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**Avian blood parasite richness decreases with MHC class I  
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This article does not present research with ethical considerations

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**Data**

*It is a condition of publication that data, code and materials supporting your paper are made publicly available. Does your paper present new data?:*

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All data used in the analysis is available in Table S1 and S2.

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**Authors' contributions**

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O.V. & C.L. wrote the manuscript. O.V. performed the comparative analysis. C.L. and M.G. designed the analysis. All authors critically revised the manuscript, gave final approval for its publication and agree to be held accountable for the work performed therein.

# 1 **Avian blood parasite richness decreases with MHC class I loci number**

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19

## 20 **Abstract**

21 Major Histocompatibility Complex (MHC) genes are among the most polymorphic in the  
22 vertebrate genome. The high allele diversity is believed to be maintained primarily by  
23 sexual and pathogen-mediated balancing selection. The number of MHC loci also varies  
24 greatly across vertebrates, most notably across birds. MHC proteins play key roles in  
25 presenting antigens on the cell surface for recognition by T cells, with class I proteins

26 specifically targeting intracellular pathogens. Here we explore the hypothesis that MHC  
27 class I diversity (measured as loci number) co-evolves with haemosporidian parasite burden  
28 of the host. Using data on 54 bird species we demonstrate that high MHC class I diversity is  
29 associated with significantly lower richness of *Plasmodium*, *Haemoproteus* as well as  
30 overall haemosporidian parasite lineages, the former thus indicating a more efficient  
31 protection against intracellular pathogens. Nonetheless, the latter associations were only  
32 detected when MHC diversity was assessed using cloning and not 454 pyrosequencing-  
33 based studies, nor across all genotyping methods combined. Our results indicate that high  
34 MHC class I diversity might play a key role in providing qualitative resistance against  
35 diverse haemosporidian parasites in birds, but further clarification is needed for the origin  
36 of contrasting results when using different genotyping methods for MHC loci  
37 quantification.

38

### 39 **Keywords**

40 Haemosporidians, copy number variation, parasite richness, pathogen-mediated selection,  
41 comparative analyses, MHC diversity

42

### 43 **1. Introduction**

44 The Major Histocompatibility Complex (MHC) is known to be the most variable gene  
45 group in vertebrates, both in terms of allelic diversity and gene number [1]. MHC genes  
46 code for cell surface glycoproteins that bind to antigenic peptides and present them to cells  
47 of the immune system, initiating adaptive immune responses. Proteins encoded by the  
48 MHC genes ensure the immune presentation of a broad spectrum of peptides, both derived  
49 from the intracellular (class I proteins) or extracellular (class II proteins) space [2]. High  
50 MHC diversity, reflected by high allele diversity or loci number, is often related to the

51 ability to present a wider repertoire of antigens to the immune system, thus representing a  
52 genetic marker of disease resistance [3] but see [4]. Several, mutually non-exclusive  
53 hypotheses have been put forward to explain the maintenance of high MHC diversity, such  
54 as the heterozygote advantage (i.e., MHC diversity conferring disease resistance), negative  
55 frequency-dependent selection (benefit of rare MHC alleles in pathogen detection) or  
56 fluctuating selection (spatial or temporal change in the selective advantage of MHC alleles)  
57 [5,6].

58

59 The number of MHC loci varies greatly across vertebrates, being the result of a complex  
60 evolutionary history, involving gene duplications and translocations [7,8]. For example,  
61 species from the Galliformes order possess only two MHC class I genes [9,10], which is  
62 considerably lower than in other bird lineages [11,12], such as passerines. Several studies  
63 reported that a low number of MHC loci could represent the ancestral state in birds, and  
64 that the highest diversification in copy numbers occurred relatively late in the avian  
65 radiation, reaching its highest diversity in passerines [12]. While, parasite-mediated  
66 selection has been considered as the most prominent component driving MHC  
67 diversification [13], it is now widely accepted that in addition to the pathogen burden,  
68 sexual selection and risk of developing autoimmune diseases or immunopathologies may  
69 together shape the evolution of an optimal rather than maximal MHC diversity [3,4,13–15].

70

71 Malaria and closely related haemosporidian parasites are intensively investigated vector-  
72 borne parasites in birds, partly because of the high diversity of hosts they are able to infect,  
73 and their nearly worldwide distribution [16]. A series of experimental studies [17–21], have  
74 demonstrated the substantial costs associated with haemosporidian infection, in terms of

75 reproductive success, health condition and survival. Hence, immunological adaptations  
76 targeting and eliminating such infections can provide considerable fitness benefits. Key  
77 components of these defenses are the MHC proteins. Nonetheless, knowledge on the co-  
78 evolution of MHC diversity and avian haemosporidian parasite burden across species  
79 remains limited [22–26]. However, other host-parasite systems found strong evidences that  
80 specific MHC alleles confer resistance to pathogens [27,28].

81

82 With the increasing use of molecular tools, a large number of bird species have been  
83 recently screened for haemosporidian parasites. The MalAvi database represents a unified  
84 database compiling these data, covering genetic and geographical data of avian blood  
85 parasites (*Plasmodium*, *Haemoproteus*, *Leucocytozoon* genera). It currently encompasses  
86 more than 14,400 records with 4,520 lineages detected in more than 2,100 host species  
87 [29]. Taking advantage of this unique database, here we explore whether MHC diversity  
88 co-evolves with haemosporidian lineages richness. Given that haemosporidian parasites are  
89 obligate intracellular parasites, here we focus on the MHC class I genes, exploring  
90 relationships between gene copy numbers (hereafter MHC-I diversity) and blood parasite  
91 lineages richness. Based on earlier species-specific studies on the link between MHC-I  
92 allelic diversity and blood parasites [23,26] and according to the heterozygote advantage  
93 hypothesis, we predict a negative association between MHC-I diversity and the diversity of  
94 intracellular parasites across host species. Nonetheless, an opposite association can also be  
95 predicted since studies focusing on extracellular pathogens, and thus MHC class II genes,  
96 detected positive associations between parasite burden and MHC diversity [10,30–32].

97

## 98 **2. Material and Methods**

### 99 (a) Database

100 We collected estimates of MHC-I diversity (i.e. loci number) from published databases  
101 [3,12,33]. In total, we compiled 81 different estimates of MHC-I diversity with  
102 corresponding blood parasite information (see final database in Table S1). For each bird  
103 species with MHC-I diversity, we collected data on the number of detected blood parasite  
104 lineages (hereafter lineages richness) of *Plasmodium*, *Haemoproteus* and *Leucocytozoon*  
105 parasites using the MalAvi database [29], (accessed on 11<sup>th</sup> June 2021. Moreover, given  
106 that the number of parasite lineages detected in a host is strongly dependent on sampling  
107 effort, we also extracted the number of publications (i.e. total number of studies exploring  
108 the given parasite genus in each particular host, see Table S2) and the total number of  
109 individuals tested for the given haemosporidian genus in each bird species. When data  
110 about the number of individuals tested was missing in the MalAvi database, this  
111 information was collected directly from the referenced publications. Given that body size is  
112 correlated with both parasite diversity and MHC diversity [12], we also collected  
113 information on species-specific body masses from literature [12].

114

#### 115 (b) Statistical analyses

116 In order to explore the effect of sampling effort, we tested the association between parasite  
117 lineages richness and indicators of sampling effort (i.e. number of individuals or number of  
118 publications) for each parasite genus separately and overall (i.e. combining all three genera  
119 of blood parasites). Linear regressions indicated that the number of publications was a  
120 considerably and consistently better predictor of parasite lineages richness than the number  
121 of individuals inspected in all three inspected parasite genera, as well as for overall  
122 haemosporidian lineages richness (appendix SI: figure S1). Therefore, we used the number  
123 of publications as covariate in all subsequent analysis to control for sampling effort.

124

125 To test for associations between MHC-I diversity and blood parasite diversity, we  
126 constructed phylogenetic generalized linear mixed model, using a Bayesian approximation  
127 in the R package ‘*MCMCglmm*’ [34]. We used parasite lineages richness as dependent  
128 variables (separately for each genus, and overall haemosporidian lineages richness) in  
129 models with poisson error distribution [34]. Models included MHC-I diversity, species  
130 body mass and the number of publication of the respective group of blood parasites as  
131 covariates. MHC-I diversity data was methodologically heterogeneous, due to a large  
132 variety of genotyping methods used to quantify it (e.g., cloning, 454 pyrosequencing, long-  
133 reads, see table S1). Given that these methods frequently provide divergent results [33,35],  
134 we included genotyping method as a random factor in the models. Moreover, MHC-I  
135 diversity was repeatedly assessed in some species using the same genotyping method,  
136 providing slightly conflicting estimates. Given that the accuracy of these estimates is  
137 difficult to determine, we included all estimates in the models, but using species as a  
138 random factor to control for pseudoreplication. Phylogeny was included in all models to  
139 control for the shared evolutionary history of the host species. We used weakly informative  
140 priors in all models [34]. Model convergence and absence of auto-correlation was assessed  
141 by visual examination of the posterior parameter distributions. Since MHC diversity might  
142 not be linearly related to parasite burden, we also tested quadratic effects in these models,  
143 by incorporating MHC class I diversity as second-degree orthogonal polynomials. In  
144 addition, because it is often ill-advised to combine data from various genotyping methods  
145 [11,12,36], we also performed models separately for the two most frequently used  
146 genotyping methods (cloning: n = 22 records, 21 species; 454 pyrosequencing: n = 35  
147 records, 22 species). The limited number of species assessed by the other genotyping  
148 methods did not allow separate analysis (n species  $\leq$  9 for all other methods). For models,  
149 only using cloning or pyrosequencing-based studies, we used the same MCMCglmm model

150 structure as described above but without the genotyping method random factor. Genotyping  
151 effort can influence estimates of loci diversity, therefore to confirm the lack of such bias in  
152 our results, we performed sensitivity analysis using the number of genotyped individuals as  
153 model weights. All results were highly consistent irrespective of control for genotyping  
154 effort (Table S6).

155

156 To account for phylogenetic non-independence we used trees from <http://birdtree.org>  
157 [37]. We downloaded 1000 random trees using the Hackett backbone tree [38], and  
158 calculated a rooted, ultrametric consensus tree using the SumTrees software [39]. All  
159 statistical analyses were conducted using R version 3.6.3 [40].

160

### 161 **3. Results**

162 We compiled complete data on parasite lineages richness and MHC-I diversity for 54 bird  
163 species, from 29 families and 7 orders, with *Passeriformes* being the most represented ( $n =$   
164 39 species; appendix SI: table S1). MHC class I loci numbers in our database varied widely,  
165 from one to 33 loci. MHC-I diversity was highly divergent in species repeatedly genotyped  
166 using different methods (e.g. *Acrocephalus arundinaceus*, cloning: 4 loci, 454  
167 pyrosequencing: 9-10 loci). Cloning generally detected less MHC class I loci than 454  
168 pyrosequencing (1-6, and 4-23 respectively). Observed parasite lineages richness also  
169 varied greatly across host species (*Plasmodium*: 0-38; *Haemoproteus*: 0-42; *Leucocytozoon*  
170 0-45; All blood parasites genera: 1-78).

171

172 The overall analysis, with genotyping method included as random factor (appendix SI: table  
173 S3a) revealed no association between MHC-I diversity and any of the three haemosporidian  
174 genera (*Plasmodium*:  $p = 0.578$ , *Haemoproteus*:  $p = 0.467$ , *Leucocytozoon*:  $p = 0.356$ ), or in

175 overall blood parasite lineages richness ( $p=0.533$ ). None of the quadratic effects of MHC  
176 class I diversity were significant (Table S3b).

177

178 Models based on cloning assessment of MHC-I diversity (appendix SI: table S4) indicated  
179 that a higher MHC-I diversity was associated with a significantly lower lineages richness of  
180 *Plasmodium* (posterior mean = - 0.47,  $p = 0.0161$ ; figure 1a), *Haemoproteus* (posterior  
181 mean = -0.51,  $p = 0.0148$ ; figure 1b), and overall haemosporidian lineages richness  
182 (posterior mean = -0.39,  $p = 0.0120$ , figure 1d). No association was found between MHC-I  
183 diversity and *Leucocytozoon* lineages richness (posterior mean = -0.05,  $p = 0.9047$ ; figure  
184 1c). In contrast, MHC diversity quantified using 454 pyrosequencing, revealed no  
185 correlation between MHC-I diversity and *Plasmodium* (posterior mean = -0.01,  $p =$   
186  $0.5843$ ), *Haemoproteus* (posterior mean = -0.01,  $p = 0.5897$ ), *Leucocytozoon* (posterior  
187 mean = -0.05,  $p = 0.1446$ ), or overall haemosporidian lineages richness (posterior mean = -  
188  $0.01$ ,  $p = 0.4264$ , appendix SI: figure S2a-d, table S5).

189

#### 190 **4. Discussion**

191 Here we performed a cross-species comparative analysis on MHC-I loci diversity in birds  
192 and explored its association with the diversity of intracellular blood parasites infecting  
193 these hosts. We did not detect an overall association between MHC-I diversity and blood  
194 parasite diversity across species, but methodological heterogeneity in MHC genotyping  
195 limits the reliability of these combined analyses [41]. On the contrary, analysis restricted to  
196 cloning assessment of MHC-I diversity (but not 454 pyrosequencing) indicated that high  
197 MHC-I diversity is associated with lower blood parasite diversity, regarding overall  
198 haemosporidian, *Plasmodium* and *Haemoproteus*, but not *Leucocytozoon* lineages richness.

199

200 Comparative studies exploring the link between MHC class II and extracellular parasites  
201 (helminths or ectoparasites) indicated that high parasite diversity was positively associated  
202 with MHC nucleotide [30], or allelic diversity [31]. Bolnik et al. [42] however found a  
203 negative correlation between MHC class II diversity and overall microbial diversity. One  
204 recent study focusing for the first time on MHC loci number found a positive association  
205 between helminth diversity and MHC class II [32]. Positive associations are commonly  
206 explained by the benefit of maintaining high MHC diversity under strong parasite pressure.  
207 Contrary to these results, our study points out a different dynamic linking intracellular  
208 blood parasite burden and MHC-I diversity. We show that MHC-I diversity and blood  
209 parasite richness is not positively, but rather negatively correlated, indicating that high  
210 MHC-I diversity may provide qualitative resistance against diverse avian haemosporidian  
211 parasites. Experimental studies support the possibility of full resistance against strains of  
212 blood parasites [17]. In fact, after inoculation with a generalist *Plasmodium* lineage (SGS1),  
213 bird species showed strikingly different parasite burdens, varying from full resistance or to  
214 high susceptibility [17]. The role of MHC protein contributing to this resistance is  
215 supported by the fact that individual flycatchers with high number of functional MHC  
216 alleles were shown to have lower probability of malarial infection [43]. Other studies  
217 demonstrate that the presence of specific MHC alleles could significantly decrease  
218 probabilities of being infected by certain blood parasite strains [23,24]. Contrasting results  
219 between parasite burden and MHC class I or class II diversity might be explained by  
220 various non-exclusive factors: (i) the distinct evolutionary history of these genes and their  
221 capacity to affect resistance and/or tolerance [12,41,44], (ii) the highly divergent species  
222 composition of the mentioned studies (e.g. patterns detected across broad taxonomic scales  
223 might not be present with narrow taxonomic coverage and vice versa), and (iii) the different

224 association of MHC diversity to different parasite groups (e.g. MHC class II diversity  
225 relates negatively to bacterial but positively to helminth diversity). Additional studies are  
226 needed to elucidate the mechanisms behind these divergent associations between MHC and  
227 pathogen diversity.

228

229 Our results indicating fewer intracellular pathogens in host species with more MHC-I loci  
230 support the heterozygote advantage hypothesis, which relies on the advantage provided by  
231 heterozygosity of the MHC alleles, through the recognition of a wider range of antigens  
232 compared to homozygotes. While it was suggested that pathogen richness alone seems  
233 insufficient to explain the maintenance of extremely high MHC diversity (especially in  
234 some passerines) [25], our results (restricted to cloning assessment of MHC) show a strong  
235 correlation between MHC and parasite diversity. Importantly however, our results indicate  
236 that the association between MHC-I diversity and parasite lineages richness is not  
237 consistent when different genotyping techniques are used. The reason for this discrepancy  
238 might be manifold. First, cloning quantification of MHC-I diversity in our database covered  
239 a relatively wide taxonomic range (*Galliformes* to *Passeriformes*), while 454  
240 pyrosequencing was only applied in passerines. It is thus possible that pathogen-mediated  
241 evolution contributed to major taxonomic differences in MHC loci numbers, but different  
242 selection processes are at play at the narrower taxonomic scales of e.g. passerines. The  
243 latter is also supported by the greatly accelerated MHC duplication rate in songbirds, which  
244 is unlikely to be the result of parasite-mediated selection alone [12]. Second, it is now  
245 known that significantly fewer MHC class I alleles are expressed than present in the  
246 genome [3], which is particularly concerning in species with highly duplicated MHC genes  
247 such as many songbirds [3,11,12]. Non-expressed MHC alleles (pseudogenes) are  
248 particularly problematic while using genotyping methods relying on genomic DNA

249 assembled from short segments [3], such as pyrosequencing, which potentially inflates  
250 estimated of copy numbers. Consistently, pyrosequencing detected higher MHC loci than  
251 cloning in all species genotyped by both techniques. Third, the association between MHC-I  
252 diversity and parasite burden is not always linear [26], sometimes indicating a negative  
253 association with moderate increase in MHC diversity, but not across high MHC diversity  
254 ranges. Consequently, an initial decline in parasite burden with moderate increase in MHC  
255 diversity (e.g. cloning analyses: loci number varies from 1 to 6) does not exclude the  
256 possibility of a different association at high loci numbers (e.g., analyses restricted to  
257 pyrosequencing: loci number varies from 4 to 23). Indeed, high MHC diversity can lead to  
258 a significant reduction of the T cell receptor repertoire, having detrimental effects on the  
259 efficiency of the immune response [45].

260

261 Overall, these results indicate a potentially important role of intracellular parasites in  
262 shaping cross-species variance in MHC-I diversity. Nonetheless, evidence is mixed when  
263 using different genotyping techniques, highlighting the need for better quality data on MHC  
264 diversity to better understand immune gene evolution and selection shaping this. We urge  
265 future research to quantify diversity in expressed MHC loci and to benefit from advanced  
266 genotyping technologies, such as long-read sequencing.

267

#### 268 **Data accessibility.**

269 All data are available in Table S1. Data and all code used to generate the results will be  
270 made available in the Dryad Digital Repository upon acceptance.

271

#### 272 **Authors' contributions.**

273 O.V. & C.L. wrote the manuscript. O.V. performed the comparative analysis. C.L. and  
274 M.G. designed the analysis. All authors contributed to revisions and approved the final  
275 version.

276

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288

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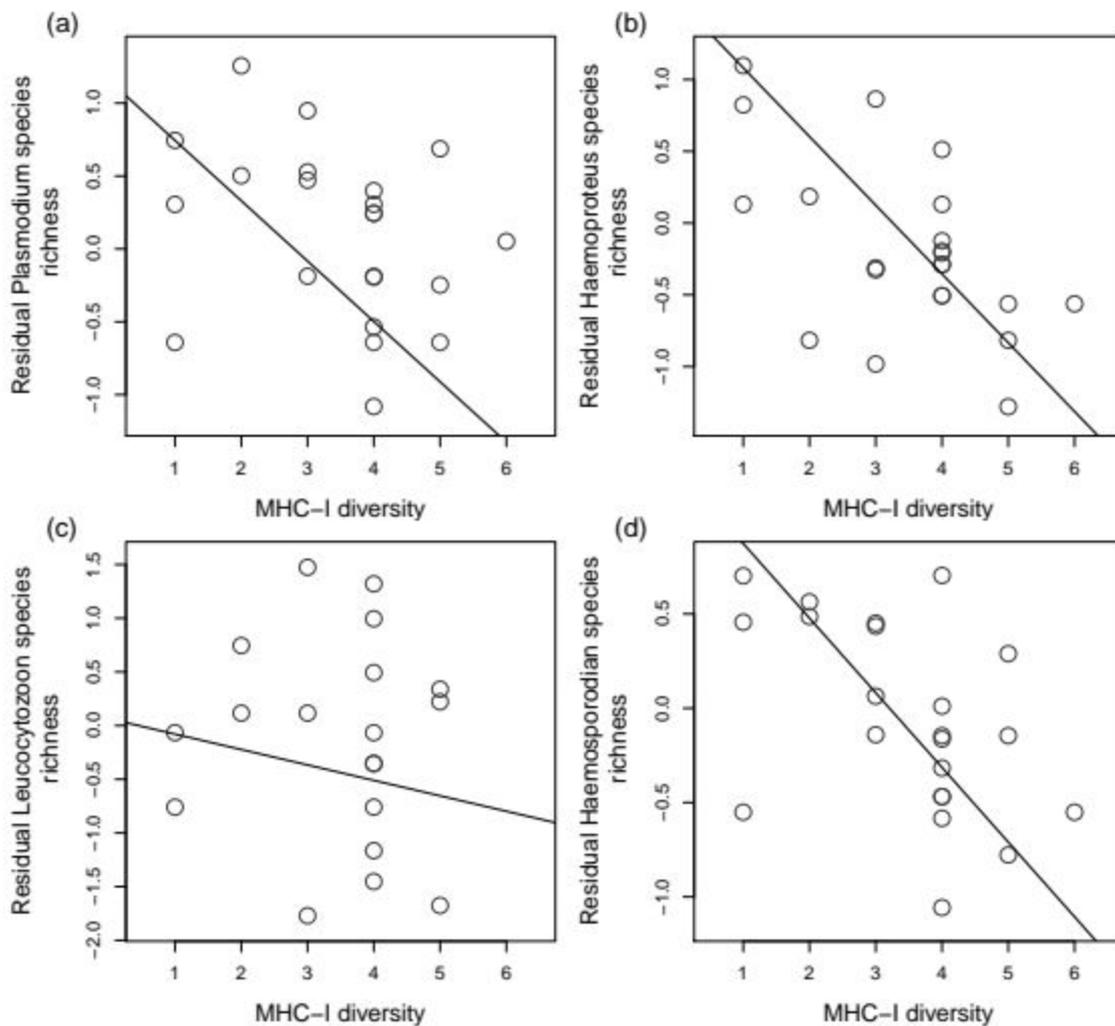
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- 420
- 421

422 **Figure 1.** Association between residual blood parasite species richness and MHC-I  
423 diversity for (a) *Plasmodium*, (b) *Haemoproteus*, (c) *Leucocytozoon* and (d) overall  
424 haemosporidian lineages richness. Parasite diversity residuals were obtained from a log-log  
425 linear regressions between parasite lineages richness (dependent) and research effort  
426 (explanatory). Slopes were obtained from phylogenetic MCMCglmms with poisson error  
427 distribution between parasite lineages richness and research effort as well as MHC-I  
428 diversity.



430

# 1 **Avian blood parasite richness decreases with MHC class I loci number**

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19

## 20 **Abstract**

21 Major Histocompatibility Complex (MHC) genes are among the most polymorphic in the  
22 vertebrate genome. The high allele diversity is believed to be maintained primarily by  
23 sexual and pathogen-mediated balancing selection. The number of MHC loci also varies  
24 greatly across vertebrates, most notably across birds. MHC proteins play key roles in  
25 presenting antigens on the cell surface for recognition by T cells, with class I proteins

26 specifically targeting intracellular pathogens. Here we explore the hypothesis that MHC  
27 class I diversity (measured as loci number) co-evolves with haemosporidian parasite burden  
28 of the host. Using data on 54 bird species we demonstrate that high MHC class I diversity is  
29 associated with significantly lower richness of *Plasmodium*, *Haemoproteus* as well as  
30 overall haemosporidian parasite lineages, the former thus indicating a more efficient  
31 protection against intracellular pathogens. Nonetheless, the latter associations were only  
32 detected when MHC diversity was assessed using cloning and not 454 pyrosequencing-  
33 based studies, nor across all genotyping methods combined. Our results indicate that high  
34 MHC class I diversity might play a key role in providing qualitative resistance against  
35 diverse haemosporidian parasites in birds, but further clarification is needed for the origin  
36 of contrasting results when using different genotyping methods for MHC loci  
37 quantification.

38

### 39 **Keywords**

40 Haemosporidians, copy number variation, parasite richness, pathogen-mediated selection,  
41 comparative analyses, MHC diversity

42

### 43 **1. Introduction**

44 The Major Histocompatibility Complex (MHC) is known to be the most variable gene  
45 group in vertebrates, both in terms of allelic diversity and gene number [1]. MHC genes  
46 code for cell surface glycoproteins that bind to antigenic peptides and present them to cells  
47 of the immune system, initiating adaptive immune responses. Proteins encoded by the  
48 MHC genes ensure the immune presentation of a broad spectrum of peptides, both derived  
49 from the intracellular (class I proteins) or extracellular (class II proteins) space [2]. High  
50 MHC diversity, reflected by high allele diversity or loci number, is often related to the

51 ability to present a wider repertoire of antigens to the immune system, thus representing a  
52 genetic marker of disease resistance [3] but see [4]. Several, mutually non-exclusive  
53 hypotheses have been put forward to explain the maintenance of high MHC diversity, such  
54 as the heterozygote advantage (i.e., MHC diversity conferring disease resistance), negative  
55 frequency-dependent selection (benefit of rare MHC alleles in pathogen detection) or  
56 fluctuating selection (spatial or temporal change in the selective advantage of MHC alleles)  
57 [5,6].

58

59 The number of MHC loci varies greatly across vertebrates, being the result of a complex  
60 evolutionary history, involving gene duplications and translocations [7,8]. For example,  
61 species from the Galliformes order possess only two MHC class I genes [9,10], which is  
62 considerably lower than in other bird lineages [11,12], such as passerines. Several studies  
63 reported that a low number of MHC loci could represent the ancestral state in birds, and  
64 that the highest diversification in copy numbers occurred relatively late in the avian  
65 radiation, reaching its highest diversity in passerines [12]. While, parasite-mediated  
66 selection has been considered as the most prominent component driving MHC  
67 diversification [13], it is now widely accepted that in addition to the pathogen burden,  
68 sexual selection and risk of developing autoimmune diseases or immunopathologies may  
69 together shape the evolution of an optimal rather than maximal MHC diversity [3,4,13–15].

70

71 Malaria and closely related haemosporidian parasites are intensively investigated vector-  
72 borne parasites in birds, partly because of the high diversity of hosts they are able to infect,  
73 and their nearly worldwide distribution [16]. A series of experimental studies [17–21], have  
74 demonstrated the substantial costs associated with haemosporidian infection, in terms of

75 reproductive success, health condition and survival. Hence, immunological adaptations  
76 targeting and eliminating such infections can provide considerable fitness benefits. Key  
77 components of these defenses are the MHC proteins. Nonetheless, knowledge on the co-  
78 evolution of MHC diversity and avian haemosporidian parasite burden across species  
79 remains limited [22–26]. However, other host-parasite systems found strong evidences that  
80 specific MHC alleles confer resistance to pathogens [27,28].

81

82 With the increasing use of molecular tools, a large number of bird species have been  
83 recently screened for haemosporidian parasites. The MalAvi database represents a unified  
84 database compiling these data, covering genetic and geographical data of avian blood  
85 parasites (*Plasmodium*, *Haemoproteus*, *Leucocytozoon* genera). It currently encompasses  
86 more than 14,400 records with 4,520 lineages detected in more than 2,100 host species  
87 [29]. Taking advantage of this unique database, here we explore whether MHC diversity  
88 co-evolves with haemosporidian lineages richness. Given that haemosporidian parasites are  
89 obligate intracellular parasites, here we focus on the MHC class I genes, exploring  
90 relationships between gene copy numbers (hereafter MHC-I diversity) and blood parasite  
91 lineages richness. Based on earlier species-specific studies on the link between MHC-I  
92 allelic diversity and blood parasites [23,26] and according to the heterozygote advantage  
93 hypothesis, we predict a negative association between MHC-I diversity and the diversity of  
94 intracellular parasites across host species. Nonetheless, an opposite association can also be  
95 predicted since studies focusing on extracellular pathogens, and thus MHC class II genes,  
96 detected positive associations between parasite burden and MHC diversity [10,30–32].

97

## 98 **2. Material and Methods**

### 99 (a) Database

100 We collected estimates of MHC-I diversity (i.e. loci number) from published databases  
101 [3,12,33]. In total, we compiled 81 different estimates of MHC-I diversity with  
102 corresponding blood parasite information (see final database in Table S1). For each bird  
103 species with MHC-I diversity, we collected data on the number of detected blood parasite  
104 lineages (hereafter lineages richness) of *Plasmodium*, *Haemoproteus* and *Leucocytozoon*  
105 parasites using the MalAvi database [29], (accessed on 11<sup>th</sup> June 2021. Moreover, given  
106 that the number of parasite lineages detected in a host is strongly dependent on sampling  
107 effort, we also extracted the number of publications (i.e. total number of studies exploring  
108 the given parasite genus in each particular host, see Table S2) and the total number of  
109 individuals tested for the given haemosporidian genus in each bird species. When data  
110 about the number of individuals tested was missing in the MalAvi database, this  
111 information was collected directly from the referenced publications. Given that body size is  
112 correlated with both parasite diversity and MHC diversity [12], we also collected  
113 information on species-specific body masses from literature [12].

114

#### 115 (b) Statistical analyses

116 In order to explore the effect of sampling effort, we tested the association between parasite  
117 lineages richness and indicators of sampling effort (i.e. number of individuals or number of  
118 publications) for each parasite genus separately and overall (i.e. combining all three genera  
119 of blood parasites). Linear regressions indicated that the number of publications was a  
120 considerably and consistently better predictor of parasite lineages richness than the number  
121 of individuals inspected in all three inspected parasite genera, as well as for overall  
122 haemosporidian lineages richness (appendix SI: figure S1). Therefore, we used the number  
123 of publications as covariate in all subsequent analysis to control for sampling effort.

124

125 To test for associations between MHC-I diversity and blood parasite diversity, we  
126 constructed phylogenetic generalized linear mixed model, using a Bayesian approximation  
127 in the R package ‘*MCMCglmm*’ [34]. We used parasite lineages richness as dependent  
128 variables (separately for each genus, and overall haemosporidian lineages richness) in  
129 models with poisson error distribution [34]. Models included MHC-I diversity, species  
130 body mass and the number of publication of the respective group of blood parasites as  
131 covariates. MHC-I diversity data was methodologically heterogeneous, due to a large  
132 variety of genotyping methods used to quantify it (e.g., cloning, 454 pyrosequencing, long-  
133 reads, see table S1). Given that these methods frequently provide divergent results [33,35],  
134 we included genotyping method as a random factor in the models. Moreover, MHC-I  
135 diversity was repeatedly assessed in some species using the same genotyping method,  
136 providing slightly conflicting estimates. Given that the accuracy of these estimates is  
137 difficult to determine, we included all estimates in the models, but using species as a  
138 random factor to control for pseudoreplication. Phylogeny was included in all models to  
139 control for the shared evolutionary history of the host species. We used weakly informative  
140 priors in all models [34]. Model convergence and absence of auto-correlation was assessed  
141 by visual examination of the posterior parameter distributions. Since MHC diversity might  
142 not be linearly related to parasite burden, we also tested quadratic effects in these models,  
143 by incorporating MHC class I diversity as second-degree orthogonal polynomials. In  
144 addition, because it is often ill-advised to combine data from various genotyping methods  
145 [11,12,36], we also performed models separately for the two most frequently used  
146 genotyping methods (cloning: n = 22 records, 21 species; 454 pyrosequencing: n = 35  
147 records, 22 species). The limited number of species assessed by the other genotyping  
148 methods did not allow separate analysis (n species  $\leq$  9 for all other methods). For models,  
149 only using cloning or pyrosequencing-based studies, we used the same MCMCglmm model

150 structure as described above but without the genotyping method random factor. Genotyping  
151 effort can influence estimates of loci diversity, therefore to confirm the lack of such bias in  
152 our results, we performed sensitivity analysis using the number of genotyped individuals as  
153 model weights. All results were highly consistent irrespective of control for genotyping  
154 effort (Table S6).

155

156 To account for phylogenetic non-independence we used trees from <http://birdtree.org>  
157 [37]. We downloaded 1000 random trees using the Hackett backbone tree [38], and  
158 calculated a rooted, ultrametric consensus tree using the SumTrees software [39]. All  
159 statistical analyses were conducted using R version 3.6.3 [40].

160

### 161 **3. Results**

162 We compiled complete data on parasite lineages richness and MHC-I diversity for 54 bird  
163 species, from 29 families and 7 orders, with *Passeriformes* being the most represented ( $n =$   
164 39 species; appendix SI: table S1). MHC class I loci numbers in our database varied widely,  
165 from one to 33 loci. MHC-I diversity was highly divergent in species repeatedly genotyped  
166 using different methods (e.g. *Acrocephalus arundinaceus*, cloning: 4 loci, 454  
167 pyrosequencing: 9-10 loci). Cloning generally detected less MHC class I loci than 454  
168 pyrosequencing (1-6, and 4-23 respectively). Observed parasite lineages richness also  
169 varied greatly across host species (*Plasmodium*: 0-38; *Haemoproteus*: 0-42; *Leucocytozoon*  
170 0-45; All blood parasites genera: 1-78).

171

172 The overall analysis, with genotyping method included as random factor (appendix SI: table  
173 S3a) revealed no association between MHC-I diversity and any of the three haemosporidian  
174 genera (*Plasmodium*:  $p = 0.578$ , *Haemoproteus*:  $p = 0.467$ , *Leucocytozoon*:  $p = 0.356$ ), or in

175 overall blood parasite lineages richness ( $p=0.533$ ). None of the quadratic effects of MHC  
176 class I diversity were significant (Table S3b).

177

178 Models based on cloning assessment of MHC-I diversity (appendix SI: table S4) indicated  
179 that a higher MHC-I diversity was associated with a significantly lower lineages richness of  
180 *Plasmodium* (posterior mean = - 0.47,  $p = 0.0161$ ; figure 1a), *Haemoproteus* (posterior  
181 mean = -0.51,  $p = 0.0148$ ; figure 1b), and overall haemosporidian lineages richness  
182 (posterior mean = -0.39,  $p = 0.0120$ , figure 1d). No association was found between MHC-I  
183 diversity and *Leucocytozoon* lineages richness (posterior mean = -0.05,  $p = 0.9047$ ; figure  
184 1c). In contrast, MHC diversity quantified using 454 pyrosequencing, revealed no  
185 correlation between MHC-I diversity and *Plasmodium* (posterior mean = -0.01,  $p =$   
186  $0.5843$ ), *Haemoproteus* (posterior mean = -0.01,  $p = 0.5897$ ), *Leucocytozoon* (posterior  
187 mean = -0.05,  $p = 0.1446$ ), or overall haemosporidian lineages richness (posterior mean = -  
188  $0.01$ ,  $p = 0.4264$ , appendix SI: figure S2a-d, table S5).

189

#### 190 **4. Discussion**

191 Here we performed a cross-species comparative analysis on MHC-I loci diversity in birds  
192 and explored its association with the diversity of intracellular blood parasites infecting  
193 these hosts. We did not detect an overall association between MHC-I diversity and blood  
194 parasite diversity across species, but methodological heterogeneity in MHC genotyping  
195 limits the reliability of these combined analyses [41]. On the contrary, analysis restricted to  
196 cloning assessment of MHC-I diversity (but not 454 pyrosequencing) indicated that high  
197 MHC-I diversity is associated with lower blood parasite diversity, regarding overall  
198 haemosporidian, *Plasmodium* and *Haemoproteus*, but not *Leucocytozoon* lineages richness.

199

200 Comparative studies exploring the link between MHC class II and extracellular parasites  
201 (helminths or ectoparasites) indicated that high parasite diversity was positively associated  
202 with MHC nucleotide [30], or allelic diversity [31]. Bolnik et al. [42] however found a  
203 negative correlation between MHC class II diversity and overall microbial diversity. One  
204 recent study also focusing for the first time on MHC loci number found a positive  
205 association between helminth diversity and MHC class II loci number and MHC class II  
206 [32]. The Positive associations are commonly explained by the benefit of maintaining  
207 high MHC diversity under strong parasite pressure. Contrary to these results, our study  
208 points out a different dynamic linking intracellular blood parasite burden and MHC-I  
209 diversity. We show that MHC-I diversity and blood parasite richness is not positively, but  
210 rather negatively correlated, indicating that high MHC-I diversity may provide qualitative  
211 resistance against diverse avian haemosporidian parasites. Experimental studies support the  
212 possibility of full resistance against strains of blood parasites [17]. In fact, after inoculation  
213 with a generalist *Plasmodium* lineage (SGS1), bird species showed strikingly different  
214 parasite burdens, varying from full resistance or to high susceptibility [17]. The role of  
215 MHC protein contributing to this resistance is supported by the fact that individual  
216 flycatchers with high number of functional MHC alleles were shown to have lower  
217 probability of malarial infection [43]. Other studies demonstrate that the presence of  
218 specific MHC alleles could significantly decrease probabilities of being infected by certain  
219 blood parasite strains [23,24]. Finally, contrasting results between parasite burden and  
220 MHC class I or class II diversity might be explained by various non-exclusive factors: (i)  
221 the distinct evolutionary history of these genes and their capacity to affect resistance and/or  
222 tolerance [12,41,44], (ii) the highly divergent species composition of the mentioned studies  
223 (e.g. patterns detected across broad taxonomic scales might not be present with narrow

224 ~~taxonomic coverage and vice versa), and (iii) the different association of MHC diversity to~~  
225 ~~different parasite groups (e.g. MHC class II diversity relates negatively to bacterial but~~  
226 ~~positively to helminth diversity). Additional studies are needed to elucidate the mechanisms~~  
227 ~~behind these divergent associations between MHC and pathogen diversity. It appears that~~  
228 ~~MHC class I copy numbers were primarily shaped by accelerated evolution ('burst') and~~  
229 ~~stabilizing selection, while the evolution of MHC class II loci were likely governed by~~  
230 ~~fluctuating selection and drift [12].~~

231

232 Our results indicating fewer intracellular pathogens in host species with more MHC-I loci  
233 support the heterozygote advantage hypothesis, which relies on the advantage provided by  
234 heterozygosity of the MHC alleles, through the recognition of a wider range of antigens  
235 compared to homozygotes. While it was suggested that pathogen richness alone seems  
236 insufficient to explain the maintenance of extremely high MHC diversity (especially in  
237 some passerines) [25], our results (restricted to cloning assessment of MHC) show a strong  
238 correlation between MHC and parasite diversity. Importantly however, our results indicate  
239 that the association between MHC-I diversity and parasite lineages richness is not  
240 consistent when different genotyping techniques are used. The reason for this discrepancy  
241 might be manifold. First, cloning quantification of MHC-I diversity in our database covered  
242 a relatively wide taxonomic range (*Galliformes* to *Passeriformes*), while 454  
243 pyrosequencing was only applied in passerines. It is thus possible that pathogen-mediated  
244 evolution contributed to major taxonomic differences in MHC loci numbers, but different  
245 selection processes are at play at the narrower taxonomic scales of e.g. passerines. The  
246 latter is also supported by the greatly accelerated MHC duplication rate in songbirds, which  
247 is unlikely to be the result of parasite-mediated selection alone [12]. Second, it is now  
248 known that significantly fewer MHC class I alleles are expressed than present in the

249 genome [3], which is particularly concerning in species with highly duplicated MHC genes  
250 such as many songbirds [3,11,12]. Non-expressed MHC alleles (pseudogenes) are  
251 particularly problematic while using genotyping methods relying on genomic DNA  
252 assembled from short segments [3], such as pyrosequencing, which potentially inflates  
253 estimated of copy numbers. Consistently, pyrosequencing detected higher MHC loci than  
254 cloning in all species genotyped by both techniques. Third, the association between MHC-I  
255 diversity and parasite burden is not always linear [26], sometimes indicating a negative  
256 association with moderate increase in MHC diversity, but not across high MHC diversity  
257 ranges. Consequently, an initial decline in parasite burden with moderate increase in MHC  
258 diversity (e.g. cloning analyses: loci number varies from 1 to 6) does not exclude the  
259 possibility of a different association at high loci numbers (e.g., analyses restricted to  
260 pyrosequencing: loci number varies from 4 to 23). Indeed, high MHC diversity can lead to  
261 a significant reduction of the T cell receptor repertoire, having detrimental effects on the  
262 efficiency of the immune response [453].

263

264 Overall, these results indicate a potentially important role of intracellular parasites in  
265 shaping cross-species variance in MHC-I diversity. Nonetheless, evidence is mixed when  
266 using different genotyping techniques, highlighting the need for better quality data on MHC  
267 diversity to better understand immune gene evolution and selection shaping this. We urge  
268 future research to quantify diversity in expressed MHC loci and to benefit from advanced  
269 genotyping technologies, such as long-read sequencing.

270

271 **Data accessibility.**

272 All data are available in Table S1. Data and all code used to generate the results will be  
273 made available in the Dryad Digital Repository upon acceptance.

274

#### 275 **Authors' contributions.**

276 O.V. & C.L. wrote the manuscript. O.V. performed the comparative analysis. C.L. and  
277 M.G. designed the analysis. All authors contributed to revisions and approved the final  
278 version.

279

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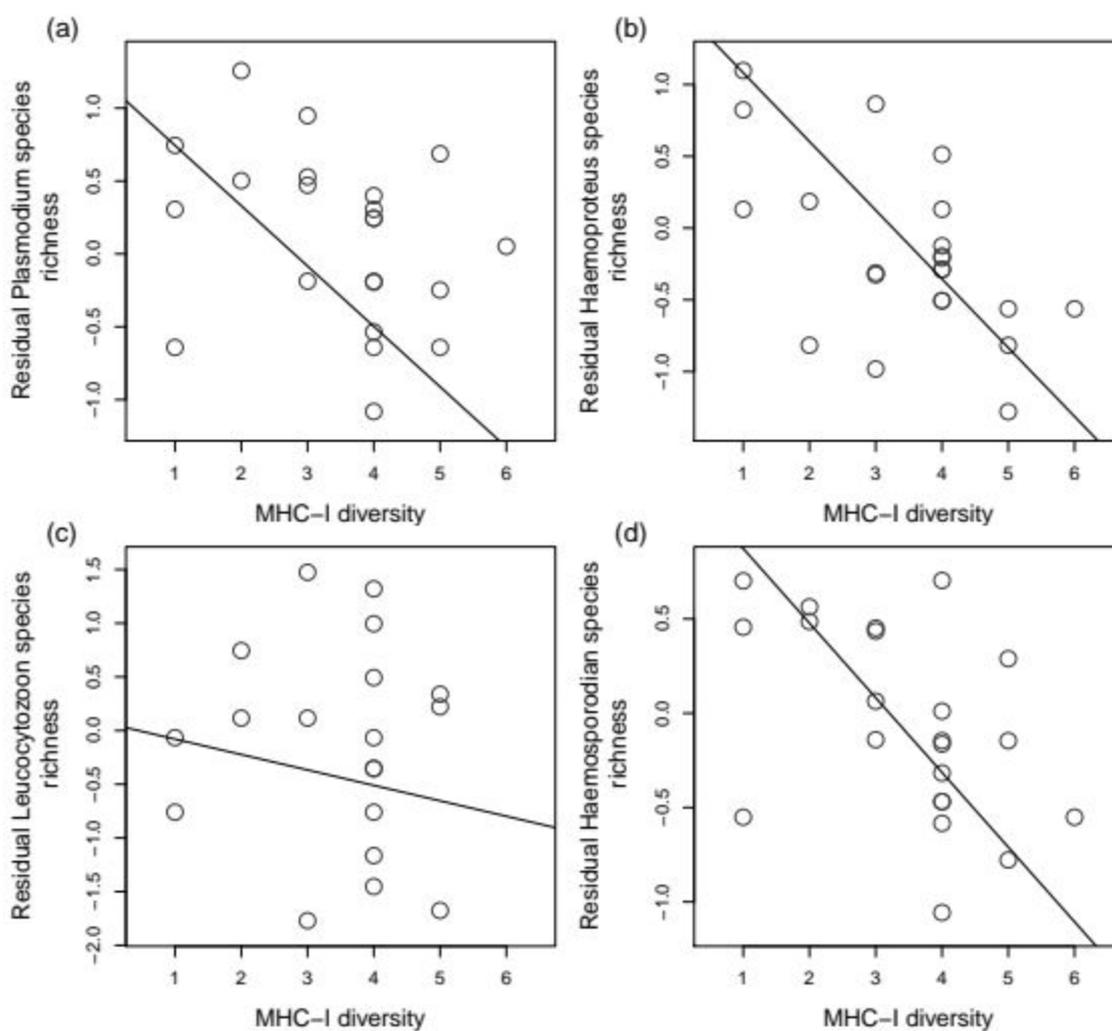
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425 **Figure 1.** Association between residual blood parasite species richness and MHC-I  
426 diversity for (a) *Plasmodium*, (b) *Haemoproteus*, (c) *Leucocytozoon* and (d) overall  
427 haemosporidian lineages richness. Parasite diversity residuals were obtained from a log-log  
428 linear regressions between parasite lineages richness (dependent) and research effort  
429 (explanatory). Slopes were obtained from phylogenetic MCMCglmms with poisson error  
430 distribution between parasite lineages richness and research effort as well as MHC-I  
431 diversity.



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