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Life-history and ecological variables as drivers of the evolution of avian innate immune defences

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Animals rely on immune defences to counteract pathogens and parasites, but investing in immune defences limits the use of shared resources for other functions. Therefore, the strength of immune defences is hypothesized to be shaped by life history or broader ecology. To better understand ecological and evolutionary patterns in innate humoral immune defences in birds, we compiled more than 400 immunological records of more than 100 avian species from about 100 previous studies that applied a standardized protocol to measure haemolysis and haemagglutination. We extracted data on 15 life-history and ecological variables and built phylogenetically informed comparative models to determine how these variables can explain variation in both immunological indices. We also inferred evolutionary patterns by selecting the best-fitting macroevolutionary models. Our comparative models indicated that several ecological variables, including seasonal stage, sex, age, migration distance, diet type and climatic factors, and only one life-history variable, body mass, were able to explain the immunological variation. The best macroevolutionary models suggest that both immune indices evolved largely through gradual divergence under strong phylogenetic constraints, with evidence for stabilizing selection within Passeriformes. Overall, our results highlight the role of seasonal and demographic pressures, alongside phylogenetic history, in shaping immune variation across avian species.

1. Background

In animals, immune defences counteract pathogens and parasites (henceforth 'parasites'), but their activation imposes costs by competing for resources with other physiological and behavioural processes [1]. Accordingly, immune defences may be compromised to maximize the overall condition of the animals. The trade-offs of resources between immune defences and other physiological or behavioural traits can be underpinned by evolution or ecology (i.e. plasticity). One example of an evolutionary trade-off is the hypothesized relationships between immune defences and life-history traits. Pace-of-life theory suggests that animal species range from 'fast-living' (i.e. those with shorter lifespans, faster growth rates and earlier onset of reproduction) to 'slow-living' (i.e. those with longer lifespans, slower growth rates and later onset of reproduction). Compared with slow-living species, fast-living species are predicted to invest more resources into growth and (current) reproduction and less into self-maintenance (and thus, future reproduction), including immune defences [2,3]. Reproductive output is another life-history

trait that can correlate with immune defences. Experimentally increased brood size can decrease immune defences in parents [4,5], and experimental immune challenge can reduce reproduction [6,7]. Body mass, another variable closely related to life history, may be relevant to immune strategy. Larger animals may require higher immune defences because their larger body surface area increases the risk of parasite encounters, and these higher immune defences may be possible because their lower mass-specific metabolic rate allows resources to be invested elsewhere [8,9].

Ecological factors can also influence immune defences, both directly and indirectly [10,11]. Temperature, for example, can influence parasite pressure and general resource availability. While immune responses depend on specific nutrients that may remain scarce regardless of temperature, in many environments, higher temperatures are associated with higher primary productivity and thus increased access to caloric resources, potentially enhancing immune readiness, particularly against temperature-related increases in parasites [12]. Species with different diets and feeding behaviours are likely to differ in their parasite encounter rates and predation risks, both of which can influence immunological investments [13]. Sociality can also influence the immune system. Social animals are hypothesized to have higher parasite transmission rates than solitary ones and thus require better immune defences [14,15]. Seasonality, in terms of seasonal changes in organismal biology and in the broader environment, may also be relevant [16]. Animals often invest more in reproduction, parental care or migration during specific seasonal stages; at these times, investments in immune defences may be lower [17]. Resource availability and parasite pressures can also differ seasonally (and spatially, e.g. among stopover sites used by migratory birds), and these differences can also shape selection on immune defences.

To broadly evaluate the influence of life-history traits and ecological variables, immune defences must be quantified in a standardized way in various species and habitats. One of the most standardized and commonly used assays in ecological immunology results in two interrelated indices of innate humoral immune function: lysis and agglutination of foreign cells [18]. This assay requires a single small (*ca* 100 µl) sample of blood, which can be safely collected from many birds (i.e. those >10 g). Because this assay has proven to be informative and fieldwork-friendly, it has been routinely used worldwide for intra- and inter-specific studies of free-living and captive birds. The assay quantifies two interacting components of the innate immune system: natural antibodies (NAbs), which drive haemagglutination *in vitro*, and complement, which mediates haemolysis *in vitro*. In the context of the assay, complement, which evolved earlier than antibodies and can be activated independently via multiple pathways, is primarily activated through the classical pathway initiated by NAbs binding to antigens. NAbs are broadly defined as antibodies that can recognize common parasites without prior exposure [19] and are considered a key part of early, innate-like immune responses [20]. Once bound to foreign cells, NAbs activate the complement cascade, leading to cell lysis [19].

Understanding how evolutionary and ecological forces shape immune function is central to explaining variation in immune defences within and among species. In the current study, we focus on innate humoral immune defences in birds and ask how variation in haemagglutination and haemolysis is related to species' life-history characteristics, ecological contexts and evolutionary history. Specifically, we tested predictions about how 15 life-history and ecological variables (electronic supplementary material, table S1) are associated with variation in these immune indices. To address these questions, we compiled haemagglutination and haemolysis values from publications that used the standard assay protocol. For all included populations, we assembled corresponding data for the predictor variables. We then used phylogenetically informed comparative analyses to test these hypotheses, and macroevolutionary models to examine the evolutionary patterns of haemolysis and haemagglutination.

2. Material and methods

(a) Data collection

We collected baseline haemagglutination and haemolysis titres from a set of articles published from 2005 to 2020. Using Google Scholar and Web of Science, we limited our analysis to English-language peer-reviewed articles that cited the original assay protocol of Matson *et al.* [18]. Articles were excluded when they fitted these criteria but did not apply the assay (e.g. review studies), provided only statistical outputs but no haemagglutination or haemolysis data, or did not include data from birds. Furthermore, we excluded (sub-)studies that (i) employed chemical or intensive physical challenges before blood sampling or (ii) modified the assay protocols in ways that could be expected to affect the results (e.g. non-standard incubation temperatures and periods, exogenous red blood cells from non-standard species, non-standard ratio of test sample to exogenous red blood cells, etc.). For subsequent analyses, we excluded species with fewer than five individual observations for either haemagglutination or haemolysis from the respective dataset. We also summarized the sample sizes reported in the eligible studies (electronic supplementary material, table S2) to provide an overview of the underlying sampling effort; these values do not necessarily correspond to the number of observations used in the further analyses.

Haemagglutination and haemolysis titres were mostly reported as means (sometimes medians) of (sub-)populations in the main or supplemental texts, tables, figures or some combination thereof. We used or extracted the mean values when possible; otherwise, we extracted and used median values. When values were presented only graphically, we used GetData Graph Digitizer 2.26 (http://getdata-graph-digitizer.com/) to extract the values. When individual raw data rather than mean or median values were provided, we grouped values into sub-populations based on sex, age group, seasonal stage, moult condition and captivity status, as possible, and calculated mean values per sub-population. In our study, 'sub-population' therefore refers to groups distinguished by such demographic or ecological attributes within a dataset, rather than to geographical sampling localities.

We collected data for 15 explanatory variables (electronic supplementary material, table S1) from both the original articles and external databases. The databases (Birds of the World [21], AnAge [22] and BirdLife International (https://www.birdlife.org/)) were used as sources for species averages for body mass, clutch size, maximum lifespan, diet type and social structure, based on the taxonomic nomenclature (i.e. genus and species) used by BirdTree [23]. Migration distance and absolute breeding latitude of each species came from Minias *et al.* [24]. Data on seasonal stage, moult condition, captivity, sex, age group, average monthly temperature and average monthly precipitation of the sampling location were obtained per (sub-)population (electronic supplementary material). Of these, data on seasonal stage, moult condition, captivity, sex and age group were collected directly from the original haemagglutination–haemolysis studies. Average monthly temperature and average monthly precipitation of the sampling location were extracted from WorldClim [25].

We constructed species-level and population-level datasets. The species-level dataset includes the species-specific mean values of haemagglutination and haemolysis, and this dataset was used to investigate the evolutionary patterns in haemagglutination and haemolysis. The population-level dataset includes the (sub-)population-specific mean/median values of haemagglutination and haemolysis as well as the eight species- and the seven population-level variables (electronic supplementary material, table S1). In the population dataset, we prioritized data from the original articles over data from databases for the eight species-level variables. We made separate consensus phylogenies for the haemagglutination species list and the haemolysis species list, respectively, since species composition differed between the datasets as a result of available data. Each consensus tree was based on 1000 phylogenies downloaded from the Ericson All Species source on the BirdTree Web server [23].

(b) Comparative analyses

All analyses were conducted in R version 4.4.2 [26]. We constructed Bayesian phylogenetic mixed models (BPMMs) to explore the relationships between all explanatory variables and the two immunological parameters. BPMMs are phylogenetically informed comparative methods that allow multiple observations for each species [27]. Therefore, we used haemagglutination and haemolysis titres and all explanatory variables from the population-level datasets to build full BPMMs with the consensus phylogenies, which were reconstructed entirely from sequenced species in the Ericson backbone and thus did not rely on taxonomic imputations. We log-transformed body mass, clutch size, average monthly precipitation and maximum lifespan in all datasets. Following Minias *et al.* [24], we extracted the residuals of log-transformed lifespan against log-transformed body mass and type of lifespan source as categorized in the AnAge database (three categories: free-living, captive and unknown) and used these residuals as a measure of relative longevity in our models. BPMMs were run using the *MCMCglmm* function in the *MCMCglmm* R package [28]. The reference category of each categorical variable was rotated in the BPMMs to generate all pair-wise *post hoc* comparisons. For each model, we ran three independent chains, each with 500 000 iterations, a thinning interval of 200 and a burn-in period of 100 000 iterations. To ensure model reliability and convergence, we assessed Gelman–Rubin diagnostics ($\widehat{R}$) and effective sample size (ESS) for all parameters. All models showed sufficient convergence ($\widehat{R} \approx 1.00$) and adequate ESS values. Posterior summaries were obtained from the combined chains.

To evaluate the robustness of our results, we re-ran the BPMMs after excluding the categories 'no data (n.d.)' and 'mixed' from the variables sex and age group, as they carry limited ecological interpretability. This allowed us to verify whether the results of pair-wise comparisons between meaningful biological categories (i.e. female versus male for sex, and adult, fledlging and newly hatched for age group) remained consistent.

Given the high representation of Passeriformes in our dataset (see §3), we conducted additional passerine-restricted BPMMs to examine whether the significance of explanatory variables is retained for this clade. Data processing followed the same procedures as in the main BPMMs, except for two differences: habitat type was excluded, as all Passeriformes species in the dataset were terrestrial, and monthly precipitation was not log-transformed owing to a more symmetrical normal distribution within the subset. These supplementary analyses helped assess the robustness of our main findings and the potential influence of uninformative predictors or taxonomic overrepresentation.

(c) Evolutionary patterns in haemagglutination and haemolysis

First, using the species-level datasets and the *fitContinuous* function in the R package *geiger* [29], we built seven macroevolutionary models to determine evolutionary patterns of haemagglutination and haemolysis [29–32]. The seven models were as follows: (i) Brownian motion model, (ii) Brownian motion model adjusted for the phylogenetic scaling parameter λ, (iii) Ornstein–Uhlenbeck (OU) model, (iv) 'early-burst' model (EB), (v) time-dependent 'delta' model, (vi) 'trend' model and (vii) 'white noise' model (electronic supplementary material). We used the Akaike information criterion corrected for small sample sizes (AIC_c) to select the best-fitting models ($\Delta AIC_c < 2$).

Second, we calculated the phylogenetic signal in haemagglutination and haemolysis. Phylogenetic signal represents the similarity of a trait between phylogenetically close species. Because both immune parameters evolved following λ-adjusted Brownian motion models (see §3), we used Pagel's λ to estimate the phylogenetic signal [33]. We calculated Moran's I to assess the strength of autocorrelation in haemagglutination and haemolysis at genus, family and order levels [34]. The R packages *phytools* [35] and *ape* [36] were used for these calculations.

Third, we assessed how variation in haemagglutination and haemolysis was partitioned among different taxonomic levels using a taxonomically nested analysis of variance. We implemented a Bayesian variance-partitioning approach using the *MCMCglmm* package in R. For each immune variable, we fitted a null model with taxonomic hierarchy (order, family, genus and species) specified as random effects. This allowed us to estimate the proportion of total variance attributable to each taxonomic

level. The models were run for 1 000 000 iterations, with a burn-in of 200 000 and a thinning interval of 200. Convergence was assessed using autocorrelation plots and Heidelberger–Welch diagnostics. The posterior means of the variance components were used to calculate the proportion of variance explained by each taxonomic level.

Fourth, we reconstructed ancestral states of haemagglutination and haemolysis. Because both defences evolved following λ-adjusted Brownian motion models (see §3), we used the *fastAnc* function in the phytools R package [35] to reconstruct their ancestral states. We used the *contMap* function in the same package to map the estimated ancestral states of both defences on their consensus phylogenies. These reconstructions were performed for the visualization of phylogenetic patterns in haemagglutination and haemolysis.

To examine whether the evolutionary patterns observed in the full dataset also held for the Passeriformes, we repeated the phylogenetic analyses for this subset. Specifically, we recalculated Pagel's λ and Blomberg's K to assess phylogenetic signals in haemagglutination and haemolysis, and re-ran the seven macroevolutionary models. However, we did not repeat the nested taxonomic variance partitioning or Moran's I analysis within Passeriformes, as such methods rely on hierarchical comparisons across multiple taxonomic levels. In particular, the absence of inter-order variation in a single-order subset would preclude estimation of variance explained at the order level and weaken the interpretability of phylogenetic structuring. These analyses were therefore retained only for the full dataset.

3. Results

To provide context, electronic supplementary material, table S2 summarizes the sample sizes reported in the original literature, which reflect the number of individual birds sampled rather than the number of observations in our analyses. For haemagglutination, we collected 421 aggregated observations from 104 species in 75 genera (representing 40 families and 13 orders; electronic supplementary material, table S2); for haemolysis, we collected 341 aggregated observations from 85 species in 64 genera (representing 35 families and 12 orders; electronic supplementary material, table S2). More than half the species were from the order Passeriformes, with 271 aggregated observations from 61 species for haemagglutination and 212 aggregated observations from 47 species for haemolysis, but Anseriformes and Charadriiformes were also well represented. Haemagglutination and haemolysis were positively correlated at the species (average) level (correlation coefficient = 0.44; $n = 84$; $t = 4.45$; $p < 0.001$).

(a) Comparative analyses

The BPMM revealed significant relationships of haemagglutination with 6 of the 15 predictor variables (table 1, which includes Gelman diagnostic values and effective sample sizes for all parameters), although the apparent effect of age group was driven only by the biologically meaningless 'mixed' category. First, body mass correlated positively with haemagglutination. Second, resident populations had higher haemagglutination than both long-distance and short-distance migrants, whereas the latter two did not differ from each other. Third, breeding and resting populations (electronic supplementary material) had lower haemagglutination than populations during autumn migration, but the other populations shared similar values. Fourth, no differences in haemagglutination were found between free-living and captive-bred birds, while both groups had lower haemagglutination than wild-caught birds. Fifth, females had higher haemagglutination than males, and this result remained unchanged when we reran the model, excluding the 'no data' and 'mixed sex' categories. Sixth, there were no differences in haemagglutination between adults, fledglings and newly hatched birds. The relationship among the three defined age classes also did not differ when the 'no data' and 'mixed age' categories were excluded from the analysis.

When we conducted a BPMM for haemagglutination restricted to Passeriformes, several results differed from those in the full model (electronic supplementary material, table S3). First, resident birds still showed higher haemagglutination than short-distance migrants, but long-distance migrants no longer differed from either group. Second, breeding populations had significantly higher haemagglutination than resting populations, although relationships among the remaining seasonal stages remained unchanged. Third, wild-caught birds still had higher haemagglutination than free-living birds, but captive-bred birds turned out to have intermediate haemagglutination between them. Fourth, in contrast to the full-species model, age group showed biologically meaningful differences: while adults did not differ from newly hatched birds, both groups exhibited higher haemagglutination than fledglings. Fifth, average monthly temperature showed a positive correlation with haemagglutination, while average monthly precipitation was negatively correlated with haemagglutination.

Overall, for haemolysis, the BPMM revealed significant relationships with or differences in 6 of the 15 variables (table 2, which includes Gelman diagnostic values and effective sample sizes for all parameters), although the apparent effects of sex and age were driven only by the biologically meaningless 'mixed' categories. First, omnivores had higher haemolysis than herbivores, whereas carnivores did not differ from either of them. Second, short-distance migrants had higher haemolysis than resident birds, while long-distance migrants showed no differences from other categories. Third, breeding, wintering and resting populations had lower haemolysis than populations during autumn migration, while populations during spring migration and indoor populations had intermediate values, which were not significantly different from all the other populations. Fourth, no differences in haemolysis were found between males and females. Males and females also did not differ when we re-ran the model excluding the 'n.d.' and 'mixed' sex categories. Fifth, there were no differences in haemolysis between adults, fledglings, and newly hatched birds. The three defined age classes also did not differ when we ran the model excluding the 'n.d.' and 'mixed' age categories. Sixth, average monthly precipitation correlated positively with haemolysis.

The results from the passeriform-only BPMM for haemolysis (electronic supplementary material, table S4) showed differences from the results from full BPMM. First, no differences in haemolysis were found among the migration distance categories.

Table 1. Bayesian phylogenetic mixed model (BPMM) for population-level measurements of haemagglutination. Consensus phylogeny was included as a random factor. Continuous and categorical predictors, as well as categories of categorical predictors and their reference categories, are listed. Estimates and 95% confidence intervals (CIs), effective sample size (ESS) and Gelman–Rubin diagnostic ($\widehat{R}$) value for continuous predictors and categories of categorical predictors are listed. Superscript letters next to categories ('a', lowest value) indicate significant differences among categories within variables where applicable; categories without significant differences are unmarked. Significance involving 'n.d.' and 'mixed' is not indicated. Significant results, which are represented by the confidence intervals that do not cover 0, are in bold type. The reference category of each categorical variable was rotated to generate all pair-wise *post hoc* comparisons.

predictor	categories	estimate	95% CI	ESS	$\widehat{R}$
(intercept)		2.44	(−1.94, 6.94)	6147	1.00
body mass		0.54	(**0.13; 0.95**)	6242	1.00
clutch size		−0.48	(−1.52, 0.61)	6116	1.00
lifespan		0.04	(−0.43, 0.52)	6082	1.00
diet type	(versus carnivore)				
	herbivore	−1.04	(−2.46, 0.36)	6000	1.00
	omnivore	−0.49	(−1.59, 0.59)	6129	1.00
social type	(versus social)				
	solitary	0.57	(−0.34, 1.45)	5773	1.00
habitat type	(versus freshwater)				
	terrestrial	0.53	(−1.46, 2.50)	6201	1.00
	saline	0.14	(−1.23, 1.51)	6549	1.00
migration distance	(versus long-distance[a])				
	short-distance[a]	0.59	(−0.41, 1.56)	6000	1.00
	resident[b]	1.98	(**0.55, 3.38**)	6000	1.00
absolute breeding latitude		0.03	(−0.00, 0.07)	5756	1.00
seasonal stage	(versus breeding[a])				
	spring migration[a,b]	0.69	(−0.62, 2.08)	5610	1.00
	autumn migration[b]	1.44	(**0.59, 2.32**)	6000	1.00
	wintering[a,b]	0.43	(−0.61, 1.37)	6000	1.00
	resting[a]	−0.23	(−0.79, 0.34)	5866	1.00
	indoors[a,b]	0.32	(−0.82, 1.50)	6211	1.00
	n.d.	0.73	(**0.14, 1.32**)	5749	1.00
moult condition	(versus moult)				
	non-moult or n.d.	−0.16	(−0.85, 0.50)	6000	1.00
captivity condition	(versus wild-caught[b])				
	captive-bred[a]	−1.19	(**−2.36, −0.03**)	5824	1.00
	free-living[a]	−1.12	(**−1.88, −0.37**)	5805	1.00
sex	(versus female[b])				
	male[a]	−1.15	(**−1.80, −0.49**)	5848	1.01
	mixed	0.69	(**0.01, 1.39**)	6000	1.00
	n.d.	0.29	(−0.60, 1.20)	6000	1.00
age	(versus adult)				
	fledgling	−0.49	(−1.16, 0.18)	6000	1.00
	newly hatched	0.25	(−0.63, 1.15)	6155	1.00
	mixed	1.31	(**0.46, 2.17**)	6154	1.00
	n.d.	0.67	(−0.08, 1.41)	5615	1.00
average monthly temperature		0.02	(−0.02, 0.06)	6000	1.00
average monthly precipitation		−0.21	(−0.57, 0.14)	5543	1.00

Table 2. Bayesian phylogenetic mixed model (BPMM) for population-level measurements of haemolysis. Consensus phylogeny was included as a random factor. Continuous and categorical predictors, as well as categories of categorical predictors and their reference categories, are listed. Estimates and 95% confidence intervals (CIs), effective sample size (ESS) and Gelman–Rubin diagnostic ($\widehat{R}$) value for continuous predictors and categories of categorical predictors are listed. Letters next to categories ('a', lowest value) indicate significant differences among categories within variables where applicable; categories without significant differences are unmarked. Significance involving 'n.d.' and 'mixed' is not indicated. Significant results, which are represented by the confidence intervals that do not cover 0, are in bold type. The reference category of each categorical variable was rotated to generate all pair-wise *post hoc* comparisons.

predictor	categories	estimate	95% CI	ESS	$\widehat{R}$
(intercept)		1.18	(−2.03; 4.34)	5863	1.00
body mass		0.27	(−0.02, 0.57)	6026	1.00
clutch size		0.12	(−0.58, 0.86)	5617	1.00
lifespan		−0.12	(−0.42, 0.18)	5822	1.00
diet type	(versus carnivore[a,b])				
	herbivore[a]	−0.83	(−1.84, 0.11)	6036	1.01
	omnivore[b]	0.20	(−0.51, 0.90)	6000	1.00
social type	(versus social)				
	solitary	−0.36	(−1.03, 0.30)	6000	1.00
habitat type	(versus freshwater)				
	terrestrial	−0.55	(−1.87, 0.73)	5886	1.00
	saline	−0.55	(−1.43, 0.34)	6016	1.00
migration distance	(versus long-distance[a,b])				
	short-distance[b]	0.43	(−0.21, 1.08)	6148	1.00
	resident[a]	−0.41	(−1.30, 0.46)	6000	1.00
absolute breeding latitude		−0.01	(−0.03, 0.01)	6138	1.01
seasonal stage	(versus breeding[a])				
	spring migration[a,b]	0.45	(−0.46, 1.33)	6120	1.00
	autumn migration[b]	1.14	(**0.52, 1.75**)	5822	1.00
	wintering[a]	−0.23	(−0.94, 0.47)	6346	1.01
	resting[a]	−0.08	(−0.47, 0.32)	6000	1.01
	indoors[a,b]	0.37	(−0.27, 1.02)	6000	1.00
	n.d.	0.05	(−0.40, 0.50)	6000	1.00
moult condition	(versus moult)				
	non-moult or n.d.	0.02	(−0.50, 0.55)	6000	1.00
captivity condition	(versus wild-caught)				
	captive-bred	0.22	(−0.50, 0.91)	6388	1.00
	free-living	−0.19	(−0.69, 0.33)	6000	1.00
sex	(versus female)				
	male	0.08	(−0.41, 0.58)	6000	1.00
	mixed	−0.60	(**−1.14, −0.07**)	6000	1.00
	n.d.	−0.07	(−0.72, 0.57)	6000	1.00
age	(versus adult)				
	fledgling	−0.00	(−0.57, 0.56)	5708	1.00
	newly hatched	−0.17	(−1.09, 0.73)	6145	1.00
	mixed	1.36	(**0.63, 2.08**)	6000	1.00
	n.d.	−0.02	(−0.52, 0.48)	6183	1.00
average monthly temperature		0.01	(−0.02, 0.03)	6183	1.00
average monthly precipitation		0.24	(**0.01, 0.47**)	6000	1.01

Table 3. Comparison of six macro-evolutionary models of haemagglutination and haemolysis, with AIC_c, ΔAIC_c and relative importance (ω_i) of each model. Higher relative importance indicates better-fitting models.

	evolutionary model	AIC_c	ΔAIC_c	relative importance, ω_i
haemagglutination	Brownian motion adjusted with λ	474.1	0.0	0.84
	Ornstein–Uhlenbeck	477.3	3.3	0.16
	white noise	483.8	9.7	<0.001
	delta	516.4	42.3	<0.001
	trend	526.7	52.7	<0.001
	Brownian motion	538.8	64.7	<0.001
	early-burst	540.9	66.8	<0.001
haemolysis	Brownian motion adjusted with λ	298.9	0.0	1.000
	Ornstein–Uhlenbeck	310.1	11.2	<0.001
	white noise	322.6	23.7	<0.001
	delta	340.4	41.5	<0.001
	trend	349.7	50.8	<0.001
	Brownian motion	360.3	61.4	<0.001
	early-burst	362.5	63.6	<0.001

Table 4. Proportion of variance in haemagglutination and haemolysis explained by different taxonomic levels based on a taxonomically nested analysis and phylogenetic autocorrelation of haemagglutination and haemolysis at different taxonomic levels. Values indicate the proportion of variance (PoV) attributable to each taxonomic level, 95% credible intervals (CI), posterior means (PM), effective sample sizes (ESS), Moran's *I*, and its associated *p* values. Higher Moran's *I* indicates that haemagglutination and haemolysis are more similar in phylogenetically closer species.

taxonomic level	PoV (%)	95% CI (lower)	95% CI (upper)	PM	ESS	Moran's *I*	*p*-value
haemagglutination							
order	4.2	0.00	1.45	0.29	4000	−0.03	>0.1
family	15.9	0.00	3.31	1.10	2195	0.21	<0.05
genus	23.8	0.00	3.68	1.65	1631	0.42	<0.01
species	24.3	0.43	3.17	1.69	2779	—	—
residual	31.7	—	—	—	—	—	—
haemolysis							
order	12.8	0.00	1.87	0.41	3780	0.01	>0.1
family	35.8	0.14	2.34	1.15	3716	0.47	<0.01
genus	3.4	0.00	0.49	0.11	3282	0.48	<0.1
species	21.4	0.25	1.20	0.69	4767	—	—
residual	26.6	—	—	—	—	—	—

Second, within the seasonal stage, only populations sampled during autumn migration had higher haemolysis, while the other groups no longer differed from each other. Third, average monthly precipitation was no longer associated with haemolysis.

(b) Evolutionary patterns in haemagglutination and haemolysis

Based on ΔAIC_c, Brownian motion models adjusted with λ best represented the evolutionary patterns in haemagglutination and haemolysis across all species (table 3). These best-fitting evolutionary models for haemagglutination and haemolysis had high Pagel's λ (0.66 and 0.67, respectively) and low Blomberg's K (0.15 and 0.17, respectively [37]). For both haemagglutination and haemolysis, moderately high phylogenetic autocorrelation was observed at lower taxonomic levels, such as family and genus (table 4). In line with these results, a taxonomically nested variance analysis showed that variation in both traits was primarily explained at the levels of family, genus and species (table 4).

Within Passeriformes, model selection yielded slightly different results. The OU model best represented the evolutionary patterns in haemagglutination, while the Brownian motion model adjusted with λ remained the best-fitting model for haemolysis (electronic supplementary material, table S5). For haemagglutination, the best-fitting model had a Pagel's λ of 0.66 and a Blomberg's K of 0.57. For haemolysis, the best-fitting model had a Pagel's λ of 0.41 and a Blomberg's K of 0.22.

Mapping ancestral state reconstructions helps to visualize phylogenetic variations in haemagglutination (104 species, figure 1) and haemolysis (n = 85 species, figure 2). Among the 61 Passeriformes species, the Fringillidae had relatively

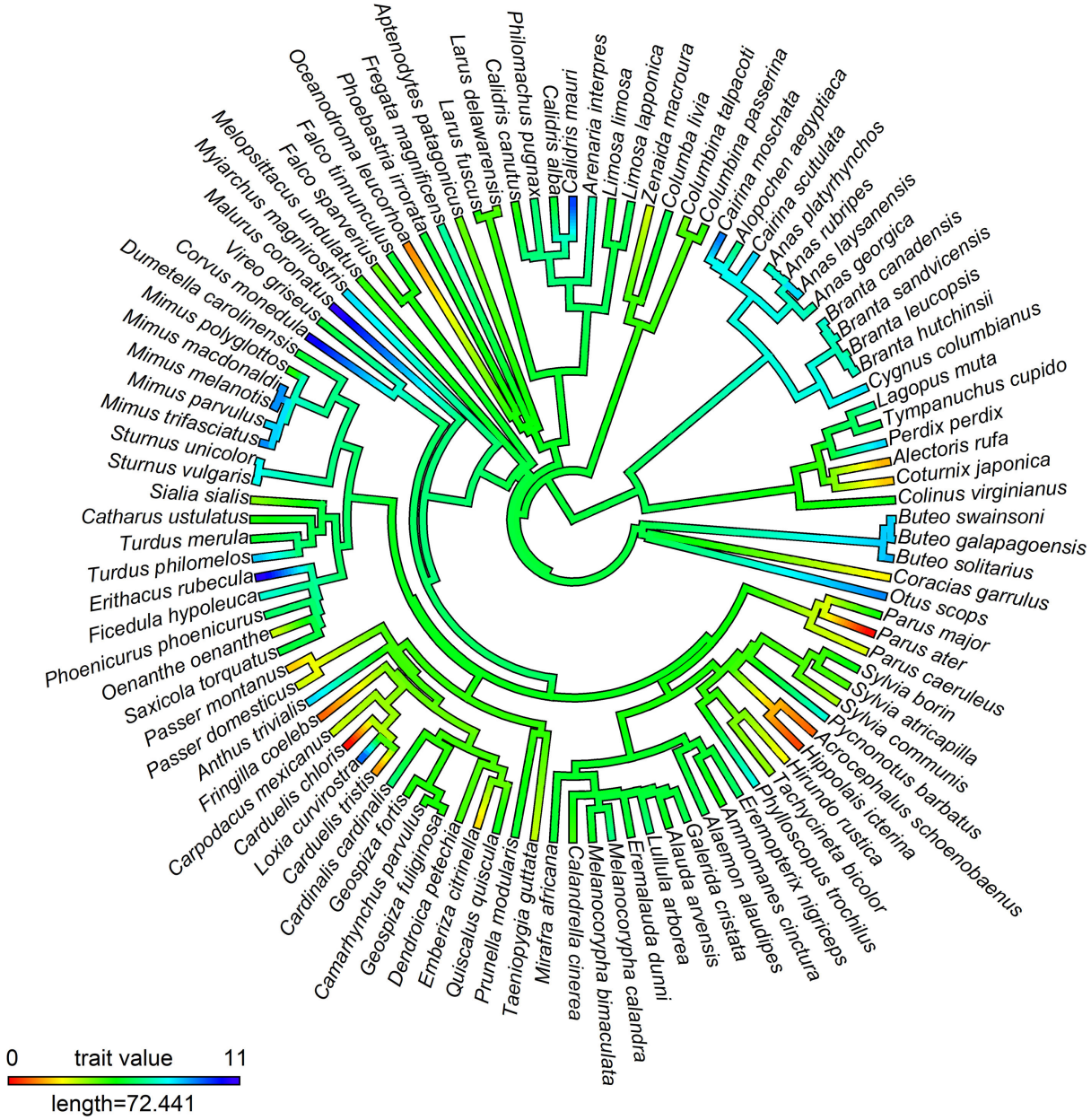

Figure 1. Fan figure of the average haemagglutination of each species through a consensus phylogeny. Low values are shown in red and high values in blue.

low haemagglutination, while the Sturnidae and neighbouring Mimidae had high haemagglutination. All Anseriformes, 12 Anatidae species and Accipitriformes (three *Buteo* species) had relatively high haemagglutination. Passeriformes exhibited large between-family variation in haemolysis. Low haemolysis was notable in four phylogenetically close families: Pycnonotidae, Phylloscopidae, Hirundinidae and Alaudidae. All four Columbiformes species also had low haemolysis. The 12 Anatidae species exhibited high haemolysis (consistent with high haemagglutination), contributing to an overall positive correlation between both immune traits (see above). Exceptions to this positive correlation were also apparent, e.g. two of the three *Buteo* species had low haemolysis contrasting with high haemagglutination values.

4. Discussion

Our study aimed to explain variation in two interrelated innate humoral immune defences in birds: NAb-driven haemagglutination and complement-driven haemolysis. We used 15 variables related to the avian life history and ecology to construct phylogenetically informed comparative models. These models indicated that relationships between the innate immune indices and some explanatory variables were in line with previous hypotheses listed in electronic supplementary material, table S1, including body mass [9], diet type [13], sex [38] and climatic factors [11,39]. Results of other variables did not match our initial predictions, but these mismatches can still provide new insights and shape new predictions.

Instead of the hypothesized lower immune defences in captive-bred and wild-caught birds owing to captivity stress [40], we found higher levels of haemagglutination in wild-caught birds than in free-living birds. This suggests that captive conditions with ample resources and reduced competition may support immunological investment by animals that were previously free-living. In our full-species datasets, no immunological differences were found among age groups; however, in

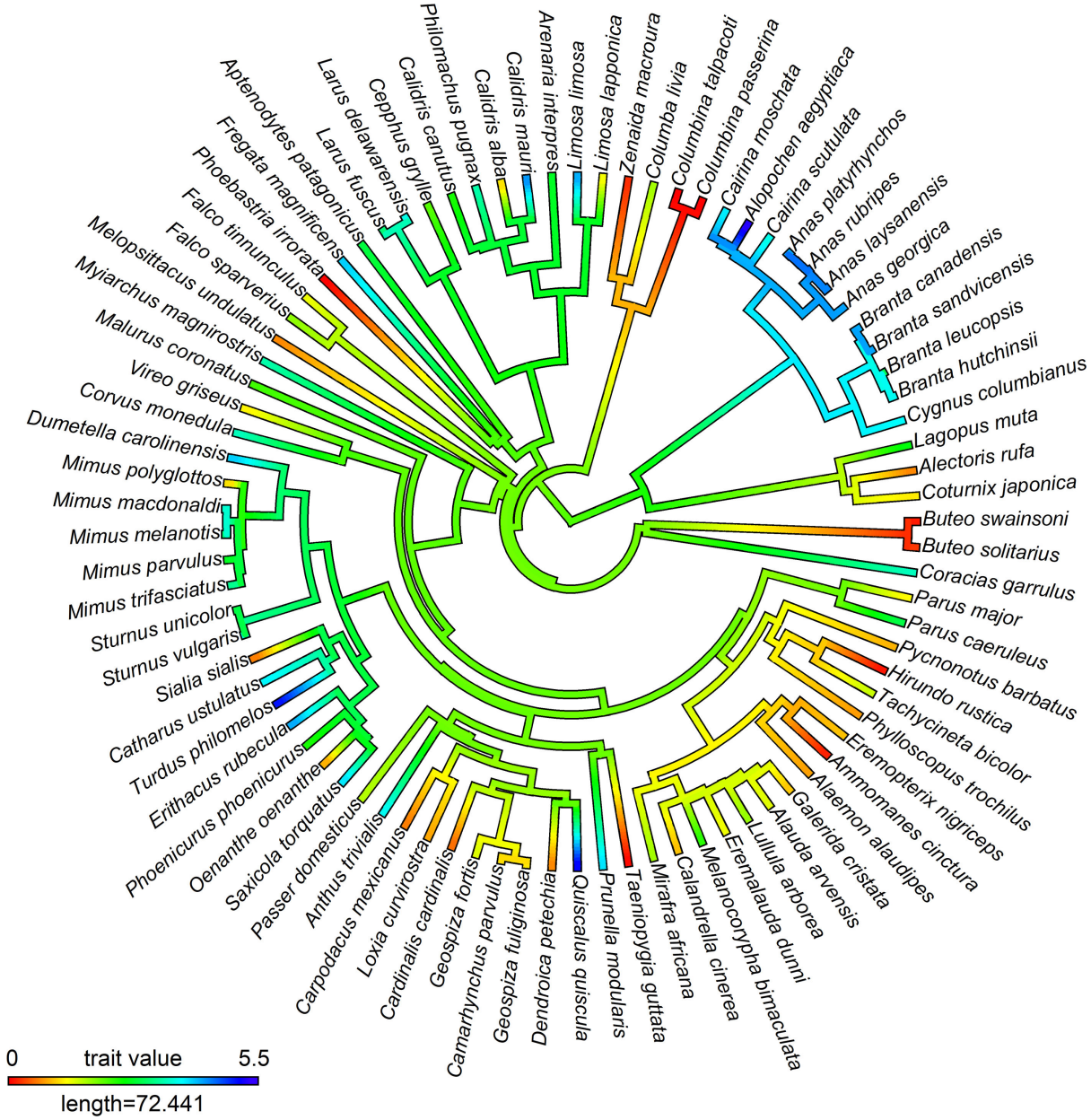

Figure 2. Fan figure of the average haemolysis of each species through a consensus phylogeny. Low values are shown in red and high values in blue.

our Passeriformes datasets, fledglings exhibited lower haemagglutination levels than both newly hatched birds and adults. These slightly different results both point towards the relatively strong innate immunity of newly hatched birds. A possible explanation is that the newly hatched birds may have maternally transferred NAbs, which can help provide innate humoral immune defences before young fledgling birds develop their own immune defence [20,41]. These results signal the complexity of innate humoral immune defences, which may not change in straightforward ways that match ecological theory.

Immunological variation associated with migration may reflect the multifaceted effects that are common with ecological factors. Migration involves tremendous changes in parasite risks, food availability and resource consumption, resulting in different immunological predictions [12,42]. Our two migration-related explanatory variables represented different traits: migration distance represented migratory capacity, while seasonal stage represented ecological and physiological conditions during sampling. For migration distance, both long-distance and short-distance migratory birds had lower haemagglutination than resident birds, but resident birds had lower haemolysis than short-distance migratory birds in our full-species datasets. The former result supports existing ideas that migrants are less able to spare resources for innate humoral immune defences [12]. However, the higher haemolysis in short-distance migrants matches better with predictions from the parasite-exposure hypothesis, which suggests individuals facing higher environmental parasite pressures need higher immune defences [43]. The lower haemolysis in the long-distance migrants suggests that this effect is context-dependent: long migrations may force trade-offs, while short migrations do not. Notably, the effects of migration distance on haemagglutination and haemolysis were not found within the Passeriformes dataset, suggesting that these associations may not be general across clades and could be driven by taxa outside of Passeriformes. For the seasonal stage, we predicted similar (depressed) levels during breeding and migration, since both are thought to be demanding in terms of energy and other resources. While birds during the breeding periods maintained low levels of haemagglutination and haemolysis, birds during autumn migration had higher levels of both indices. Thus, resource allocation can explain immune variation during breeding, but not during migration [42]. The

parasite-exposure theory can again help to explain these differences. Birds may need to invest less in innate immune defences in familiar breeding habitats (where acquired defences may be more effective and less costly) and invest more in innate defences as preparation for exposure to novel parasites during migration [43].

Overall, our haemolysis results matched our *a priori* predictions less well than haemagglutination results did. Such differences have also been reported in previous studies [15,44–46]. One possible explanation is that haemolysis may be more sensitive to short-term perturbations. For example, key elements of the complement system that drive haemolysis are known to act as acute-phase proteins, changing in concentration in response to infection and inflammation [47]. Moreover, haemolysis was shown to be more strongly negatively correlated with stress than was haemagglutination [48]. The higher costs of maintaining complement compared with those of maintaining natural antibodies may also help explain the labile nature of haemolysis [49].

Across all species, both haemagglutination and haemolysis were best described by Brownian motion models adjusted with Pagel's λ. This indicates a gradual divergence of these traits along the avian phylogeny, with patterns strongly shaped by phylogenetic relatedness. In contrast, within the largest clade (Passeriformes), the evolution of haemagglutination was better explained by an Ornstein–Uhlenbeck model, suggesting stabilizing selection around a lineage-specific optimum. These contrasting outcomes imply that different evolutionary dynamics may operate on the same immune trait across avian clades and at different evolutionary scales. High Pagel's λ values and low Blomberg's K further confirm that both immune indices exhibit a strong phylogenetic signal, meaning that closely related species tend to resemble each other more than expected under independent evolution. Notably, the substantially higher Blomberg's K for haemagglutination within Passeriformes points to stronger clustering among close relatives in this clade. These results were consistent with our nested taxonomic models and Moran's I analyses, which showed that most variance in haemagglutination was explained at the genus and species levels, whereas haemolysis was more structured at the family level.

In conclusion, our study revealed several ecological factors, but notably only one life-history trait (body mass), that correlated with either haemolysis or haemagglutination. The combined results of phylogenetically informed comparative models and macroevolutionary models suggest that these two innate humoral immune defences have been shaped by avian ecological diversity and retain signatures of phylogenetic history. Future work linking these immune indices to functional outcomes, such as host susceptibility or competence, will help clarify their role in structuring host–parasite interactions across the avian tree of life.

Ethics. Our study is a comparative analysis of avian immune defences, which collected data from peer-reviewed publications. Therefore, we do not need new ethical approval.

Data accessibility. We have submitted the data and code and made them publicly available via the repository Dryad [50].

Supplementary material is available online [51].

Declaration of AI use. Artificial intelligence (AI)-assisted tools (ChatGPT, OpenAI) were used exclusively to improve the accuracy of terminology, sentence fluency, and vocabulary translation, and to debug code used for data analysis. All scientific reasoning, study design, choice of analytical methods, data interpretation and conclusions were carried out solely by the authors, who take full responsibility for the content of this work.

Authors' contributions. W.-X.V.-H.P.: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing—original draft; A.G.d.C.: formal analysis, investigation, visualization; P.M.: methodology, resources, validation, visualization; W.F.d.B.: conceptualization, methodology, project administration, supervision, validation, writing—review and editing; K.D.M.: conceptualization, methodology, project administration, resources, supervision, validation, writing—review and editing.

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