

Physiological pace of life: the link between constitutive immunity, developmental period, and metabolic rate in European birds

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Abstract Constitutive innate immunity is the first lined of defence against infections, but the causes determining its variability among species are poorly understood. The pace of life hypothesis predicts that species with a fast speed of life, characterized by high energy turnover and short developmental time, invest relatively little in defence in favour of growth and early reproduction, whereas ‘slow-living’ species are predicted to invest more resources into costly defence. We conducted phylogenetic comparative analysis on 105 European bird species and determined that the number of leukocytes, and the levels of natural antibodies (NABs) and complement, measured on adult birds, increased or tended to positively correlate with the length of incubation period. However, we found that the length of

incubation and fledging periods have opposite effects on immune defence (i.e. immune parameters show a negative association with the length of fledging period). Our results suggest that the contrasting effects of the incubation and fledging periods are related to the timing of the development of immune cells and of NABs and complement, which largely mature during the embryonic phase of development. In support of this hypothesis, we found that species with a long relative incubation period [i.e. whose total pre-fledging developmental time (incubation plus fledging) consists largely of the incubation period] invested more in constitutive innate immunity. Finally, in support of the pace of life hypothesis, for a subsample of 63 species, we found that the basal metabolic rate significantly or tended to negatively correlate with immune measures.

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Introduction

Studies of ecological immunology addressing the causes of diversity in immune function have revealed a range of factors associated with immune system variation among populations and species (e.g. Nunn et al. 2003; Lee et al. 2008; Šimková et al. 2008; Sparkman and Palacios 2009). The effective distinction between self and non-self is in the first line of immunological protection against parasite attack, and has indisputable fitness benefits. However, developing and maintaining an effective immune system drains resources from the limited energy pool available for competing life history traits (e.g. Knowles et al. 2009; Bourgeon et al. 2010; Previtali et al. 2012), therefore

individuals have to optimize the net pay-off of such compromises. Because fitness costs and benefits of immune investment differ across individuals and species, and since these are contingent upon life history and ecological traits, a large intra- and interspecific variation in immune function is expected (e.g. Tella et al. 2002; Nunn et al. 2003; Tieleman et al. 2005; Matson 2006; Lee et al. 2008).

Recently, there has been growing interest in the utility of the pace of life syndrome for explaining interspecific variation in defence and the outcome of host–parasite interactions (e.g. Tieleman et al. 2005; Martin et al. 2007; Lee et al. 2008; Johnson et al. 2012). Species with a ‘fast-paced’ life are characterized by a high mass-specific metabolic rate, rapid growth and large investment in reproduction. Life history theory proposes that such species invest relatively little in self-maintenance (e.g. immune function, stress response) in favour of growth and early maturation, as opposed to those with a ‘slow-paced’ life (Ricklefs and Wikelski 2002; Lee 2006). Comparative studies on energetics and stress physiology are consistent with this hypothesis (e.g. Wiersma et al. 2007; Bókony et al. 2009; Hau et al. 2010). However, studies scrutinizing the pace of life hypothesis from the perspective of immune function have produced conflicting results (e.g. Tella et al. 2002; Tieleman et al. 2005; Palacios and Martin 2006; Lee et al. 2008; Addison et al. 2009; Edwards 2012). The ambiguous results regarding the covariation of immune defences and life histories may stem from the complexity of the immune system. Because all aspects of the immune system bear some energetic cost, we expect a reduced investment in immune function in species with a fast speed of life characterized by a high metabolic rate. On the other hand, because the developmental and defence trajectories of the innate and adaptive immune system differ (see below), we may expect that the relationship between immune function and life history pace of life, measured as the embryonic and post-hatching developmental period, varies in function of immune parameters.

In most bird species, a significant proportion of mass gain occurs during the embryonic (i.e. incubation) period before hatching and chicks reach near adult mass during fledging (Remeš and Martin 2002). However, species may vary in the relative time allocated to the embryonic and post-embryonic (i.e. fledging) development during the pre-fledging period, as precocial and large-sized birds gain more mass in the post-hatching period, while in altricial and small species the incubation part of the total developmental period is longer (Remeš and Martin 2002). Unlike adaptive immunity, components of the innate immunity are directly encoded by germ-line genes in the absence of somatic rearrangement (Avrameas 1991; Boes 2000). Therefore, their concentration in blood is not dependent on previous exposure to specific antigens and they are functional at hatching,

albeit not all immune measures are fully developed at this stage of growth (Apanius 1998). Also, because the number of cycles of cell proliferation of stem cells determines the diversity of cell lines, and these cell cycles apparently do not extend beyond the end of the incubation period in chickens (Weill and Reynaud 1987), a relatively longer incubation period suggests a larger number of cell cycles, a more diverse array of cell lines and hence a more responsive immune defence (Ricklefs 1992). Therefore, we predict that species with a long relative embryonic period (i.e. longer portion of incubation out of the total development time) have a more developed innate immune system, while those species with a long relative post-embryonic period (i.e. longer portion of fledging out of the total development time) invest less in this defence.

The negative correlation between the mass-adjusted metabolic rate and the activity of one component of the constitutive innate immunity (bactericidal activity of the whole blood) is consistent with the pace of life hypothesis (Tieleman et al. 2005). However, apparently contradictory results were found for life history pace of life and immune investment. The high investment in immune cells and reactive natural antibodies (NABs) of species with a long developmental period supports the hypothesis of life history pace of life (Lee et al. 2008; Martin et al. 2011; Edwards 2012). On the other hand, opposing results were found for other measures of the innate immune system (Palacios et al. 2011; Horrocks et al. 2012). Tella et al. (2002) and Martin et al. (2011) found that slow-paced species had higher cell-mediated immunity assayed by phytohaemagglutinin (PHA) wing web swelling, whereas Palacios and Martin (2006) found the opposite relationship using the same measure of immunity.

Here, we used the phylogenetic comparative approach in order to test how pace of life relates to the evolution and diversity of immune defence strategy across 105 temperate European bird species. Using a diverse assemblage of host species, we collected data on the concentration of different leukocytes in the blood, and the activity of NABs and the complement system, which are known to be important components of the constitutive immune function in birds (Lee 2006; Boughton et al. 2011; Demas et al. 2011). These cells and proteins are constitutively present at low levels in the blood stream and provide rapid first-line defences (Davison et al. 2008). NABs have been defined as antigen-binding antibody present in non-immunized individuals with broad specificity but low binding affinity. It has been suggested that in mammals NABs cooperate with the complement system (Thornton et al. 1994). White blood cells (WBCs) take part in the constitutive innate immune system with non-specific, broad-range activity. Heterophils are phagocytic immune cells and form the first line of cellular defence against invading microbial pathogens. Lymphocytes are

responsible for the production of immunoglobulin-associated humoral immunity (B-cells) and are part of the cellular-mediated immune system (T-cells), together constituting the adaptive immune system (Davison et al. 2008). Because the lymphocytes are constitutively present in the blood stream, in our study we considered them as part of the constitutive innate immune system. Moreover, natural killer cells, which are innate immune cells, are morphologically identical to lymphocytes. Because the total number of WBCs is a general measure of the amount of investment in defence (Semple et al. 2002; Blount et al. 2003; Nunn et al. 2003; Demas et al. 2011), and represents the sum of different immune cells (heterophils, lymphocytes, monocytes, basophils, eosinophils), we used this variable as a proxy for constitutive innate immune function.

We predict that the developmental period is an important component of the pace of life, which determines the immune function. However, because the developmental trajectories of different immune branches differ, and the time available is important for the development of immune cells and proteins, we predict that the relationship between innate immune function and the embryonic and post-hatching developmental periods vary. Further, we test the prediction that the metabolic rate, which is a good proxy for the pace of life (Wiersma et al. 2007; Pontzer et al. 2014), explains the immune function in birds. This is the first study to test the association between life history and metabolic pace of life and constitutive innate immune function in a large group of temperate birds. Therefore, it fills important gaps in our understanding on the evolution and diversity of innate defence.

Materials and methods

Bird capture and blood sampling

We captured adult birds ($n = 699$ individuals, 105 species) between 1 April and 15 July, from 2009 to 2013, across Romania, with a few bird specimens captured in central Hungary. Captured species consisted of temperate European birds that varied greatly in body size (5.6–1,324.5 g; see Supplementary Appendix S1) and covered a large taxonomic range (35 families according to the BirdLife International checklist; URL: www.birdlife.org/datazone/info/taxonomy). We captured individuals with mist nets (Ecotone, Poland) and various traps recommended for birds of different sizes. All birds were captured during their breeding season except for the following spring migrants: Bohemian waxwing (*Bombycilla garrulus*), pied flycatcher (*Ficedula hypoleuca*), and red-breasted flycatcher (*Ficedula parva*). Excluding the data of these three species from the analyses did not change the results (data not shown). Therefore,

we decided to include the data of these three species in our study. Depending on body size, 50–250 μ L blood was collected in heparinized capillary tubes by venipuncture of the brachial vein. Samples were drawn mostly within 10–15 min but not later than 20 min following capture. A drop of blood was smeared on a microscope slide for the leukocyte count. Blood samples were kept on ice in a cooling box for a maximum of 10 h, when they were centrifuged at ca. 6,200g for 5 min. The plasma was separated from red blood cells and was stored at -20° C until analysis. After bleeding, birds were individually marked with a numbered aluminium ring and standard biometry measures were taken.

Immunological characterization

Haemolysis–haemagglutination assay

We assessed the levels of NABs and complement using a modified haemolysis–haemagglutination assay (Matson et al. 2005). We have described this method in detail elsewhere (i.e. Pap et al. 2011). Briefly, the only modification related to the original protocol was that here we used freshly collected rat instead of rabbit red blood cell suspension (see Seto and Henderson 1968). In this assay, agglutination reflects the activity of the NABs, while lysis represents the interaction between the NABs and the complement (Matson et al. 2005).

Total and differential leukocyte counts

To count leukocytes, we prepared a blood smear, air-dried, fixed in Dia-Fix, and stained with Dia-Red and -Blue Papanotic (Diagon, Hungary). Smears were examined at 1,000 $\times$ magnification with oil immersion, using the same protocol as in our previous studies (Pap et al. 2011, 2014), and the proportion of different types of leukocytes was assessed by counting 50 leukocytes. The number of WBCs of different types was expressed to approximately 10^4 erythrocytes. Leukocytes were counted by two workers (G. O. and J. V.-S.), and were highly repeatable within ($R > 0.85$ and $P < 0.0001$ for all WBC types) and between data collected by each ($R > 0.80$ and $P < 0.0001$ in all cases). Monocyte, eosinophil and basophil counts were not analysed separately because of their low concentration in the blood (see Supplementary Appendix S1); however, they were used in calculating the total number of WBCs in blood.

Life history traits

All data on species' immune parameters, body mass, life history, ecology, and basal metabolic rate (BMR) are presented in Appendices S1 and S2 of the online

supplementary information. Life history variables included in the analyses were clutch size, length of incubation (the period between laying the last egg of a clutch and hatching), and length of fledging period [the period between hatching and fledging; see Cramp and Perrins (1977–1994)]. Migratory, breeding and feeding behaviour, sociality, and habitat type have been found to be responsible for the risk of infestation (e.g. Møller and Erritzøe 1996; Tella et al. 1999; Figuerola and Green 2000; Waldenström et al. 2002; Blount et al. 2003; Garamszegi and Møller 2007), and species with a high infestation rate invest more in their immune system (e.g. Tella et al. 2002; Garamszegi and Møller 2007). Therefore, we included all these variables (habitat type, migratory behaviour, nest type, coloniality, and food type) in our models in order to control for their effect on immune parameters. We collated data on species' life history and ecology from Cramp and Perrins (1977–1994). We scored breeding habitat as terrestrial or wet (i.e. species living in marshes, wetlands, humid grasslands with or without direct contact with water). We categorized species as resident, short-distance (winter quarters north of the Sahara), or long-distance migrants (winter grounds in tropical Africa) based on the migratory behaviour of the species' Eastern European populations (Végvári et al. 2009). This categorization may slightly differ from similar classifications of the Western European birds, but our criteria take into account that eastern populations of the same bird species (where the samples come from) are more often migratory due to the colder continental winter, while Western European populations are more often resident (e.g. European starling *Sturnus vulgaris*). Nest type was categorized as cavity (natural or artificial holes), or open (for species breeding in nest cups or open nesting places). The social behaviour during the reproductive season was classified as colonial or solitary. Because the immune function may largely depend on the protein content of the food consumed (Pap et al. 2008), we assigned the summer diet to one of two categories ranked according to the proportion of animal and plant materials in food: species feeding mainly (>50 %) on vertebrates and/or invertebrates, and omnivorous species or those feeding mainly on plants and invertebrates. We included body mass extracted from Dunning (2008), completed with data from Cramp and Perrins (1977–1994), as an additional predictor variable in the analyses because the physiological and life history traits scaled to body size, but still showed considerable residual variation. If a range was reported by Cramp and Perrins (1977–1994), we used the mean of the reported minimum and maximum values; if multiple estimates were reported, we used the one representing the breeding season of Western Palearctic populations. We used the measures of BMR (kJ/h) from McNab (2009) for a total of 63 species.

Statistical and phylogenetic analyses

All statistical analyses were carried out with the R statistical environment version 3.1.0 (R Development Core Team 2014). To account for phylogeny, we used the phylogenetic tree published by Thuiller et al. (2011) with original branch lengths (see Supplementary Appendix S3). To investigate the relationship between immunological parameters and potential explanatory variables (see below) we used phylogenetic generalized least squares (PGLS) models as implemented in R package *caper* (Orme 2011). The PGLS approach controls for non-independence among species by incorporating a matrix of covariances among species, based on their phylogenetic relationships (Martins and Hansen 1997; Pagel 1997, 1999). In all analyses, we set the degree of phylogenetic dependence (Pagel's λ) to the most appropriate degree evaluated for each model by likelihood ratio statistics. Habitat, migratory behaviour, nest type, coloniality, and food type were entered into the models as fixed factors, whereas all other continuous variables (body mass, incubation period, fledging period, clutch size) were included as covariates. To assess the minimum model best describing variation in the dependent variables we used stepwise selection, eliminating non-significant predictors from the full model in a backward stepwise manner based on the largest P -value (retention criterion, $P \leq 0.05$).

Prior to statistical analyses, BMR, heterophil, lymphocyte, total WBC counts and body mass were log transformed, while agglutination and lysis scores were square root transformed in order to achieve model residual normality. Different and total WBC counts and plasma parameters were used as response variables in separate PGLS models with body mass, life history and ecology traits as explanatory variables. The strong positive correlation between incubation and fledging periods across bird species (PGLS, $F = 48.09$, $df = 1,103$, $P < 0.0001$) and the opposite effect of the incubation and fledging periods on immune parameters raised the possibility of multicollinearity problems in the case of multivariate models. Therefore, we repeated analyses in which we used the relative incubation [i.e. incubation/(incubation + fledging period)] and (absolute) incubation period, which allowed us to test in the same models the effect of developmental period and trajectory on immune parameters (see Supplementary Appendix S6). This approach is appropriate, since percent incubation period indicates both the relative length of incubation and fledging period (i.e. high percent incubation period values mean both long incubation and short fledging). Since the latter analyses resulted in similar conclusions when the incubation and fledging periods were included separately in the models (see “Results”), we concluded that the opposite relationship of the incubation and fledging periods

with the immune variables are real and are not statistical by-products.

We report the minimal models, in which the body mass was retained to control for allometry. Also, we retained in the minimal models the incubation and fledging periods if they reached near statistical significance ($P \leq 0.1$). Additionally, we provide full models for the first set of analyses (i.e. where the incubation and fledging periods are introduced in the models as separate explanatory variables; see Supplementary Appendix S5) as a verification of the results obtained by minimal models. Sampling effort differs across species, which is known to be a source of bias in comparative studies, since estimates are not given with similar precision for different species (Garamszegi and Møller 2010, 2011). However, if within-species variance is particularly small compared to between-species variance, then ignoring this measurement error has no effect on the type I error of phylogenetic analyses (Harmon and Losos 2005). To test whether immune parameters measured here are species-specific and thus suitable for multispecies comparison, we tested the intraspecific repeatabilities of these traits by assessing the importance of between- compared with within-species variance (function *icc* from R package *irr*; Gamer et al. 2012). Since variance is sample size dependent, the estimation of repeatability in comparative studies with unbalanced sample sizes across species is equivocal. To overcome this limitation we repeated 1,000 times the repeatability test that randomly selected two individuals per species in each run. Intra-class correlation coefficients (ICCs) and corresponding confidence intervals (CIs) were calculated as the means of the 1,000 estimates. The repeatability test only used species with at least two individuals sampled and were tested using raw, non-transformed data. Immune variables were found to be species-specific as conspecific values were highly similar for species with at least two individuals sampled (see “Results”); therefore, we present the models unweighted to sampling effort. Furthermore, unweighted PGLS models had the lowest Akaike information criterion (AIC) values when compared with models weighted to sample size (results not presented). Finally, to ensure that our results were not affected by the large variance of immune measures of species with very few individuals sampled, we repeated the analyses on a subset of data limited to species with more than one individual sampled (see Supplementary Appendix S4). We quantified the amount of phylogenetic dependence of the five physiological variables by calculating the metric λ as implemented in *phylosig* function in the R package *phytools* (Revell 2012). We report mean $\pm$ SE values and two-tailed statistical tests with a 5 % significance level. Sample sizes differ across tests because data are incomplete for some species.

Results

Repeatability, covariation and phylogenetic signal of immune parameters

Immune parameters varied widely among species: heterophils (per 10^4 erythrocytes), range 0.65–52.07; lymphocytes, 0.00–33.82; WBC, 0.95–84.07; agglutination, 0.00–7.29; lysis, 0.00–6.00. Including only species with at least two individuals sampled, and randomly selecting two samples from each species, the repeatability was moderate and significant in all cases (heterophils, ICC = 0.48, 95 % CI = 0.31–0.61, $F = 3.21$, $P = 0.0001$; lymphocytes, ICC = 0.30, 95 % CI = 0.11–0.47, $F = 2.01$, $P = 0.0318$; WBC, ICC = 0.43, 95 % CI = 0.26–0.58, $F = 2.74$, $P = 0.0013$; agglutination, ICC = 0.32, 95 % CI = 0.12–0.48, $F = 1.97$, $P = 0.0078$; lysis, ICC = 0.38, 95 % CI = 0.19–0.55, $F = 2.31$, $P = 0.0017$). This indicates that our measures of the immune system are species specific and thus suitable for multispecies comparison. The number of total and differential leukocytes significantly and positively correlated to each other (all $P < 0.0001$), and the agglutination positively correlated with lysis ($P < 0.0001$). However, none of the total and differential leukocyte numbers were significantly related to plasma parameters (all $P > 0.2790$). Pagel's λ varied between 0.53 and 0.92, suggesting a moderate to high degree of phylogenetic dependence (heterophils, $\lambda = 0.92$, $P < 0.0001$; lymphocytes, $\lambda = 0.58$, $P < 0.0001$; WBC, $\lambda = 0.87$, $P < 0.0001$; agglutination, $\lambda = 0.53$, $P = 0.0015$; lysis, $\lambda = 0.66$, $P < 0.0001$).

Covariation of life history traits

The length of relative incubation period was negatively correlated with both body mass [β (SE) = -0.02 (0.01), $t = -2.43$, $P = 0.0168$] and total developmental period (i.e. incubation + fledging period) [β (SE) = -0.15 (0.02), $t = -7.26$, $P < 0.0001$]. In a multivariate PGLS model where body mass and total developmental period were included together as explanatory factors, only the latter proved to be a significant predictor of relative incubation period [body mass, β (SE) = 0.00 (0.01), $t = 0.38$, $P = 0.7047$; total developmental period, β (SE) = -0.16 (0.02), $t = -6.55$, $P < 0.0001$], showing that species with a longer total growth period spend a shorter relative time in the embryonic stage.

Incubation and fledging periods

The number of leukocytes (total and differential) and scores of plasma parameters were significantly explained by body mass; species with a large body size had a higher concentration of leukocytes, and higher agglutination and lysis scores

Table 1 Minimal phylogenetic generalized least squares (PGLS) models explaining the variation in immune variables between species in relation to incubation period, fledging period, body mass, life history and ecological traits

	Heterophils	Lymphocytes	WBCs	Agglutination	Lysis
Intercept	0.80 (1.07), 0.74	−1.66 (0.88), −1.88 ^{0.07}	1.15 (1.49), 0.77	−0.57 (0.73), −0.78	−1.82 (0.94), −1.94
Body mass	0.26 (0.07), 3.59***	0.38 (0.09), 4.31***	0.29 (0.10), 2.90**	0.21 (0.07), 2.74**	0.24 (0.08), 3.24**
Incubation period	0.70 (0.39), 1.78 ^{0.08}	2.04 (0.48), 4.25***	0.97 (0.56), 1.73 ^{0.09}	1.24 (0.40), 3.08**	1.20 (0.40), 3.02**
Fledging period	−0.57 (0.24), −2.36*	−1.37 (0.33), −4.19***	−0.85 (0.34), −2.49*	−0.71 (0.26), −2.71**	−0.57 (0.26), −2.20*
Habitat					
Riparian	–	0.37 (0.22), 1.67	–	–	–
Aquatic ^a	–	−0.35 (0.25), −1.39	–	–	–
Migration					
Short	0.35 (0.11), 3.05**	–	0.28 (0.17), 1.61	–	0.17 (0.14), 1.16
Long ^b	−0.15 (0.12), −1.23	–	−0.23 (0.18), −1.32	–	0.37 (0.14), 2.67**
Nest type: open	–	−0.36 (0.16), −2.24*	–	–	–

Minimal models were obtained by eliminating non-significant predictors from full models in a backward stepwise manner based on the largest *P*-value. Model intercepts implement the first level of each factor (i.e. terrestrial species for habitat, and resident species for migratory behaviour). All other levels of candidate factors are compared to the level implemented in the intercept. Values are β (SE) and *t*

WBCs White blood cells

Significant *P*-values are in *italic* (* *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001); nearly significant *P*-values (*P* < 0.1) are shown as *superscripts*

^a Significant difference between aquatic and riparian groups for lymphocytes [β (SE) = −0.72 (0.33), *t* = −2.22, *P* = 0.0287]

^b Significant difference between short- and long-distance migrant groups for heterophils [β (SE) = −0.49 (0.10), *t* = −5.05, *P* < 0.0001] and WBCs [β (SE) = −0.51 (0.15), *t* = −3.40, *P* < 0.01] and non-significant difference for lysis [β (SE) = 0.20 (0.13), *t* = 1.53, *P* = 0.1296]

(Table 1). The minimal PGLS models show that the incubation period had a significant and positive effect on lymphocytes and plasma parameters (Table 1; Fig. 1a–e). The number of heterophils and WBCs was marginally significantly and positively related to the incubation period (Table 1; Fig. 1a–e). In contrast, the fledging period was negatively related to all immune parameters (Table 1; Fig. 1a–e). Habitat and nest type proved to significantly affect the number of lymphocytes, and migration significantly explained the number of heterophils, WBCs and lysis scores (Table 1). The effect of the incubation and fledging periods on differential and total WBCs, and plasma parameters, was qualitatively similar in the models based on the subset of data limited to species with more than one observation and in the full models (Appendices S4 and S5).

Finally, to demonstrate that the opposing effects of the incubation and fledging period on immune parameters were not confounded by the possible multicollinearity between these two explanatory variables, we repeated the analysis where we included the immune parameters as dependent variables in separate PGLS models and the body mass, the absolute incubation period, relative incubation period, and ecological traits as explanatory variables (Supplementary Appendix S6). These results confirmed the significant and opposing effect of the incubation and fledging periods on immune parameters. The relative length of the incubation period significantly and positively correlated with all WBC and plasma parameters (Supplementary Appendix S6).

Basal metabolic rate

Because incubation and fledging periods are correlated (see above), we estimated their partial effects on BMR in a PGLS model, where we included the absolute developmental periods and body mass. The overall phylogenetic model showed significant effects of all predictors on BMR, except fledging period [β (SE) = 0.17 (0.14), *t* = 1.24, *P* = 0.2220]. BMR increased with increasing body mass [β (SE) = 0.71 (0.04), *t* = 17.98, *P* < 0.0001], but decreased with the length of the incubation period [β (SE) = −0.48 (0.20), *t* = −2.40, *P* = 0.0199].

The PGLS models show that BMR had a significant and negative effect on heterophils, WBCs, agglutination and lysis scores (Table 2; Fig. 2a–e). Also, the effect of BMR on lymphocytes was negative, albeit marginally significant (*P* = 0.0645).

Finally, to analyse the relative importance of the incubation period, fledging period and BMR on immune parameters, we performed a multivariate PGLS analysis where we included the immune parameters as dependent variables in separate PGLS models and the body mass, developmental periods, and BMR as explanatory variables (Table 3). These results confirmed the significant and opposing effect of the incubation and fledging periods, and the negative effect of BMR on immune parameters. However, significance levels for all explanatory variables were reduced and were found to be non-significant in many cases (Table 3).

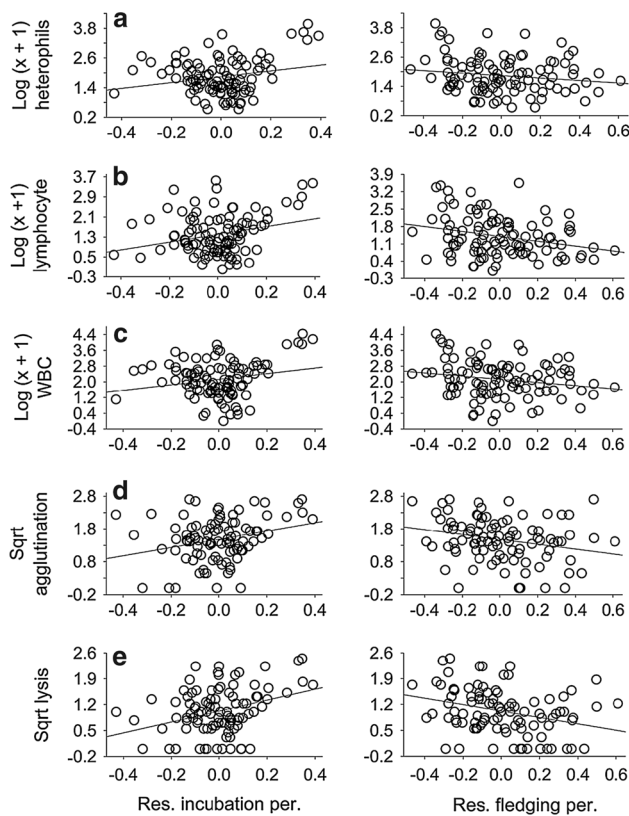


Fig. 1 Relationship of white blood cells (WBCs) (a–c) and plasma parameters (d–e) with incubation and fledging period residuals (i.e. corrected to body mass). Among the immune measures, lymphocytes, agglutination and lysis are significantly and positively related to incubation period residuals on body mass. All immune measures are significantly and negatively correlated to fledging period residuals. For details, see Table 1. Values are calculated from ordinary least squares models, and regression lines are from predictions of each model

Discussion

Immune function and developmental period

An important axis of the pace of life syndrome is the length of the developmental period, with fast-living species spending a shorter time in the egg and nest. The length of the developmental period has also been suggested as a driver of the diversification of immunological profiles of species

with different life histories (Ricklefs 1992; Lee 2006). Although the viability of the pace of life hypothesis was already tested for certain components of the immune system (e.g. PHA-induced immune response), other components have received less attention (different leukocytes, NAb, complement). Leukocytes have rarely been used in avian comparative studies (Blount et al. 2003; Edwards 2012), but are frequently used in mammalian studies where WBC counts are often explained by life history and ecological traits related to disease risk and sexual selection (e.g. Nunn 2002; Semple et al. 2002; Nunn et al. 2003; Schneeberger et al. 2013). Our study shows that the number of different leukocytes and the activity of NAb and complement are developmentally constrained. We found that the length of the embryonic period marginally correlates with or significantly and positively predicts the number of differential and total WBCs and the levels of NAb and complement. In contrast, the post-embryonic developmental period significantly and negatively correlates with all immune parameters. This relationship was significant or near-significant in the minimal models (see Table 1) and after controlling for potentially confounding variables, such as clutch size, migratory behaviour, habitat, nest type, coloniality and diet (see Supplementary Appendix S5). The increased number of WBCs and high activity of NAb and the complement in species with long embryonic developmental period support the prediction that slow-living species with a long developmental period invest more resources into immune defence, irrespective of the associated costs of different ‘arms’ of the immune system (see Klasing and Leshchinsky 1999; Lee 2006; Davison et al. 2008; Previtali et al. 2012); whereas species with a fast pace of life characterized by rapid growth invest relatively less in defence in favour of growth and early reproduction (Ricklefs 1992; Ricklefs and Wikelski 2002; Lee 2006). These results corroborate our findings on the reduced immune defence of species with high BMR (see below).

Interestingly, we found that the incubation and fledging periods have opposite effects on constitutive immune defence, and that besides the absolute incubation period, the relative incubation period consistently explains the immune parameters. This finding demonstrates that species with a long relative incubation period (i.e. whose

Table 2 PGLS models explaining the variation in immune variables between species in relation to basal metabolic rate (BMR) and body mass

	Heterophils	Lymphocytes	WBCs	Agglutination	Lysis
Intercept	−1.22 (0.48), −2.55*	−1.00 (0.61), −1.64	−1.22 (0.63), −1.94 ^{0.06}	−1.10 (0.76), −1.46	−2.26 (0.76), −2.96**
Body mass	0.99 (0.17), 5.96***	0.79 (0.21), 3.70***	1.07 (0.22), 4.93***	0.78 (0.22), 3.48**	0.94 (0.22), 4.32***
BMR	−0.89 (0.27), −3.35**	−0.64 (0.34), −1.88 ^{0.07}	−0.89 (0.35), −2.54*	−0.88 (0.32), −2.72**	−1.05 (0.31), −3.41**

Values are β (SE) and t

Significant P -values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) are shown in *italic*; nearly significant P -values ($P < 0.1$) are shown as *superscripts*

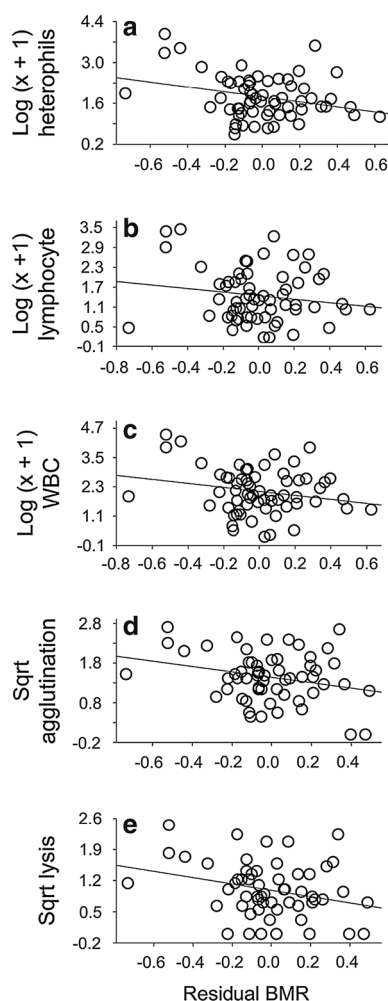


Fig. 2 Measures of immune function relative to basal metabolic rate (BMR). Among the immune measures, heterophils, WBCs, agglutination and lysis are significantly and negatively related to BMR residuals (i.e. corrected to body mass). For details, see Table 2. Values are calculated from ordinary least squares models, and *regression lines* are from predictions of each model

pre-fledging development falls largely into the embryonic period) have more developed innate immune traits as adults because this part of the immune system largely develops

during the embryonic phase. At the same time, species with relatively short relative incubation periods invest less in the innate immune system, most likely because of energetic and nutritional limitations imposed by rapid embryonic growth and/or due to the limited time available for the development of immune cells and function. Species with a large size and long total developmental period (characteristic of the slow pace of life) have relatively short incubation periods and long fledging periods (Remeš and Martin 2002; our data set). Studies on poultry and more recently on wild birds show that the adaptive immune defence develops largely following hatching, because it needs antigenic stimuli to generate a broad repertoire of specific paratopes (Apanius 1998; Mauck et al. 2005; Davison et al. 2008). In contrast, the innate immune system is active already at hatching (Apanius 1998; Davison et al. 2008; Arriero et al. 2013) and can be compromised by the somatic growth rate during embryonic development. Therefore, we argue that the variation in relative incubation period observed between species with small and large body masses and between short and long total developmental periods, respectively, may explain the contrasting effects of incubation and fledging periods on measures of immune defence. Our results show that the incubation period is more strongly related to BMR (species growing fast during incubation have a high BMR) than the fledging period. This suggests that the incubation period is indeed an important aspect of the pace of life. The absence of a relationship between fledging period and BMR indicates that the post-hatching developmental period affects immune function through the time available for the immune system to develop and less through energetic limitations due to growth. The significant correlation between BMR and incubation period can explain the reduced partial effect of developmental periods and BMR on immune measures when all these explanatory variables were included in the same model (see Table 3). However, the reduced partial effect of developmental periods and BMR on immune measures was probably affected by the increased number of explanatory variables included in the models (four). Therefore, at this level we cannot infer

Table 3 Full PGLS models explaining the variation in immune variables between species in relation to body mass, incubation period, fledging period and BMR

	Heterophils	Lymphocytes	WBCs	Agglutination	Lysis
Intercept	−2.45 (0.81), −3.02**	−2.51 (0.98), −2.57*	−2.44 (1.06), −2.31*	−1.91 (1.10), −1.74 ^{0.09}	−0.74 (0.86), −0.85
Body mass	0.79 (0.23), 3.47**	0.60 (0.27), 2.19*	0.87 (0.30), 2.93**	0.95 (0.25), 3.87***	0.62 (0.24), 2.55*
Incubation period	0.80 (0.46), 1.72 ^{0.09}	1.78 (0.55), 3.22**	1.12 (0.60), 1.87 ^{0.07}	0.49 (0.49), 1.00	0.68 (0.49), 1.38
Fledging period	−0.10 (0.33), −0.29	−0.96 (0.39), −2.45*	−0.41 (0.42), −0.96	−0.58 (0.35), −1.65	−0.54 (0.35), −1.55
BMR	−0.72 (0.32), −2.26*	−0.37 (0.38), −0.97	−0.67 (0.41), −1.62	−1.01 (0.33), −3.06**	−0.66 (0.34), −1.95 ^{0.06}

Values are β (SE) and t . For abbreviations, see Tables 1 and 2

Significant P -values (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$) are shown in *italic*; nearly significant P -values ($P < 0.1$) are shown as *superscripts*

which of the traits (incubation period and BMR) are more important in determining the differential and total WBCs and plasma parameters in adult birds. A further increase in sample size combined with path analysis could reveal this. It is interesting to note that all three comparative studies published on birds have found a positive effect of incubation period on different components of the innate immune system (Lee et al. 2008; Martin et al. 2011; Edwards 2012). These results, in line with our findings, strongly suggest that the length of the embryonic period has an important effect on the development of the avian immune system. However, studies on reptiles and small mammals show that the pace of life may differently affect the innate immune response relative to what has been found in birds (Martin et al. 2007; Sparkman and Palacios 2009), which suggests that different ontogenetic peculiarities of these groups can have contrasting effects on the activity of the immune system.

The opposing association of immunity with incubation period and with fledging period can be a consequence of factors other than the timing of the development of the immune function (Martin et al. 2001; Remeš and Martin 2002; Arriero et al. 2013) because the embryos and nestlings may be subject to different selection pressures that shape their developmental rates. There are at least two prevailing risks during the developmental period: predators and parasites. High predation mortality selects for fast developmental rates in both the incubation and the fledging periods, since the benefits of predation avoidance outpace the benefits of effective immune function developed during a slow-pace ontogeny. However, because embryos are protected by a hardly penetrable eggshell, parasite pressure might be low during incubation in contrast with the nestling period. Furthermore, if parasite pressure is high throughout the developmental period, then it selects for a slow embryonic rate to allow the ontogeny of a powerful immune function, but might select for fast post-embryonic rate to allow escape from the attack of (ecto)parasites. We suggest that a next step in the study of the development of immune function and evolution of the physiological pace of life should include extrinsic factors, such as predation and parasitism (Martin et al. 2001; Remeš and Martin 2002; Arriero et al. 2013).

We found that immune variables are species specific as conspecific values were highly similar for species with at least two individuals sampled. Also, we found that the effect of phylogenetic history on immunological traits was moderate to strong, suggesting that it is highly conservative across the evolutionary tree. Because there is a substantial level of inter-individual variation within species, which can be related to random sampling (i.e. sampling immune-suppressed or parasitized individuals), the species-specific values of immune measures are an approximation. However, our results are probably not affected by statistical noise due

to differential sampling of species, because unweighted PGLS models had the lowest AIC values when compared to models weighted to sample size. Therefore, we conclude that our results are conservative, reaching significant levels only for highly correlated traits. Finally, the significant and positive correlation between the number of different and total WBCs, and between agglutination and lysis, indicates that our results are at least partially influenced by the covariation between immune traits. However, we found that WBCs and plasma parameters are non-significantly correlated, which suggests that at least for two groups of immune measures, developmental period and BMR have a significant and independent effect.

The effect of body mass on immune parameters

In general, WBCs and the activity of NAb and complement were strongly positively correlated with body mass, which is known to be one of the most important traits explaining the pace of life (Bennett and Owens 2002; Sibly et al. 2012). Body mass increases from the fast-paced to the slow-paced limits of the life history continuum (Lee 2006). The allometric effect of body size on the number of WBC has been shown for primates, carnivores, and Neotropical bats (Semple et al. 2002; Nunn et al. 2003; Schneeberger et al. 2013); but, despite the wide use of leukocytes in avian immunological studies, to the best of our knowledge, ours is the first study to prove the relevance of mass dependence of the differential and total WBCs in birds. As mentioned above, the allometric effect of body mass on the number of leukocytes and the activity of NAb and complement is in line with the pace of life hypothesis (Tella et al. 2002; Addison et al. 2009). Alternatively, but not exclusively, we admit that the ‘hosts-as-islands’ and the ‘parasite-exposure’ hypotheses (Kuris et al. 1980; Johnson et al. 2012) cannot be ruled out, because large-bodied, slow-living species with long developmental periods and life spans experience an increased likelihood of parasite encounter, which may select for a diverse and effective immune system (Lee 2006).

Immune function and metabolic pace of life

Here we showed that the heterophils, WBCs and plasma parameters are significantly and negatively related to the mass-corrected BMR, which is an important component of the pace of life syndrome at individual and higher taxonomic level (Tieleman et al. 2005; Wiersma et al. 2007; McNab 2009). Also, the number of lymphocytes tended to negatively correlate with BMR. These findings demonstrate that metabolic rate has a profound effect on immunological defence and hence might connect the immune profile to life histories (Ricklefs and Wikelski 2002; Tieleman et al.

2005). Our results are in full agreement with those of Tieleman et al. (2005) in their comparative study on Neotropical birds, which shows that beside one general measure of the constitutive innate immune system (bactericidal activity of the blood), the reactivity of the NAb and the complement are constrained by the energetic turnover of the organism. Also, the number of heterophils and total WBCs, which are a general measure of the immune function (Semple et al. 2002; Blount et al. 2003; Nunn et al. 2003; Demas et al. 2011), significantly and negatively correlated with the metabolic rate, suggesting that the amount of resources invested in innate defence is constrained by the energetic expenditure of the organism.

The energetic cost of the production of differential and total WBCs, NAb and the complement has not been measured and is still debated, but is thought to be relatively low, at least during activation, in comparison with that of the adaptive immune defence. The low cost of activation of leukocytes, NAb and complement can be explained by the lack of a diversification process, such as that required for lymphocyte development, low rates of cell turnover when an immune response is not being mounted, and the small tissue mass accounted for by constitutive innate cells and proteins (Klasing and Leshchinsky 1999; Lee 2006). However, results on wild-living birds show the energetic limitation of different WBCs, NAb and the complement at the individual level (Hörak et al. 1998; Mauck et al. 2005; Bourgeon et al. 2010), supporting the prediction of the metabolic pace of life, namely that only species with a low metabolic rate can invest in energetically costly immunological defence. Thus, our study supports this view and contradicts the alternative assumption that species with a fast pace of life and high mass-corrected metabolic rate invest more in constitutive innate immunity because they are selected to sacrifice adaptive immunity and rely only on energetically less costly immune defence (Lee 2006).

Final conclusions

Our data support the hypothesis that fast-living species characterized by a high metabolic rate and fast embryonic growth invest relatively little in defence. Also, we found that the pre- and postnatal developmental periods have distinct effects on the constitutive immune function of adult birds. This advocates for further investigations on the potential adaptive role of developmental effects. The incubation period shows a positive and the fledging period a negative trend with immune parameters. Accordingly, we found that the relative length of the incubation period related to the total developmental period positively correlates with immune parameters. Thus, species with a long relative incubation period invested more in innate immunity, presumably because this part of the immune system

largely develops during the embryonic phase. These findings are interesting for the field of eco-immunology because they reveal complex associations between circulating immune cells, effectors of the innate immune system (i.e. NAb and complement) and life history traits.

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