explain adaptative processes occurring in host–parasite systems (Kaltz and Shykoff 1998; Penczykowski et al. 2016), as parasites or pathogens usually impose strong selection on their hosts, the role of phenotypic plasticity and maternal effects appears comparatively small, and single genes often have strong effects (Kawecki and Ebert 2004).

Pathogen-recognition genes, such as the major histocompatibility complex (MHC), are among the key loci that should respond to spatial variation in pathogen-driven selection via local adaptation. In general, MHC receptors bind small peptides (antigens) derived from the processing of intracellular (MHC class I) and extracellular (MHC class II) pathogens or parasites. On successful recognition, the antigens are presented to specialized immune cells (T cells), which trigger a cascade of immune responses aimed at pathogen elimination. The MHC is recognized as one of the most polymorphic regions in vertebrate genomes (Geraghty et al. 2002), and extraordinary diversity of these genes within and across populations is thought to be maintained via balancing selection, which acts through 3 major mechanisms of overdominant selection (i.e., heterozygote advantage; Hughes and Nei 1988), negative frequency-dependent selection (Takahata and Nei 1990), and fluctuating selection (Hedrick 2002). The last of these mechanisms assumes that pathogen regime fluctuates over time and in space, as so should fluctuate directional selection at the MHC (Spurgin and Richardson 2010). Consequently, in heterogeneous landscape, different subsets of MHC alleles are expected to be selected in different habitats, thus maintaining genetic diversity within populations, but across landscapes. Although empirical support for fluctuating selection and local adaptation at the MHC has been reported for different vertebrate taxa, including fish (Dionne et al. 2007, Eizaguirre and Lenz 2010), amphibians (Hernández-Gómez et al. 2019), birds (Ekblom et al. 2007, Loiseau et al. 2011), and mammals (Charbonnel and Pemberton 2005, Bryja et al. 2007), most evidence comes from between-population comparisons at large geographical scale and we still lack a thorough knowledge on how polymorphism at MHC genes respond to a fine-scale habitat variation.

The aim of this study was to examine associations of habitat structure with polymorphism at the MHC class I and class II genes in an apex avian predator, the white-tailed eagle *Haliaeetus albicilla*. For this purpose, we genotyped MHC class I and class II in ca. 150 white-tailed eagle chicks from nearly 100 territories distributed across Poland. The white-tailed eagle is a large monogamous bird of prey (body mass of 4–6 kg) with long lifespan (up to 40 years). Adults are resident and winter in their territories, breeding dispersal is uncommon, although young birds can disperse at the distances of several hundred km (Whitfield et al. 2009). White-tailed eagles are primarily associated with a mosaic of woodlands, where they nest, and aquatic habitats, such as coastal areas, river valleys, or areas rich in lakes and fish ponds, where they prey on fish and waterbirds. The size of the Polish population is currently estimated at 1400–1500 breeding pairs (Chodkiewicz et al. 2015), being one of the largest mainland populations of this species in Europe. Despite this, genetic diversity of white-tailed eagles from Poland (as assessed at neutral microsatellite loci and mtDNA) was on the lower end of the variability range among the core European populations (Langguth et al. 2013). This could have been driven by the long-term reduction in the effective population size (e.g., during glaciation period; Hailer et al. 2007) and was likely enhanced by a severe population bottleneck in early and mid-20th century. In 18th and 19th centuries, the size of Polish population gradually decreased (mostly because of persecution), reaching a minimum of ca. 20 breeding pairs at the beginning of 20th century and staying at critically low level (<50 breeding pairs) until 1950s (Zawadzka et al. 2009). Despite this recent bottleneck, our preliminary analysis of the MHC in white-tailed eagles revealed that non-negligible MHC diversity was retained in the Polish population, but greater level of polymorphism and stronger signature of balancing selection was detected at the MHC class II when compared with class I (Minias et al. 2019). It has been suggested that MHC class II diversity could have been adaptively retained through the bottleneck due to stronger selection from extracellular pathogens (Minias et al. 2019). Thus, it is possible that greater spatial variation of extracellular pathogens between habitats could result in stronger fluctuating selection acting on MHC class II than class I. Here, we hypothesized that MHC polymorphism in the white-tailed eagle may reflect fine-scale habitat variation within or across populations, and we predicted that habitat-related associations are likely to be stronger for MHC class II than class I genes.

Materials and Methods

Study Populations and Material

Samples were collected in 2017–2018 from 3 white-tailed eagle populations (Figure 1) in Poland:

(i) NW population, mostly sampled in Szczecin and Gorzów Wielkopolski districts, at the Wolin Island and around Szczecin Lagoon; this is the region with highest white-tailed eagle pair densities in Poland and one of the key European refuges for the species during the critical population bottleneck in early and mid-20th century;

(ii) NE population, sampled in a 100-km-wide zone along the middle and Eastern Polish Baltic coast up to the eastern borders of Poland, including large woodland areas and several national parks (NP), including Bory Tucholskie NP, Wigierski NP, and Białowieski NP; this region holds medium to high white-tailed eagle pair densities and it was quickly recolonized after early and mid-20th-century bottleneck; and

(iii) SE population, sampled in central, central-eastern, and southern Poland, mostly in Łódź and Lublin districts; this is a region with relatively low densities of white-tailed eagles and one of the most recently recolonized after the early and mid-20th-century decline (e.g., a viable nesting population in the Łódź region was re-established at the beginning of 21st century; Anderwald et al. 2007). The recolonization event was probably started by an influx of immigrants from more stable Polish populations, but ringing resightings indicate that recruits from other Baltic countries (e.g., Lithuania or Estonia) contributed to the process of population establishment (D. Anderwald, unpublished data).

In total, we collected samples from 98 white-tailed eagle territories (22–54 territories per population). The number of territories sampled in 2017 and 2018 was 41 and 63, respectively, including 6 territories sampled in both years. Growing body or wing covert feathers (with blood in the shafts) collected from chicks were used

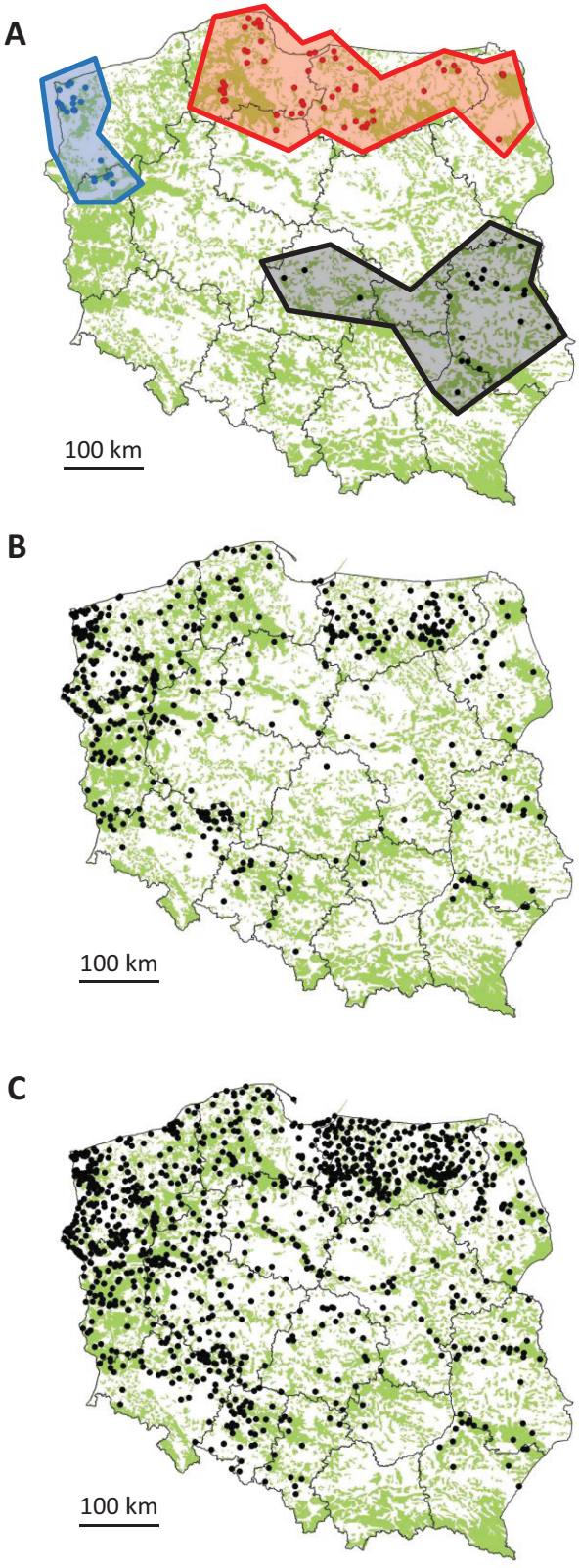

Figure 1. Location of white-tailed eagle territories: (**A**) sampled in 2017–2018; (**B**) all recorded in Poland in 1998; and (**C**) all recorded in Poland in 2018. Data on white-tailed eagle distribution in Poland was available via the Eagle Conservation Committee. Three study populations (**A**) are marked with solid lines. The map was created using publicly available Forest Data Bank information on forest distribution in Poland (https://www.bdl.lasy.gov.pl/portal/).

as a sample material. We collected 1–3 feathers per individual into Eppendorf tubes with 96% ethanol and stored them in −4 °C until laboratory analyses. In total, 152 chicks were sampled, resulting in the mean of 1.46 samples collected per brood.

Habitat Characteristics

We assessed habitat characteristics at the 2 major levels: within foraging territory and directly at the nest site. Because the reported home range radius of white-tailed eagles is between 2.5 and 15 km (Heuck et al. 2017), we have assessed foraging habitats within a radius of 8 km, which should well represent an average territory size in our study species. This threshold is also consistent with field observations of foraging white-tailed eagles in our populations (D. Anderwald, personal observation). To characterize habitats within the territories, we used the Corine Land Cover 2018 database available via the Copernicus Land Monitoring Service of the European Union. Extracted data (level 1) were grouped into 5 broader habitat categories of forests, grasslands, waters, agriculture areas, and built-up areas, and the surface of each habitat type was calculated for each white-tailed eagle territory. We also used ArcGIS software (Environmental Systems Research Institute, Redlands, CA) to calculate the distances from nests to the nearest water, hard-surfaced road, and building. Because agricultural area showed relatively strong negative correlation with forest area ($r = -0.62$, $n = 98$, $P < 0.001$; Supplementary Table S1), we excluded this variable from analyses to avoid multicollinearity of predictors. All other habitat variables showed weaker intercorrelations (Supplementary Table S1) and were retained in the modeling.

Habitat at nest sites was assessed using information retrieved from the Forest Data Bank (FDB), a public database maintained by the Bureau for Forest Management and Geodesy in Poland. The FDB contains detailed habitat data for all State and National Park forests in the country. To retrieve the data, we first used collected coordinates of white-tailed eagle nests and forest maps to identify forest subcompartments, where the nests were located. Forest subcompartments are the smallest recognizable forest land forming units that are used for forest management and represent uniform forest type patches. The average size of forest subcompartments with white-tailed eagle nests sampled in this study was 0.060 ± 0.005 [SE] km^2, so they provided information on habitat directly at the nest site. The distances between the nests and the borders of the subcompartments were not taken into account. We have collected the following information: 1) forest type with 4 categories of deciduous: mixed deciduous, mixed coniferous, and coniferous forests; 2) dominant tree species with 4 categories of pine: *Pinus* sp., birch *Betula* sp. and alder *Alnus* sp., beech *Fagus* sp., and others; 3) forest moisture with 3 categories of waterlogged, wet, and moist forests; 4) forest naturalness with 3 categories of natural (0), seminatural (1), and transformed (2) forests; 5) tree density with 3 categories of medium, loose, and interrupted density; 6) forest productivity measured as the relative standing volume (ratio of the observed to the expected volume of merchantable timber per ha); 7) presence of understory with 2 categories (present vs. absent); and 8) maximum tree age. The FDB data could not be collected for 3 nest sites located in private forests, so the analyses of nest-site habitat characteristics were limited to 95 territories. Noncategorical habitat traits collected at nest sites (forest productivity and maximum tree age) only weakly correlated with some of the habitat characteristics collected at the level of foraging territories (Supplementary Table S1).

MHC Genotyping and Allele Validation

DNA from feather blood was extracted using Bio-Trace DNA Purification Kit (EURx, Gdańsk, Poland). To genotype MHC class I and class II we used methodology originally developed for other accipitrid species (Alcaide et al. 2007, 2009), but successfully cross-applied in the white-tailed eagle (Minias et al. 2019). The MHC genes were amplified using 2 pairs of degenerate primers, MHCI-int2F/MHCI-ex4R for class I (Alcaide et al. 2009) and Acc2FC/Acc2RC for class II (Alcaide et al. 2007). The primers amplify class I exon 3 (binding to the flanking region of intron 2 and conserved region of exon 4) and class II exon 2 (binding to the flanking regions of introns 1 and 3), which code for protein fragments that form a peptide-binding groove of MHC molecules, thus having a direct role in pathogen recognition. In PCR amplifications, we used fusion primers composed of the MHC primer, a 7-bp barcode indicating sample identity, and Illumina adapter sequence. All PCR amplifications followed protocols described previously by Minias et al. (2019). PCR products were purified and Illumina libraries were prepared using NEBNext DNA Library Prep Master Mix Set for Illumina (New England Biolabs, Ipswich, MA). Illumina MiSeq (2 × 250 bp) platform was used for sequencing.

Raw Illumina sequences were processed using the Amplicon Sequencing Analysis Tools (AmlpiSAT) web server (Sebastian et al. 2016) and data processing followed Minias et al. (2019). Briefly, we used AmpliMERGE tool for pair-ended read merging and AmpliSAS tool for demultiplexing, clustering, and filtering of Illumina reads. We set default Illumina parameters for clustering (1% substitution errors, 0.001% indel errors, and 25% minimum dominant frequency) and filtering (chimeras and sequences with <3% frequency discarded). Minimum amplicon depth was set to 100 reads and maximum depth was limited to 5000 reads because of AmpliSAS performance reasons. Following our previous analyses on the MHC in white-tailed eagles (Minias et al. 2019), we set the exact read length at 412 and 269 bp for MHC class I and class II, respectively. The average allele depth (after processing) was 1997 ± 140 [SE] and 3705 ± 94 [SE] reads per sample, but we failed to obtain sequencing results for 7 and 12 individuals at the MHC class I and class II, respectively, resulting in the final sample sizes of 145 (class I) and 140 (class II) individuals. The analysis of independent technical replicates indicated >90% reproducibility of validated alleles. All validated MHC class I and class II alleles were aligned separately in Geneious v.10.0.5 (Biomatters Ltd., Auckland, New Zealand). Removal of non-exonic regions from the alignments retained a 273-bp fragment of MHC class I exon 3 (total exon length: 276 bp) and 258-bp fragment of MHC class II exon 2 (total exon length: 270 bp). Individual MHC diversity was expressed as the number of MHC class I or class II alleles recorded per individual.

Microsatellite Genotyping

To control for neutral heterozygosity in the analyses of MHC diversity, we genotyped 8 di- and tetranucleotide microsatellite markers (Hal01, Hal03, Hal04, Hal10, Hal13, IEAAAG04, IEAAAG05, and IEAAAG15) previously developed for the white-tailed eagle (Busch et al. 2005; Hailer et al. 2005). All PCR amplifications were conducted in a final volume of 20 µL containing 10 µL of DreamTaq PCR Master Mix, 1 µL of DNA template, and 0.2 µM of each primer. Annealing temperatures and PCR protocols followed original

sources (Busch et al. 2005; Hailer et al. 2005). Forward primers were labelled with 6-FAM fluorescent dye, and fragment size analysis was conducted using the 3730XL capillary sequencer (Applied Biosystems, Foster City, CA). Allele sizes were scored against GeneScan TM 600 LIZ Standard in Geneious v.10.0.5 software. We obtained information on 97.7% of microsatellite genotypes across all loci and the number of detected alleles ranged from 2 to 14 per locus. None of the markers showed evidence for genotyping errors (e.g., null alleles, stutter peaks, or large allele dropout; tested with Micro-Checker v.2.2.3, van Oosterhout et al. 2004); deviated from Hardy–Weinberg equilibrium in any of the study populations (as tested with Arlequin ver. 1.3.5.2, Excoffier and Lischer 2010); or stayed in linkage disequilibrium with other markers (as tested with Fstat ver. 2.9.3, Goudet 1995). Multilocus heterozygosity was calculated as the sum of heterozygosity values at each locus divided by the total number of loci.

Statistical Analyses

Associations between habitat characteristics and MHC were analyzed using generalized linear mixed models (GLMMs), as implemented in the *lme4* package (Bates et al. 2014) developed for R statistical environment (R Foundation for Statistical Computing, Vienna, Austria). First, we tested for associations between habitat and individual MHC diversity by entering the number of MHC class I and class II alleles as the response variables. Due to different sample sizes and to reduce the number of predictors in each model, we tested MHC–habitat associations at 2 separate levels of territories (forest area, grassland area, water area, built-up area, distance to water, distance to roads, and distance to buildings entered as covariates) and nest sites (forest type, dominant tree species, forest moisture, forest naturalness, tree density, and understory presence entered as fixed factors; forest productivity and maximum tree age entered as covariates). Heterozygosity at neutral (microsatellite) loci was entered as an additional covariate in these models because associations between MHC diversity and habitat could be primarily driven by the general level of genome-wide heterozygosity. The distribution of individual MHC class I and class II diversity was reasonably close to normal, and the models were run for the Gaussian distribution of the response variables. Brood identity, year, and population were entered as random factors in each model to avoid pseudoreplication resulting from nonindependence of data collected from siblings, as well as to control for interannual and interpopulation variation in the MHC data. Second, we tested for associations of habitat characteristics with specific MHC alleles. For these analyses, we selected a similar number of MHC class I (n = 6) and class II (n = 7) alleles, as we aimed to avoid any biases caused by overrepresentation of alleles from either class. Alleles with intermediate frequencies across all populations were selected (10–90% for class I and 20–80% for class II), as unbalanced sample sizes under extreme allele frequencies yield low statistical power to detect significant associations. The presence of each MHC allele was entered as a binary response variable, and the models were run using binomial distribution family. In each model, we included the same set of territory or nest site habitat characteristics, and the same set of random factors as described above. The *car* R package (Fox and Weisberg 2019) was used to infer Wald χ^2 statistics and P values for all predictors. P values obtained for specific MHC alleles were corrected for the positive false discovery rate (Storey 2003) using bootstrap procedure in the *qvalue* R package (Dabney et al. 2010). Between-population variation in habitat characteristics was tested in Statistica v.10.0 software (StatSoft, Tulsa, OK) using analysis of variance (ANOVA) for continuous variables and χ^2 tests for categorical variables. Between-population differentiation at the MHC and microsatellite markers was assessed with 2 different statistics. First, we calculated Jost's D statistic (Jost 2008), which is based on the effective number of alleles instead of expected heterozygosity (as in the case of traditional F statistics) and, thus, can be applied to multilocus MHC data, where individual heterozygosity is unknown. In these calculations, each MHC allele was coded as present or absent (as in a dominant marker) and Jost's D estimates were computed using the *SpadeR* software (Chao et al. 2016) for the MHC and the *diveRsity* R package (Keenan et al. 2013) for microsatellites. Second, we calculated Rho statistic (Ronfort et al. 1998), which was originally designed to measure genetic differentiation between populations that vary in ploidy level and, thus, it can be reliably applied to multilocus gene data, where it is not possible to assign specific alleles to loci (such as for the MHC). Computations of Rho values were conducted in SPAGeDi software (Hardy and Vekemans 2002), and statistical significance was assessed with 10 000 permutations. Finally, correlation between individual allelic diversity at MHC class I and class II was tested with the Pearson product moment coefficient. All values are reported as means ± SE.

Results

Territory and Nesting Site Selection

Forest was identified as the most common habitat type in white-tailed eagled territories (43.08 ± 1.98%), followed by agricultural areas (33.76 ± 2.13%) (Table 1). We recorded only 3 territories with low (<15%) share of forest areas, but there were many more territories with low share of agricultural areas (n = 38). Grassland and open water areas, which are the primary foraging

Table 1. Descriptive statistics for habitat characteristics (continuous variables only) of white-tailed eagle territories and nest sites

Habitat characteristics	Mean	Standard error	Minimum	Maximum	Skewness
Forest area (%)	43.08	1.98	2.48	83.79	0.29
Grassland area (%)	10.74	0.90	0.93	36.41	1.39
Agriculture area (%)	33.76	2.13	0.13	85.50	0.16
Water area (%)	9.11	1.47	0.00	89.71	3.38
Built-up area (%)	3.31	0.24	0.00	11.47	1.14
Distance to water (km)	2.27	0.25	0.03	13.37	2.25
Distance to roads (km)	1.13	0.08	0.15	3.93	1.56
Distance to buildings (km)	1.14	0.06	0.29	3.23	1.33
Forest productivity (%)	78.53	2.48	10	140	−0.21
Maximum tree age (years)	133.18	3.90	50	335	1.55

habitats for white-tailed eagles, on average covered 10.74 ± 0.90% and 9.11 ± 1.47% of territories, respectively (Table 1). Distribution of open water areas within territories was strongly right-skewed (skewness: 3.88; Table 1) and over half of all territories (n = 58) had very low (<5%) share of open water. Share of grassland areas within territories was much less right-skewed (skewness: 1.39; Table 1) and very low (<5%) share of grasslands was recorded in ca. one-third of territories (n = 34). Share of built-up areas within territories was generally low (below 12% in all cases) (Table 1). Most pairs (n = 74) nested in a relatively close distance (<3 km) to open water, but in several pairs (n = 5), this distance was >8 km (maximum 13.37 km) (Table 1). Most nests were located in mixed (deciduous and coniferous) forests (n = 59) with moist soils (n = 67), and pine was identified as the dominant tree species in most cases (n = 63). Maximum age of trees at the nest site ranged from 50 to 355 years (Table 1), but large majority of nests were located in stands with trees >100 years of age (n = 85). Tree density was in most cases interrupted (n = 45), forest productivity was moderate (78.5 ± 2.48% on average), and understory was present in ca. one-third of all territories (n = 31).

Between-Population Variation in Habitat Selection

We found significant between-population variation in several territory and nest-site characteristics (Supplementary Table S2). Eagles from NW population nested in territories with the highest share of grassland (NW vs. NE: $P < 0.001$; NW vs. SE: $P = 0.077$) and open water areas (NW vs. SE: $P = 0.003$). At the same time, the share of agricultural areas within the territories was lowest in this population (NW vs. NE: $P < 0.001$; NW vs. SE: $P < 0.001$). NW birds also nested in the oldest forests (NW vs. SE: $P = 0.004$) and furthest from buildings (NW vs. NE: $P = 0.043$) or roads (NW vs. NE: $P = 0.003$; NW vs. SE: $P = 0.009$). Finally, NW birds more frequently nested in forest dominated by alder/birch (40.91% vs. 0.0% in NE and 19.1% in SE) rather than pine (31.8% vs. 76.9% in NE and 76.2% in SE) and in forests with higher soil moisture (waterlogged/wet forests: 54.5% vs. 11.5% in NE). Habitat differences between NE and SE populations were less apparent, but we found that NE birds nested in territories with smaller grassland areas ($P = 0.003$), but in older forests ($P = 0.016$) than SE birds. Also, NE birds more frequently nested in forests dominated by beech (13.5% vs. 0.0%) rather than alder/birch and in forests with dryer soils (waterlogged/wet forests: 11.5% vs. 47.6%). No significant between-population variation was found for other habitat characteristics (forest area, built-up area, distance to water, forest type, forest naturalness, forest productivity, tree density, and understory presence; all $P > 0.05$; Supplementary Table S2).

MHC Diversity and Differentiation

Across all 3 populations, we recorded 13 MHC class I and 19 MHC class II alleles in the white-tailed eagles. A mean of 3.70 ± 0.10 class I and 5.52 ± 0.15 class II alleles were retrieved per individual, and the maximum number of alleles per individual was 6 and 9 for MHC class I and class II, respectively, which was consistent with the presence of 3 class I and 5 class II loci. Correlation between individual allelic diversity at MHC class I and class II was marginally nonsignificant ($r = 0.16$, $n = 133$, $P = 0.069$). We found no between-population differentiation at the MHC class I or class II, as measured with Jost's D and Rho (all $P > 0.05$; Supplementary Table S3). In contrast, significant differentiation between all pairs of populations was detected at the neutral microsatellite markers (Supplementary Table S3).

Between-Habitat Variation in MHC

We found that MHC class II diversity was associated with the share of forest area within the territories and forest productivity, as pairs from more forested territories, but with lower forest productivity produced offspring with more MHC class II alleles (Figure 2; Supplementary Table S4). Both forest area and forest productivity were also among the major habitat predictors for the presence of specific MHC class II alleles. The presence of 3 alleles (DAB*03, DAB*08, and DAB*09) showed significant positive associations with forest area (Figure 3A; Supplementary Table S5) and negative associations with forest productivity at the nest sites (Figure 3B; Supplementary Table S6). Other significant habitat predictors for the presence of specific MHC class II alleles included distance to water (negative association for DAB*03) and understory presence at the nest site (negative association with DAB*03 and DAB*08) (Figure 3; Supplementary Tables S5 and S6). All these associations remained significant after correction for multiple comparisons (Supplementary Tables S5 and S6). The presence of allele DAB*04 was also negatively associated with grassland area within the territory and positively associated with water area within the territory, but these associations did not retain significance after correction for multiple comparisons (Supplementary Table S5).

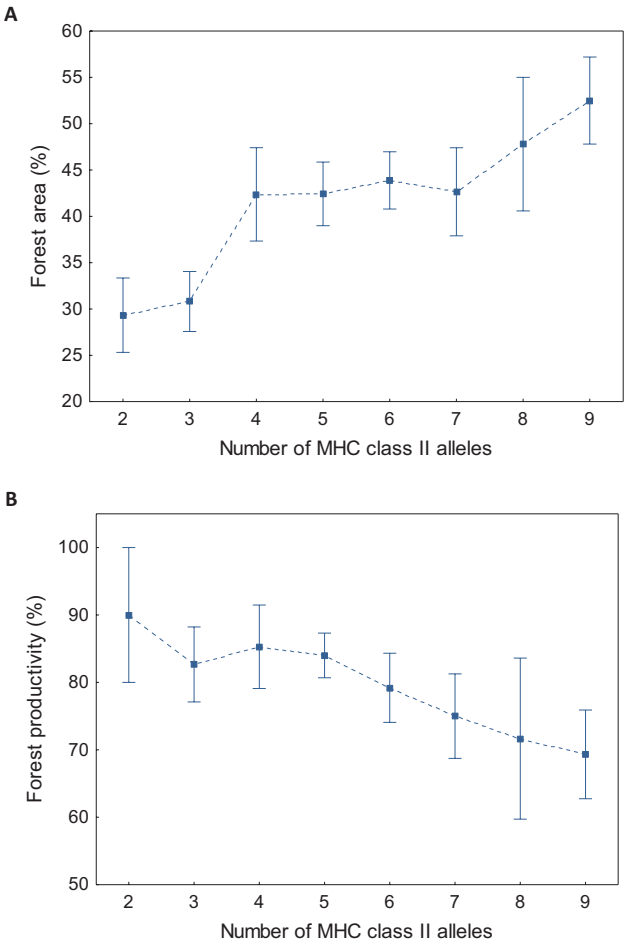

Figure 2. Associations of individual MHC class II diversity (number of alleles per individual) with the share of forest area within the territory (**A**) and forest productivity at the nest sites (**B**) in the white-tailed eagle. Means ± SE are presented for each category of MHC diversity.

We found no associations between habitat characteristics and MHC class I diversity in white-tailed eagles (Supplementary Table S4). Associations of habitat predictors with specific MHC class I alleles were infrequent, and only 2 habitat variables, understory presence and forest naturalness, were found to be associated with the presence of single alleles (Supplementary Table S7 and S8). Specifically, we recoded less-frequent occurrence of UA*04 in forests with understory presence and more frequent occurrence of UA*02 in transformed versus seminatural forests (Figure 4), but the latter association did not remain significant after correction for multiple comparisons (Supplementary Table S8).

Discussion

In this study, we found strong support for associations between MHC and habitat structure in white-tailed eagles sampled across one of the key European mainland populations. The associations were much more apparent for MHC class II genes, which are responsible for the recognition of extracellular pathogens and were previously reported to have a stronger signature of pathogen-driven selection in the white-tailed eagle when compared with MHC class I genes. We found several significant associations between MHC class II diversity or specific MHC class II alleles with multiple habitat characteristics at nest sites and foraging territories. In contrast, there was little evidence for associations of habitat with diversity and allelic composition at the MHC class I genes.

Forest area within foraging territory and forest productivity were identified as the major predictors of MHC class II polymorphism in white-tailed eagles from our study populations. We found that eagles from territories with larger forest area and nests located in forests of lower productivity had greater diversity of MHC class II genes. Consistently, 3 specific MHC class II alleles had higher frequency in territories with larger forest areas and at nest sites with lower forest productivity. It suggests that these 2 habitat traits may be important determinants for the composition of pathogen/parasite fauna of the white-tailed eagles. It also suggests that more forested territories with lower productivity may be associated with higher diversity of extracellular pathogens and that birds nesting in this kind of habitat may require specific MHC class II alleles to effectively cope with habitat-specific pathogen pressure. At the same time, these alleles may not be adaptive in other habitats, as indicated by their lower frequency in territories with contrasting habitat structure. Although forest area and forest productivity were not correlated across our sampled territories ($P > 0.05$), they were associated with a similar set of MHC class II alleles (DAB*03, DAB*08, and DAB*09). Furthermore, DAB*03 was negatively associated with distance from nest to the nearest open water, whereas DAB*03 and DAB*08 were negatively associated with understory presence at the nest site. Also, another class II allele, DAB*04 was associated positively with water area, but negatively with grassland area within territories, although these associations lost significance after correction for multiple comparisons. Taking all this into account, associations between MHC class II and habitat appear to be complex and apparent at multiple levels of habitat variation.

It is striking that key habitat predictors of MHC class II composition in white-tailed eagles showed no significant variation between

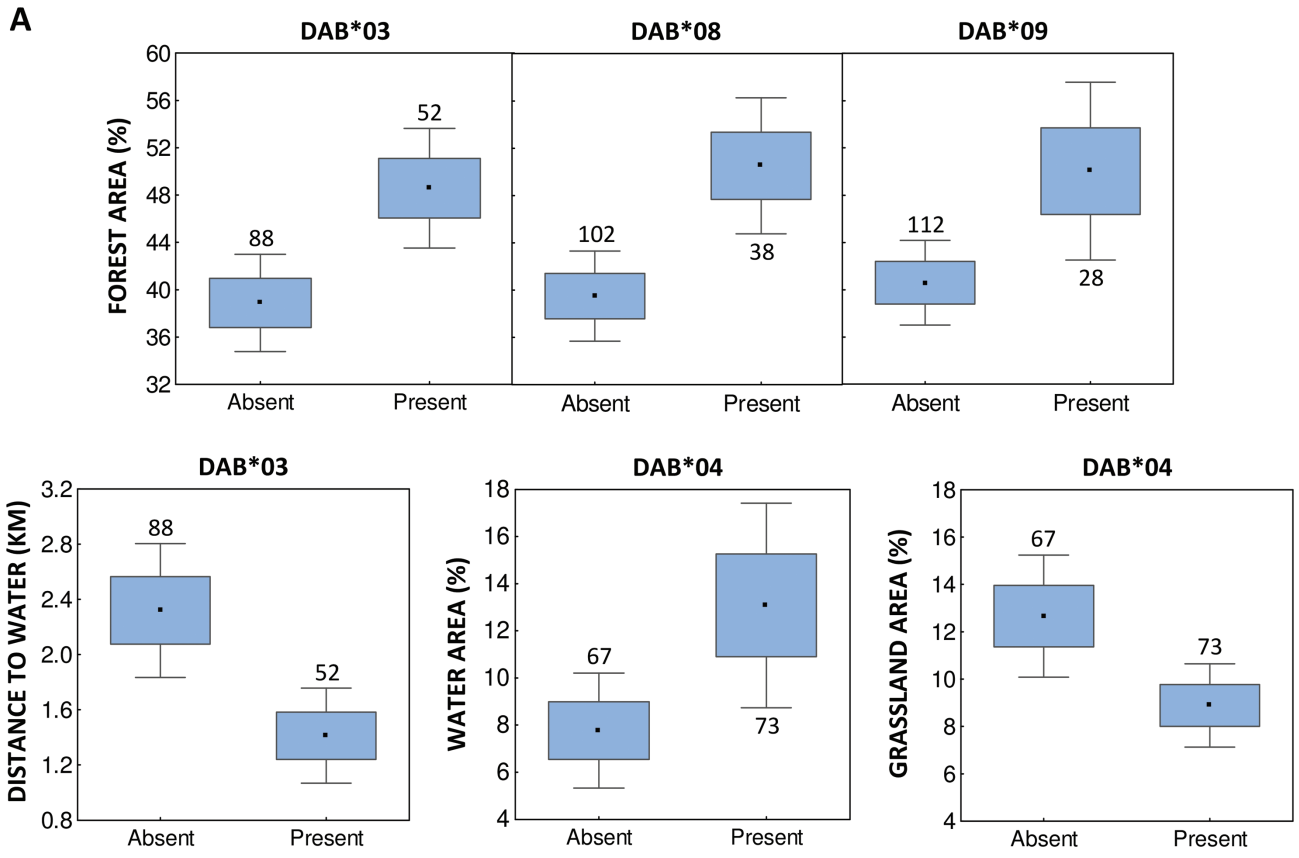

Figure 3. Associations of MHC class II alleles (DAB) with habitat characteristics within territories (**A**) and at the nest sites (**B**) of white-tailed eagles. Central point represents mean, box represents SE, and whiskers represent 95% confidence intervals. Sample sizes are shown above or below the boxes.

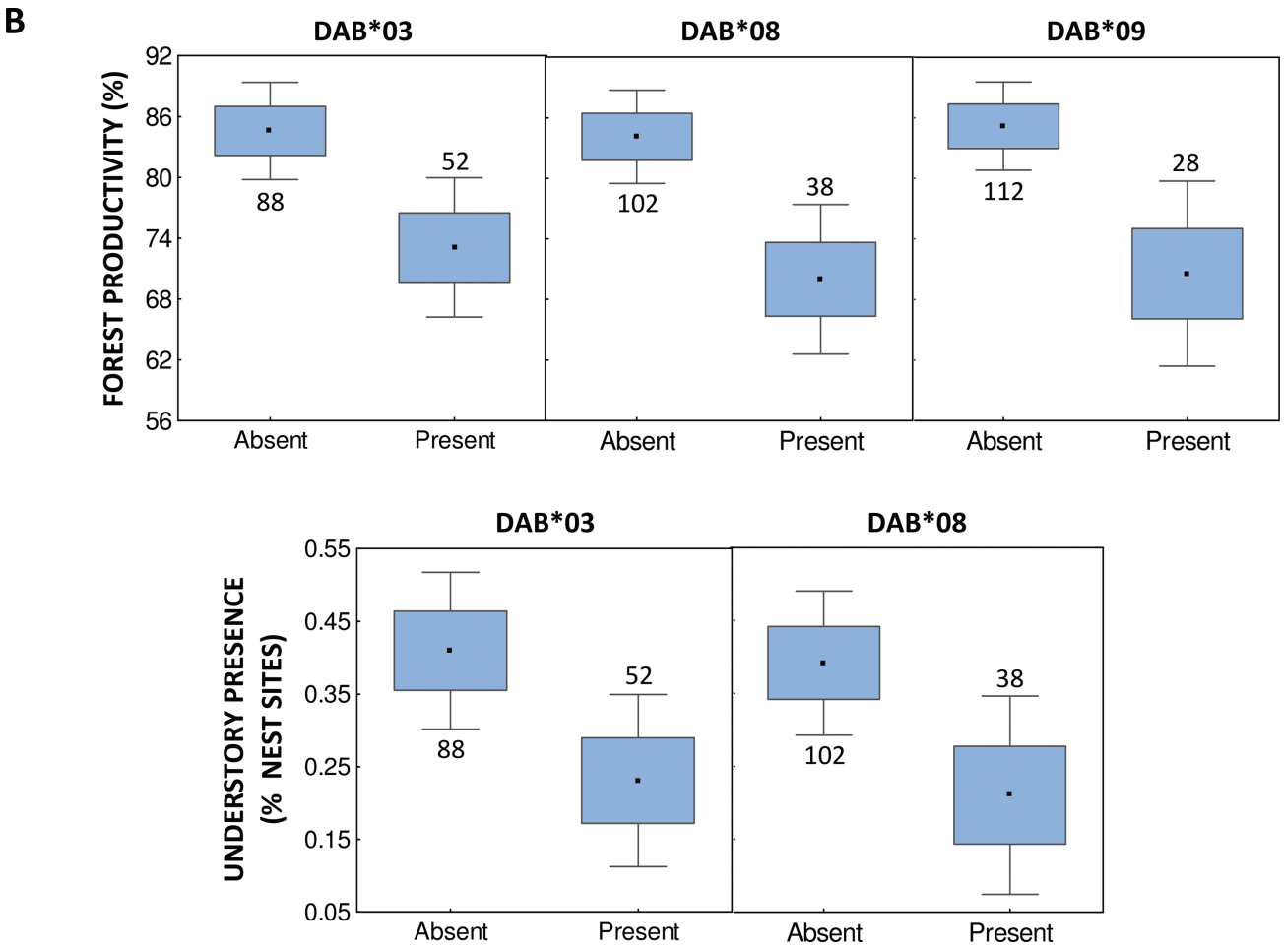

Figure 3. Continued.

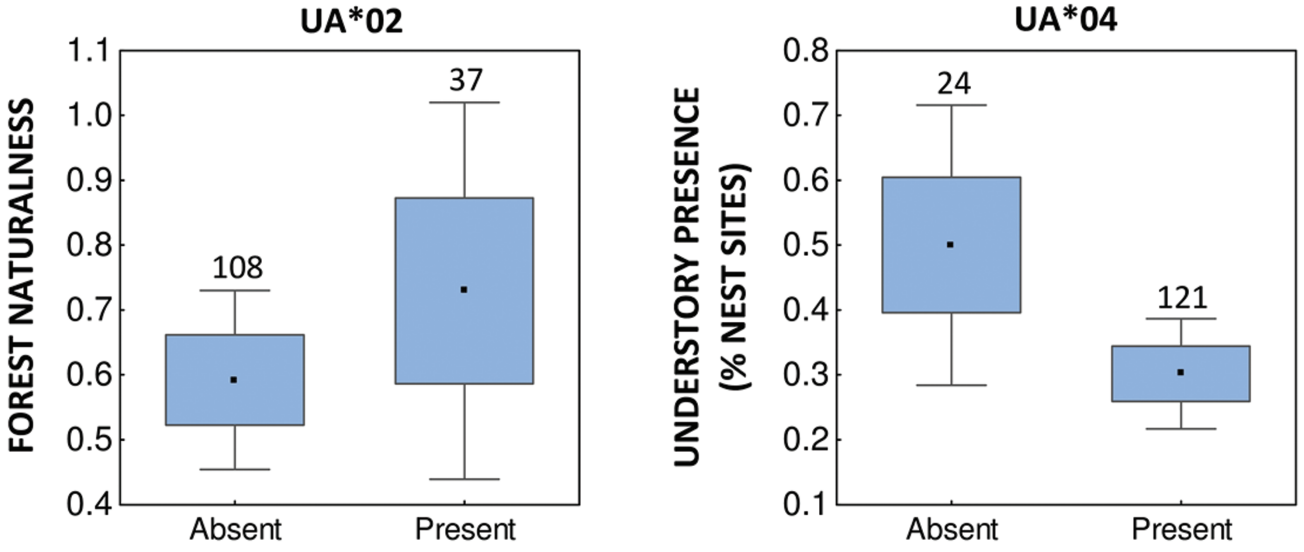

Figure 4. Associations of MHC class I alleles (UA) with habitat characteristics at the nest sites of white- tailed eagles. Central point represents mean, box represents SE, and whiskers represent 95% confidence intervals. Sample sizes are shown above or below the boxes.

our 3 study populations. Also, we found no evidence for MHC differentiation between these populations. This pattern is surprising, as all 3 populations had different demographic histories, which was expected to affect their genetic structure. The NW population remained a refuge for white-tailed eagles during the critical population bottleneck in the first half of 20th century, so it was predicted to

retain more genetic variation than the SE population, which went extinct during the bottleneck and was only recently re-established. Consistently with this prediction, we found a significant differentiation between all 3 populations at the neutral microsatellite loci, with the greatest pairwise differentiation between NW and SE population. No accompanying differentiation at the MHC indicates that associations of MHC with habitat are unlikely to be explained by divergent selection among populations resulting in local adaptations to spatially variable environmental conditions. Instead, the pattern we observed could be due to fluctuating (balancing) selection acting on MHC class II genes within our study populations, where different habitats within each population are associated with different pathogen regimes. As a result, different MHC alleles could be adaptively selected in different habitats, which should maintain greater MHC diversity within each population. We cannot, however, exclude that strong associations of MHC class II with habitat may arise through some alternative mechanism. For example, high-quality individuals that monopolize optimal habitats may carry specific MHC class II alleles and show higher MHC class II diversity. In this scenario, habitat variation in MHC polymorphism may not directly reflect habitat variation in pathogen pressure.

Our analyses provided much weaker empirical support for associations of MHC class I genes with habitat structure. Only 2 of 6 tested MHC class I alleles (33%) showed associations with single habitats traits (forest naturalness and understory presence) and only one of these associations retained statistical significance after correction for multiple comparisons. Also, no associations were found between habitat structure and individual MHC class I diversity. This contrasted with ca. 60% of MHC class II alleles (4 of 7 tested) staying in associations with diverse habitat characteristics. Contrasting patterns detected for MHC class I and class II stay in accordance with our previous molecular analysis of selection signature at the MHC of white-tailed eagles. We found greater sequence polymorphism and higher ratio of nonsynonymous to synonymous nucleotide substitutions (*dN*/*dS* ratio) at the MHC class II than class I genes, being consistent with stronger balancing selection acting on the MHC class II (Minias et al. 2019). On the one hand, this pattern agrees with general pattern of selection at the MHC of nonpasserine birds species (Minias et al. 2018) and could reflect evolutionary history of the avian MHC. On the other hand, we cannot exclude a role of recent demographic processes, especially the critical population bottleneck in the early and mid-20th century. The maintenance of MHC class II diversity through the bottleneck could be facilitated by higher number of duplicated class II loci in the MHC region of the white-tailed eagle (5 loci vs. 3 loci for class II and class I, respectively), whereas MHC class I diversity could have been more easily lost due to genetic drift. Despite this hypothesis, non-negligible variation was still retained at the MHC class I of the white-tailed eagle, and contrasting patterns of MHC class I and class II associations with habitat structure are unlikely to be explained solely by demographic history, but are expected to reflect adaptive processes.

Although we found contrasting patterns of habitat variation between MHC class I and class II, we acknowledge that our analyses were based on the data collected from offspring rather than from adults breeding in particular habitats. Although MHC allelic composition and diversity in offspring is expected to roughly reflect MHC polymorphism of their parents, it is possible that young birds may possibly select different habitats during their lifetime and we have no information on whether habitat choice is correlated between offspring and parents in the white-tailed eagles. Despite non-negligible natal dispersal potential (up to several hundred kilometers; Whitfield et al. 2009), many immature white-tailed eagle repeatedly visit their natal population before maturation (Nemesházi et al. 2018) and most recruits settle relatively close to their natal territories. For example, median natal dispersal distance was 21–45 km in males and 47–58 km in females from a reintroduced Scottish population (Whitfield et al. 2009). This may suggest that breeding habitats are generally similar to the natal ones at the landscape scale, but it does not allow to draw any conclusions on the fine-scale habitat selection. Also, our study design did not allow us to explore an interface between MHC polymorphism, habitat selection and mate choice. Recent genetic studies on the Hungarian white-tailed eagle population provided evidence for kin avoidance in mate choice (Nemesházi et al. 2018), but it remains to be studied whether white-tailed eagles choose their mates based on the MHC polymorphism (mechanisms and hypotheses associated with MHC-based mate selection reviewed by Kamiya et al. 2014), and if so, whether the processes of mate selection could affect between-habitat MHC variation.

Information on the associations between MHC and fine-scale habitat variation within populations are virtually lacking and most research that examined MHC in the context of habitat variation focused on habitat fragmentation and comparisons across broad habitat categories. For example, MHC class I allelic richness of the ornate dragon lizard *Ctenophorus ornatus* differed between populations from transformed (agricultural) and natural (native woodlands) habitats and these differences reflected parasite (tick) load between the habitats (Radwan et al. 2014). At the same time, MHC differentiation between agricultural populations was much higher than between woodland populations, which could reflect higher population fragmentation due to habitat degradation, reduced gene flow between subpopulation and loss of MHC diversity through genetic drift (Radwan et al. 2014). Exceptionally extensive research on habitat variation in the MHC has been conducted on geographically diverse populations of a small freshwater fish, the three-spined stickleback *Gasterosteus aculeatus*. An analysis of samples from Northern Germany showed that lake and river stickleback ecotypes harbored contrasting parasite communities and differed in the allelic composition of the MHC class II genes (Eizaguirre et al. 2011). This pattern was explained with a combined action of genetic drift and parasite-mediated selection, which had a homogenizing effect on the MHC within each habitat type and diverging effect between habitat types (Eizaguirre et al. 2011). A more recent study from British Columbia, Canada, supported previous findings that parasite community differed substantially between lake and stream habitats of sticklebacks (Stutz and Bolnick 2017). The authors demonstrated that lake and stream stickleback populations harbored distinct, although overlapping MHC class II genotypes and the associations between particular MHC II alleles and particular parasite taxa supported the effects of both balancing selection within habitats and divergent selection between habitats (Stutz and Bolnick 2017). Despite their relatively high popularity in MHC research, birds are clearly underrepresented in the studies on MHC-habitat associations, and to the best of our knowledge, only some large-scale between-population comparisons were conducted so far. For example, strong differentiation at the MHC class II was recorded in the great snipe *Gallinago media* from Scandinavian and South-eastern Baltic regions, which was speculated to reflect substantial habitat variation between these populations (dry mountain fens vs. floodplains and coastal meadows) (Ekblom et al. 2007).

In conclusion, our study provided convincing evidence for MHC–habitat associations in the large bird of prey, the white-tailed eagle. These associations were primarily apparent for MHC class II rather

than class I genes, which suggests a stronger selective pressure from extracellular than intracellular pathogens, being consistent with our previous molecular examination of selection signatures at the white-tailed eagle MHC (Minias et al. 2019). Because MHC–habitat associations were primarily recorded at the within-population level, we speculate that they were more likely to have arisen in response to fluctuating (balancing) selection rather than divergent selection among populations. Nevertheless, we acknowledge that our current results do not provide any direct evidence on selection patterns in the white-tailed eagle and data on pathogen/parasite pressure, and individual fitness would be required to complement our current findings.

Supplementary Material

Supplementary data are available at *Journal of Heredity* online.

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Data Availability

Raw data have been deposited at the electronic Supplementary Material. All sequences generated and used in the study have been deposited in GenBank (nos. MK186004–MK186030).

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