

Constitutive immune defences correlate with life-history variables in tropical birds

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Summary

1. It has been suggested that immune defences are shaped by life history and ecology, but few general patterns have been described across species. We hypothesized that ‘fast’ life-history traits (e.g. short development times, large clutch sizes) would be associated with developmentally inexpensive immune defences, minimizing the resource demands of young animals’ immune systems during periods of rapid growth. Conversely, ‘slow’ life histories should be associated with well developed antibody-mediated defences, which are developmentally costly.

2. We therefore predicted that ‘fast-living’ species would exhibit higher levels of complement proteins, a component of non-specific innate defence, but lower levels of constitutive (‘natural’) antibodies. Additionally, we predicted that constitutive immune defences in general would be higher in species with ecological characteristics that might increase exposure to pathogens, such as open nests, omnivorous diets, gregariousness, and closed forested habitat.

3. Across 70 Neotropical bird species, we found a strongly positive relationship between incubation period and natural antibody levels in adult birds, suggesting that longer developmental times might allow the production of a more diverse and/or more reactive adaptive immune system. Complement activity was positively, although weakly, correlated with clutch size, providing some support for the hypothesis that faster-living species rely more on innate defences, such as complement. Unexpectedly, solitary species had higher natural antibody titres than species that frequently join flocks.

4. Our results suggest that, despite probably widespread differences in the intensity and diversity of pathogen exposure, species-level variation in constitutive immune defences is understandable within the context of life-history theory.

Key-words: immunology, incubation, life history, natural antibodies, tropical birds

Introduction

Recently ecologists have begun to search for explanations for immunological variation in the ecology and life histories of organisms (Tella, Scheuerlein & Ricklefs 2002; Schmid-Hempel 2003; Lee 2006). Life-history theory predicts that trade-offs should exist between investment in current reproduction vs. self-maintenance and survival (Stearns 1992). As immune defence is an important component of self-maintenance, it has been suggested that organisms with high reproductive rates (‘fast’ life histories; Promislow & Harvey 1990) should invest less in immune defence (Sheldon & Verhulst 1996; Rigby & Moret 2000). Ecologists’ appreciation of the complexity of the immune system is growing (Martin, Weil & Nelson 2006; Norris & Evans 2000), and the idea of a nexus

between immunology and life history has been extended (Ricklefs & Wikelski 2002): life history trade-offs are likely to influence not just how vigorously an organism defends itself, but which protective activities or ‘arms’ of the immune system are emphasized.

Here we present the first taxonomically broad, large-scale study relating multiple types of immune defence to life-history traits. We measured two types of immune defence, natural antibodies and complement activity, in adults of 70 species of Neotropical bird representing a range of life-history and ecological characteristics. These measures of immune defence are especially useful for studies of evolutionary questions because of their apparent low sensitivity to current infection status (Baumgarth *et al.* 1999; Matson, Klasing & Ricklefs 2005). Antibodies are a component of adaptive immune defence: ‘natural’ or constitutive antibodies provide a first line of defence against pathogen attack (Ochsenbein *et al.* 1999).

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Complement proteins are a constitutive component of the innate immune system and are involved in a catalytic cascade important in identifying and killing microbes (Nauta, Roos & Daha 2004). Complement activity is already detectable in 7-day-old quail embryos (Kai *et al.* 1985), while natural antibodies are produced by B1 cells, which have a prolonged developmental period in the bursa of Fabricius that extends well beyond hatching (Seto & Henderson 1968). We predicted that 'slow-living' species (Promislow & Harvey 1990) with relatively long developmental periods should exhibit stronger adaptive immune defences, such as natural antibodies, because in birds the diversity of B-cells is generated only during the development of the embryo and young chick (McCormack, Tjoelker & Thompson 1991). Conversely, we predicted that species with 'fast' life histories would rely more heavily on rapidly developed complement proteins.

In addition to life-history trade-offs, the evolution of immune defences is likely to be shaped by the frequency of pathogen exposure (Shudo & Iwasa 2001). Habitat, social or mating system, and feeding ecology, among many other variables, have the potential to change pathogen exposure and thus the selective pressures on immune defences (Moller *et al.* 2001; Semple, Cowlshaw & Bennett 2002). We predicted that avian species that engage in behaviours that could increase exposure to pathogens, such as flocking, building open nests that are exposed to vectors, and eating a wide variety of foods, would exhibit higher levels of both types of constitutive immune defences. Maintaining higher levels of constitutive defences could allow species with high pathogen-encounter rates to incur fewer costs associated with repeatedly mounting induced immune responses.

With this study, we begin testing the above predictions by examining relationships between constitutive immune indices and species-level life-history and ecological characteristics. We suggest that a significant portion of among-species variation in constitutive immune defences can be explained in the context of life history and ecology.

Methods

BIRD CAPTURE AND SAMPLING

During March–April 2004 and 2005, we captured 451 individuals of 70 bird species (1–50 individuals per species, Appendix S1 in Supplementary material; for taxonomic authorities see Monroe & Sibley 1993) in and around Gamboa, Panama (9°7' N, 79°42' W) using mist nets. Mist-netting was conducted during the breeding season, but the precise age and breeding status of individuals was unknown. Any apparent juveniles (based on gape colour and/or plumage) were excluded from the analyses. Within 15 min of capture we collected 50–100 µl of blood from each bird, from the brachial vein, in heparinized capillary tubes. Blood samples were kept on ice (1–10 h) and later centrifuged and the plasma collected. Plasma samples were stored frozen at –4 °C until analyses were performed. This work was carried out under permission from Princeton University's Animal Care and Use Committee (IACUC) and the Panamanian authorities, and adhered to recommended practices for the use of wild birds in research.

NATURAL ANTIBODY AND COMPLEMENT ACTIVITY

We estimated natural antibodies and complement activity in plasma following a published method (Matson *et al.* 2005). Briefly, plasma is serially diluted twofold with phosphate-buffered saline in a 96-well assay plate, and incubated with rabbit red blood cells (Hemostat Laboratories R59169, Dixon, CA, USA) for 90 min at 37 °C. Natural antibody titres and complement activity are scored as $-\log_2(D + 1)$, where D is the highest dilution at which agglutination (natural antibodies) or lysis (complement) of the red blood cells is observed. A standard was included on each plate (chicken plasma, *Gallus gallus*); between-plate variation was 14.7%.

LIFE-HISTORY AND ECOLOGICAL CHARACTERIZATION

Life-history and ecological variables included in the analyses were: clutch size; incubation and nestling periods; nest type (open or enclosed); nest height (low \leq 3 m, medium = 3–8 m, high \geq 8 m, or 'all'); body mass; habitat (forest-dependent or open/edge habitat species); diet (primarily granivore, frugivore, insectivore or omnivore); and flocking tendency (mostly solitary vs. obligate or facultative flocking). Life-history data were collected by locating nests and observing bird behaviour. We measured incubation period as the time from onset of incubation to hatching of the first egg. Most species began incubation on the evening the penultimate egg of a clutch was laid. Nestling period was the time from hatching of the first egg to the first departure from the nest. We categorized nests as open when they were open cups, platforms or scrapes on the ground. Enclosed nests were burrows, tree cavities, gourd- or pendant-shaped nests, and cavities in termitaria. Body masses were obtained from mist-netted birds at and near Gamboa.

For species for which we lacked some data, life-history and ecological values were obtained from a number of published sources (Skutch 1945, 1969, 1985; Willis 1974; Ehrlich, Dobkin & Wheye 1988; Ridgely & Gwynne 1989; Stiles & Skutch 1989; Karr 1990; Karr *et al.* 1990; Thiollay 1994; Brawn, Karr & Nichols 1995; Stotz *et al.* 1996; Geffen & Yom-Tov 2000; Jullien & Clobert 2000; Ahumada 2001; Buehler, Castillo & Brawn 2004; Pereira, Daily & Roughgarden 2004). When multiple sources were not consistent with one another, we used the value most often reported, or if there were only two sources, the value measured nearest our study site. The range of life history and ecological values included in this study are given in Appendix S2 (see Supplementary material).

STATISTICAL ANALYSES

Phylogenetic tree

We created a phylogenetic topology from published phylogenies that used molecular data to estimate relationships between species (Sibley & Ahlquist 1990; Burns 1998; Patten & Fugate 1998; Johnson & Clayton 2000; Klicka, Johnson & Lanyon 2000; Brumfield & Braun 2001; Grapputo *et al.* 2001; Irestedt *et al.* 2001; Johnson *et al.* 2001; Yuri & Mindell 2002; Burns, Hackett & Klein 2003; Carson & Spicer 2003; Ericson & Johansson 2003; Barker 2004; Barker *et al.* 2004; Chesser 2004; Cracraft *et al.* 2004; Irestedt *et al.* 2004b; Irestedt, Fjeldsa & Ericson 2004a; Ericson *et al.* 2005; Mann *et al.* 2006) (Fig. 1). We retained polytomies in the tree when node support was poor or information lacking. Branch lengths are not specified

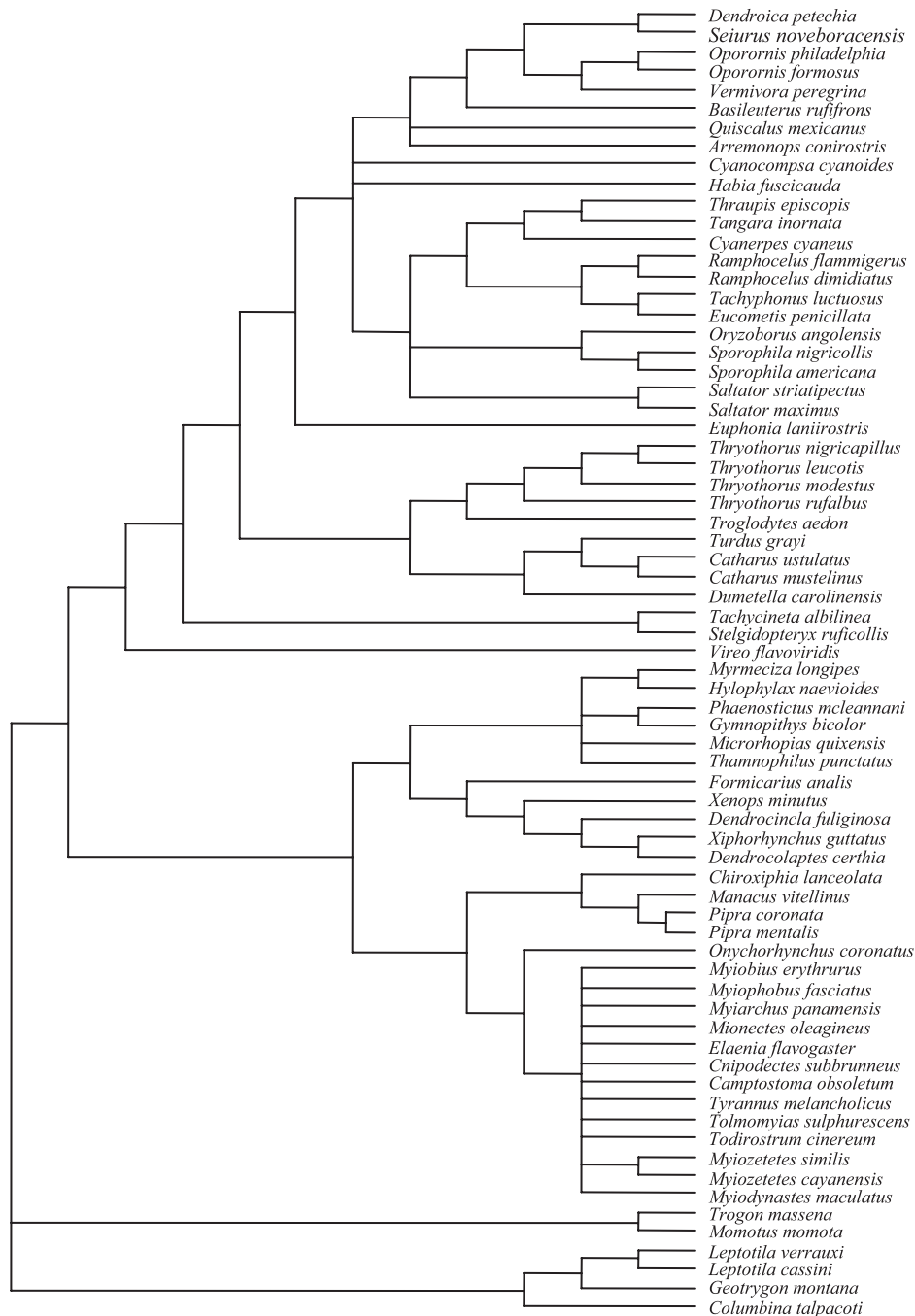


Fig. 1. Phylogenetic relationships of 70 tropical bird species used in the study.

in many of the phylogenies we used, so we conducted all analyses using an ultrametric tree (constructed in MESQUITE; Maddison & Maddison 1997), which assumes a molecular clock. We checked these results for sensitivity to changes in branch lengths and evolutionary model by comparing them with an analysis using a tree with all branch lengths = 1, which is equivalent to a punctuational model of evolution (Harvey & Pagel 1991).

Statistical models

When given in the text, all mean values are reported ± 1 SE. We log-transformed incubation and nestling periods, body mass and complement titres [$\log(\text{titre} + 0.5)$] to obtain distributions closer to the normal distribution. Species' mean antibody titres and complement activities were not correlated in our sample, and we analysed the two immune parameters

Table 1. *F* statistics and *P* values for independent variables predicting natural antibody titres and complement activity included in the best-fit phylogenetically informed models

Term	Natural antibodies	Complement activity
Clutch size		$F_{1,66} = 7.03, P = 0.01$
Incubation period	$F_{1,65} = 16.07, P < 0.001$	
Nestling period		
Body mass	$F_{1,65} = 3.56, P = 0.064$	
Habitat		
Diet		
Flocking	$F_{1,65} = 5.92, P = 0.018$	$F_{1,66} = 2.66, P = 0.11$
Nest type	$F_{1,65} = 3.14, P = 0.081$	
Nest location		

Terms not included in the best-fit models are listed without statistical values.

separately. For both dependent variables we report the results of a 'conventional' statistical analysis (assuming a star phylogeny; Felsenstein 1985), and an analysis based on the phylogeny shown in Fig. 1. We fit models by generalized least squares (GLS) using stepwise (both forward and backward) model selection according to Akaike's information criterion (AIC) under different assumptions (gradual vs. punctuated evolution; Brownian motion vs. constrained evolution). We incorporated phylogenetic information into the analyses using the 'phylogenetic generalized least squares' approach (Martins & Hansen 1997); in this approach the form of the covariance matrix is dependent on the phylogenetic tree and the model of evolution assumed. For each of the two dependent variables, we compared two phylogenetically informed models that assume different modes of evolution: Brownian motion, or 'random' evolution that is proportional to branch length (a proxy for time; Felsenstein 1985); and 'constrained' evolution, which is equivalent to stabilizing selection or an evolutionary constraint (Martins & Hansen 1997). We compared model fits using a likelihood ratio test (Pagel 1997). To obtain *F* statistics and *P* values for independent variables, we performed ANOVAs on the best-fit models selected by AIC, using marginal sums of squares (Table 1).

Some of the independent variables included in the best-fit models were significantly correlated with other variables (Appendix S3, see Supplementary material). While there is no ideal way to separate the effects of correlated predictors, we additionally fitted full models by GLS that included all nine independent variables, and tested the significance of each variable in the model after all other terms had been added, to verify that the variables selected by AIC contributed uniquely to the model's predictive power. The resulting 'full' models do not provide the best fit for the data because of the large number of non-significant independent variables included, but are provided as a verification of the results obtained by AIC (Appendix S4, see Supplementary material).

The equations and R^2 values describing the relationships shown in Figs 2 and 4 are the result of conventional bivariate linear regression, and are provided to show the quantitative relationships and explanatory power of individual significant

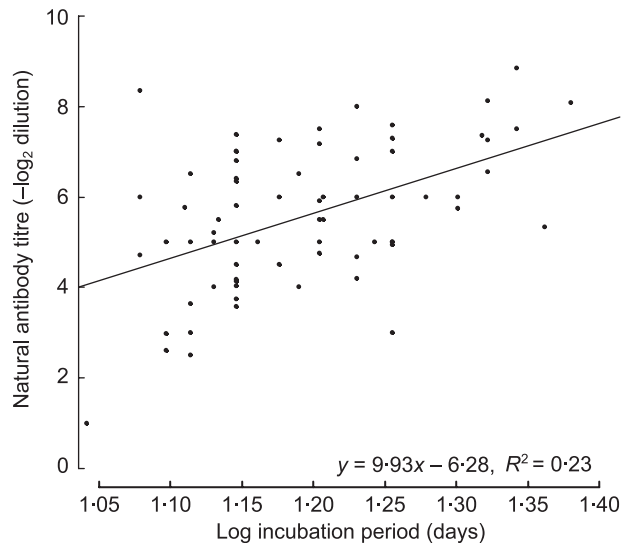


Fig. 2. Relationship between incubation period and estimates of natural antibody titre (measured as haemagglutination) across tropical bird species. Data points are species means; the line represents a conventional linear model fit.

independent variables. All analyses were carried out in R ver. 2.4.0 (R Development Core Team 2006) using packages APE (Paradis, Claude & Strimmer 2004) and MASS (Venables & Ripley 2002).

Results

NATURAL ANTIBODIES

Relationships between natural antibody titres and ecological and life-history variables were better explained by a phylogenetic model assuming 'constrained' evolution (Martins & Hansen 1997) than one assuming Brownian motion (likelihood ratio, LR, comparing best-fit models = 22.04, $P < 0.001$), although the results did not differ qualitatively. The constrained evolution model was also a better fit than the best-fit conventional analysis assuming a star phylogeny (LR = 4.11, $P = 0.043$). The alpha parameter, an index of the strength of the restraining evolutionary force, was optimized at a value of 0.51, or $\approx 6/h$, where h is total tree height (the sum of branch lengths from root to tip). This suggests weak phylogenetic signal in our data and/or a strong constraining force, according to simulation studies (Diniz-Filho 2004). Changing branch lengths did not affect results, therefore we present the results from the best-fit phylogenetically informed analysis based on an ultrametric tree in addition to results from the conventional analysis.

The strongest predictor of natural antibody titre was incubation period (Fig. 2); species with longer incubation periods had higher levels of circulating natural antibodies (conventional model, incubation term: $F_{1,65} = 21.96, P < 0.001$; phylogenetic analysis, incubation term: $F_{1,65} = 16.07, P < 0.001$). This relationship remained significant in the full model (after all eight other independent variables were included; Appendix S4). There was a non-significant trend for body

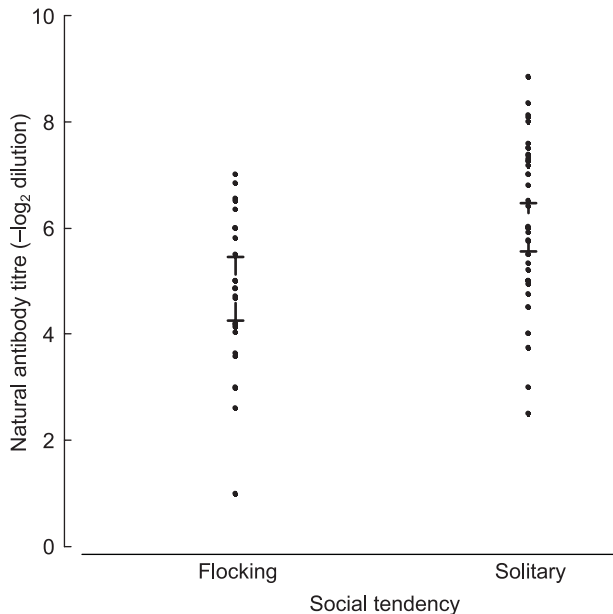


Fig. 3. Estimated natural antibody titres (haemagglutination) in flocking vs. solitary tropical bird species. Bars, 95% CI, data points are species means.

mass to be positively correlated with natural antibody titre in the best-fit models (conventional analysis, body mass term: $F_{1,65} = 3.04$, $P = 0.086$; phylogenetic analysis, $F_{1,65} = 3.56$, $P = 0.064$; linear regression $R^2 = 0.02$), but not in the full model including all independent variables (Appendix S4). The relationship between natural antibody titres and incubation period was not an artefact of a correlation between body mass and incubation period, as there was not a significant relationship between body mass and incubation period in our sample (see Appendix S3), and the relationship between incubation period and natural antibodies remained identical if the residuals of incubation on body mass were used in the model in place of unadjusted incubation period (data not shown).

Solitary species had higher natural antibody titres than flocking species (conventional analysis, flocking term: $F_{1,65} = 6.03$, $P = 0.017$; phylogenetic analysis, $F_{1,65} = 5.92$, $P = 0.018$; Fig. 3), although the range of antibody titres overlap extensively. Flocking tendency remained significant in the model when all other independent terms were included (Appendix S4).

COMPLEMENT ACTIVITY

Relationships between complement activity and life-history and ecological factors were explained marginally better by a 'constrained' evolution model (Martins & Hansen 1997) based on the phylogenetic relationships in Fig. 1, than by a conventional analysis (LR = 4.93, $P = 0.085$). A Brownian motion model provided a poor fit. The α parameter (Martins *et al.* 1997) was optimized at a value of 0.46, or $\sim 5.5/h$, where h is tree height, again reflecting weak phylogenetic signal and/or a strong stabilizing or constraining force (Diniz-Filho 2004). Again, changing branch lengths did not affect results, and we present the results obtained using an ultrametric tree.

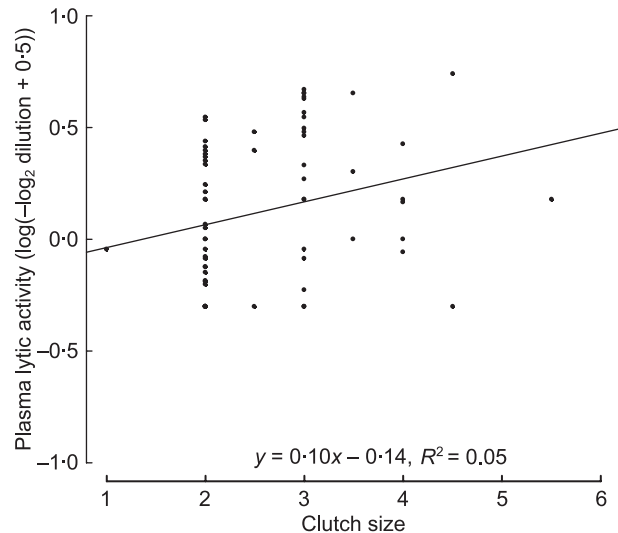


Fig. 4. Relationship between clutch size and complement haemolytic activity across tropical bird species. Data points are species means; the line represents a conventional linear model fit.

The only life-history or ecological variable that was a significant predictor of complement activity in both conventional and phylogenetic analyses was clutch size (conventional analysis, clutch size term: $F_{1,67} = 5.28$, $P = 0.025$; phylogenetic analysis, $F_{1,66} = 7.03$, $P = 0.01$), which showed a weak positive correlation with complement activity (Fig. 4). Clutch size remained a significant predictor of complement activity even when all other independent terms were first included in a model (Appendix S4).

Discussion

NATURAL ANTIBODIES AND INCUBATION PERIOD

We predicted that 'slow-living' species with long developmental periods should utilize antibody-mediated immune defences more heavily. Our results supported this prediction: we found incubation period to be the strongest predictor of natural antibody levels, explaining 23% of the variation present in adult birds. Higher natural antibody activities could result from an overall greater concentration of circulating natural antibodies, greater diversity of natural antibodies, or both. Mechanistically, a positive relationship between developmental period and antibody diversity in birds is expected, because antibody diversification is restricted to the developmental period in avian species (McCormack *et al.* 1991). Ricklefs (1992) found that blood parasite prevalence is related to incubation period across avian families (Ricklefs 1992), and speculated that this pattern might be related to greater time available for the development of antibody diversity. The development and maintenance of natural antibodies in birds is not entirely understood, but is thought to require stimulation of B-1 cells by auto-antigens (Parmentier *et al.* 2004; Haghighi *et al.* 2006). Species with longer incubation periods might develop higher levels of B-1 cell diversity, and with a

more diverse B-1 cell repertoire, a greater number of B-1 cell lines are likely to recognize and react to auto-antigens.

From a life history perspective, variation in natural antibody levels could be maintained by selective pressures acting directly on young birds and adults. In chicks, the specific antibody response takes several weeks post-hatch to fully develop, but maternal antibodies are protective for only 1–2 weeks following hatching (Klasing & Leshchinsky 1999). Incubation and nestling periods are positively related in general (Bosque & Bosque 1995; Franklin & Wilson 2003), as well as among the relatively small-bodied altricial species in our study (Appendix S3). In most altricial species, the majority of mass gain occurs in the nest as chicks fledge near adult mass (Remes & Martin 2002). Therefore species with longer incubation periods might gain greater benefits from high levels of constitutive antibody as nestlings, because slowly developing species will be without the protection of maternal antibodies, and lack a fully functional antibody response, for a greater proportion of their growing period (Klasing & Leshchinsky 1999). In contrast, a chick with a short (e.g. 10-day) nestling period is expected to be protected by maternal antibodies for the duration of its growth. Having high natural antibody titres could allow species with longer incubation and nestling periods to rely less often on expensive induced innate response during this critical time. However, data on natural antibody levels in young chicks will be required before this idea can be evaluated fully.

In adults of slow-living species, adaptive immunity could be favoured because species with longer life spans presumably face a greater number of infections overall, and are more likely to encounter the same pathogen multiple times. Although natural antibodies themselves do not confer immunological memory, natural antibody titres have been shown to be positively correlated with induced, specific antibody responses (Parmentier *et al.* 2004). Additionally, compared with innate immune defences, adaptive immunity tends to have lower costs of use in terms both of auto-immune damage and resource requirements (Klasing *et al.* 1999). Therefore high natural antibody titres might allow slow-living species to reduce the immediate costs of pathogen exposures.

The significance of developmental period for immune-related measures has now been described repeatedly in wild birds as well as in other organisms (Rolff, Van de Meutter & Stoks 2004; Palacios & Martin 2006). Tella *et al.* (2002) found a positive correlation between incubation period and T-cell-mediated inflammation in adult birds; and Moller *et al.* (2001) found a positive relationship between nestling period and nestling T- and B-cell responses among colonial and hole-nesters. Incubation period is also negatively correlated with blood parasite prevalence at the family level (Ricklefs 1992). The reported patterns suggest the existence of widespread general relationships between developmental period and immune defence, at least in birds.

NATURAL ANTIBODIES AND FLOCKING TENDENCY

Contrary to our predictions, flocking species had lower natural antibody levels than solitary species. These results appear

counterintuitive, because birds in flocks might be expected to experience higher infection rates by directly transmitted pathogens or parasites. The relationship between gregariousness and parasite infection or immunity has been examined in several study systems, and results are mixed. Both positive (Davies *et al.* 1991; Poiani 1992; Ezenwa 2004) and negative relationships (Watve & Sukumar 1995; Wilson *et al.* 2003; Kundu & Faulkes 2004) have been found between group living and immunity or parasitism. The explanation usually invoked for a negative relationship between group size and parasitism is the existence of a 'dilution' effect (Mooring & Hart 1992), for example, birds in flocks might experience decreased *per capita* vector biting rates. In the case of mixed-species flocks, such as those common in our data set, there might also be a dilution effect of biodiversity: in particular, transmission of parasites that are host species-specific might be slowed (Schmidt & Ostfeld 2001). Such a hypothesis will be testable with observational and experimental data in the future.

CLUTCH SIZE AND COMPLEMENT ACTIVITY

We hypothesized that species with 'fast' life histories should have immune defences characterized by an emphasis on developmentally inexpensive innate constitutive defences, such as complement (Lee 2006). In support of this hypothesis, we found a positive (although weak) correlation between clutch size and complement-mediated haemolytic activity across species. Parallel to our results, Tella *et al.* (2002) found a positive relationship between the phytohaemagglutinin response, which also involves aspects of innate immunity, and clutch size in southern hemisphere birds (when one outlier species was excluded), although this pattern was not found in their overall sample. The relationship between innate immune defence and clutch size, but not other life-history traits in our study, could be due to a particularly strong relationship between clutch size and parental effort: clutch size could be a reliable index of parental investment in current reproduction, as it should reflect the aggregate costs of egg laying, incubation, and feeding of chicks.

In humans, differences in complement levels can be genetically determined (Esparza-Gordillo *et al.* 2003; Esparza-Gordillo *et al.* 2004), but are also affected by infection and nutritional status (Scrimshaw & SanGiovanni 1997). In birds, complement levels appear to be little changed by an ongoing immune response (Matson *et al.* 2005), and higher levels of complement in species that produce large clutches could reflect evolved differences in complement production. However, it is also possible that complement levels could be an indirect indicator of other immune parameters that change with clutch size and affect the response to infection. Experiments inducing adaptive and innate immune responses across species with varying clutch sizes will help to clarify this issue.

Overall, our data suggest that there are general relationships between life history and immune defences across species. In birds, incubation period and clutch size appear to be important correlates of constitutive immune defences. Among ecological factors, we found sociality to be significantly

correlated with constitutive natural antibody levels. The species in our data set were mostly passerines, and some species were represented by a small number of individuals; additional relationships between immunity and life history might come to light as a greater number of both individuals and species are sampled. The number of studies demonstrating life history–immunology relationships within species is growing rapidly (Ardia 2005; Martin, Hasselquist & Wikelski 2006). Now we are beginning to discover that larger-scale patterns in immunological variation across multiple species can be explained using an evolutionary and ecological approach.

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Supplementary material

The following supplementary material is available for this article:

Appendix S1. Species and sample sizes used in measurements of the immune response.

Appendix S2. Summary of life history and ecological traits used in this study.

Appendix S3. Correlation coefficients between independent variables used in the analyses.

Appendix S4. Results of generalized least squared analyses including all independent variables.

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