doi:10.1111/j.1420-9101.2008.01503.x

Immune-challenged house wren broods differ in the relative strengths of their responses among different axes of the immune system

A. M. FORSMAN, L. A. VOGEL, S. K. SAKALUK, J. L. GRINDSTAFF[1] & C. F. THOMPSON

Department of Biological Sciences, Illinois State University, Normal, IL, USA

Keywords:

bactericidal activity;
ecological immunology;
house wren;
humoral immunity;
phytohaemagglutinin;
Troglodytes aedon.

Abstract

Single components of the immune system are widely used to assess immune function in free-living vertebrates. However, as different immunological components are triggered by different types of threats and may be regulated independently, there is little reason to assume that they should respond similarly if challenged. We investigated whether three commonly assayed immune responses, cutaneous immune activity (phytohaemagglutinin assay), antibody response (tetanus toxoid immunization), and plasma bactericidal activity (*Escherichia coli* killing) are positively related in nestling house wrens (*Troglodytes aedon*). Multivariate analysis revealed significant differences in overall immune responsiveness among broods (i.e. nests), primarily attributable to differences in plasma bactericidal activity. Among broods, humoral immune response was negatively related to cutaneous immune activity and positively related to plasma bactericidal activity. We found no significant relationships among these measures of immunity among individual nestlings within broods. Our results suggest that different broods (i.e. families) invest differentially in the various branches of the immune system. Further study is needed to characterize the roles of maternal, genetic and environmental effects on the expression of this physiological bias.

Introduction

Immunocompetence of individuals in wild populations is frequently assessed with a single immune assay, but reliance on one component of immunity to measure overall strength and efficacy of the immune system can be misleading (Adamo, 2004). Apparent immunosuppression, as assessed by one immune assay, may actually reflect concurrent physiological investment in a second, negatively correlated, unmeasured response (Braude *et al.*, 1999; Adamo, 2004; Martin *et al.*, 2006c). Individuals may be selected to invest differentially in various components of the immune system (Adamo, 2004; Martin *et al.*, 2006b), resulting in an underlying bias towards one type of immune response over another, regardless of previous antigen exposure. This bias in immunological investment may be caused by genetic or environmental factors that affect the ontogeny of correlated immune responses, or it may be a result of temporary immunoredistribution (*sensu* Braude *et al.*, 1999) of immune cells from one tissue type to another mediated by hormones (Dhabhar *et al.*, 1995; Dhabhar & McEwen, 1996; Dhabhar, 1998).

Alternatively, activation of one component of the immune system may have antagonistic effects on the activation and strength of other types of responses, resulting in trade-offs within the immune system (Martin *et al.*, 2006b). As a result, the magnitude of a response to one immunological challenge may be modulated by ongoing immune responses when individuals are exposed to multiple challenges over a short period of time. In this case, activation of one component, such as humoral immunity, may limit the expression of another, such as cutaneous immune activity, in response to a subsequent challenge, such as a phytohaemagglutinin (PHA) injection. Navarro *et al.* (2003), for example, found that house sparrows (*Passer domesticus*) with ongoing *Haemoproteus* infections exhibited weaker immune

Correspondence: Anna M. Forsman, Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY, 14853, USA.
Tel.: +1 607 254 4306; fax: +1 607 255 8088;
e-mail: amf226@cornell.edu
[1]Present address: Department of Zoology, Oklahoma State University, Stillwater, OK, USA.

responses to PHA challenge than uninfected birds. Thus, a weak response to a single immune assay may be misinterpreted as diminished immunocompetence if the response is attenuated by previous infection (Adamo, 2004). Assessing only a single component of the immune system may, therefore, lead to spurious conclusions regarding overall immune capacity.

The PHA skin test (Smits *et al.*, 1999), which measures cutaneous immune activity, and the quantification of antibody production in response to immunization, which measures humoral immunity (Fairbrother *et al.*, 2004), are frequently used to assess immunocompetence. Studies in free-living birds comparing humoral response and cutaneous immune activity provide support for the existence of a full range of relationships among different components of the immune system, positive (Møller *et al.*, 2001), negative (Gonzalez *et al.*, 1999) and non-significant (Blount *et al.*, 2003; Faivre *et al.*, 2003). Selection studies in domestic chickens (*Gallus g. domesticus*) have also shown variable relationships among immune responses within individuals (e.g. Kean *et al.*, 1994). However, as the majority of such studies have either been conducted under controlled aviary conditions or across individuals, there is little available information regarding the relationship among contemporaneous immune responses of individual, free-living birds.

We therefore asked the question: Do nestling house wrens (*Troglodytes aedon*) within and among broods respond similarly to three, contemporaneously applied immune challenges? To our knowledge this is the first study to examine the relationships among immune responses measured with three frequently used immune assays (PHA skin test, *in vivo* antibody production assay and plasma bactericidal assay) in the same individuals in a free-living population of birds under natural conditions.

Materials and methods

For this study we used 13 broods with a total of 49 nestlings from a nestbox-breeding population of house wrens on the Mackinaw study site (Eckerle & Thompson, 2006) in McLean County, Illinois (40°40′N, 88°53′W) in June–August 2006. For each nestling, we assessed three different components of immune activity, with all nestlings receiving the immunological challenges in the same order. We stimulated the humoral branch of the adaptive immune system by immunization with tetanus toxoid, an inactivated form of the neurotoxin tetanospasmin produced by the bacterium *Clostridium tetani*. We immunized nestlings by intraperitoneal injection on brood-day 7 (the first egg of the clutch hatches on brood-day 0) with 100 μL of commercially available, alum-precipitated tetanus toxoid (Cat no. 202773; Iowa Vet Supply, Iowa Falls, IA, USA). Approximately 50 μL of blood were collected from the brachial vein immediately prior to injection and 6 days post-injection (brood-day 13) to quantify pre- and post-injection relative antibody concentrations. After return from the field site each day, we centrifuged chilled (*c.* 4 °C) blood samples to separate plasma for use in antibody and bactericidal assays. Relative antibody concentrations were quantified by enzyme-linked immunosorbent assay (ELISA) using 96-well plates coated with tetanus toxoid in phosphate-buffered saline (PBS; 1 : 5 dilution) to capture only those antibodies with specific binding capacity to the injected antigen. We added one plasma sample to each tetanus-coated well at a 1 : 20 dilution with ELISA wash buffer (1x PBS; 1% bovine serum albumin, BSA, Fisher Scientific (Pittsburg, PA, USA), BP1605; 0.05% Tween-20, Fisher Scientific, BP337). Two aliquots of plasma were analysed for each nestling. We removed unassociated reagents with wash buffer and blocked any unbound well surface with BSA to avoid nonspecific antibody interference. To detect nestling antibodies, we used a horseradish peroxidase-conjugated secondary antibody (1 : 1000 dilution; anti-bird IgG, Bethyl Laboratories, Montgomery, TX, USA, A140-110P) that cross-reacts with house wren immunoglobulin (A.M. Forsman, unpublished data). We included chicken plasma, which contains antibodies that recognize tetanus toxoid, as a standard (1 : 80 dilution; Sigma-Aldrich, St. Louis, MO, USA, C5405) on all ELISA plates, and report relative antibody concentrations (post minus pre) as percent of standard (Viney *et al.*, 2005).

Cutaneous immune activity was induced in nestlings by a standardized intradermal injection in the left wing-web of 50 μL of PHA (5 mg mL^{-1}; Sigma-Aldrich, L8754) dissolved in sterile PBS (Smits *et al.*, 1999) on brood-day 12. PHA, a plant-derived mitogen, stimulates the recruitment to the site of injection of leucocytes involved in both adaptive and innate immune responses, producing a measurable tissue swelling in the wing-web (McCorkle *et al.*, 1980; Martin *et al.*, 2006a). We used a digital thickness gauge (Mitutoyo, Aurora, IL, USA; no. 547-500) to measure wing-web thickness (mean of three measures) prior to and c. 24 h after PHA injection. Post-injection swelling was measured prior to blood collection from the left brachial vein. Change in wing-web thickness was used as a measure of generalized immune cell activity.

We measured bactericidal activity of nestling plasma, which encompasses multiple components of the innate immune system, including natural antibodies, complement proteins, and lysozyme (Matson *et al.*, 2006). On the day of collection (brood-day 13), 5 μL of fresh plasma were incubated with approximately 200 colony-forming units of *E. coli* (American Type Culture Collection, strain 8739) at 41 °C for 45 min following Matson *et al.* (2006). Control samples of bacteria were plated without the addition of plasma. Samples were plated in duplicate on tryptic soy agar plates and stored overnight in an incubator at 37 °C to allow colony formation by surviving bacteria. The following day we counted visible *E. coli*

colonies and calculated the mean number of surviving colonies for the duplicates. The numbers of colonies from control samples were used to calculate percentage of total bacteria killed for each nestling sample.

We analysed the data using one-way multivariate analysis of variance (MANOVA, SAS Institute, Inc., 2002) to test for differences among broods in overall immune responsiveness and the relationships between the three dependent variables (PHA response, antibody production and bactericidal activity). Immune response data from 49 individual nestlings were used in this analysis. Brood identity was incorporated in the MANOVA model as the main fixed effect (13 different broods). All three dependent variables met the assumption of equal variance; however, antibody production data had to be square-root transformed to meet the assumption of normality (Kolmogorov–Smirnov; $P = 0.87$), but the bactericidal activity data did not (K-S; $P = 0.02$). Although no transformation succeeded in normalizing bactericidal activity, MANOVA is robust to departures from normality, especially when using Pillai's trace (Scheiner, 2001). To test for correlations between the dependent immune variables among nestlings within broods, we used linear regression analyses of individual nestling responses ($n = 49$). As individual nestlings did not represent independent observations, we calculated residual immune response of each nestling from its brood mean to control for genetic, maternal, and common environment effects associated with nest of origin (i.e. brood effect). The residual regression analyses allowed us to investigate relationships between dependent variables among individual nestlings as opposed to among broods. A univariate mixed model approach would have required us to assign one or more immune parameters as independent variables; however, we have no *a priori* reason to assume that immune responses are independent or that any one response may have a causative effect on any other response.

Results

We used MANOVA and residual analyses to partition correlations among the three immune variables into among-brood and within-brood components. Nest of origin significantly affected overall nestling immune responsiveness (MANOVA: $F_{36,108} = 4.14$, $P < 0.0001$). Two significant axes of variation in immune response were detected among broods. The first significant canonical variate (Eigenvalue = 6.092, $P < 0.0001$) explained 71.4% of the overall variation in immune response among broods and was primarily a function of bactericidal activity of plasma samples (range = 32.9–98.7% killing) and, to a lesser extent, antibody response to tetanus toxoid immunization (range = 0–52.6% of standard; Table 1). The standardized canonical coefficients for bactericidal activity and antibody response were of the same sign indicating a weak positive relationship between these two measures of immunity across broods (Fig. 1a). The second significant canonical variate (Eigenvalue = 2.207, $P = 0.0001$) was mostly a function of antibody response and PHA response (range = 0.05–1.21 mm; Table 1), and explained 25.9% of the overall variation in immune response among broods. PHA response and antibody production were of opposite signs (Table 1), indicating a negative relationship between these two immune responses among broods (Fig. 1c). Bactericidal activity and PHA response (Fig. 1b) vary mostly along separate axes of variation (Table 1); therefore, any bivariate relationship between these responses is weak (Fig. 1b).

There were no significant univariate correlations between any of the measures of immune responsiveness among the 49 individual nestlings within the 13 broods (residual correlations; PHA vs. tetanus, $r = 0.092$, $P = 0.531$; PHA vs. bactericidal, $r = 0.218$, $P = 0.133$; tetanus vs. bactericidal, $r = -0.227$, $P = 0.117$).

Discussion

Mean antibody production in response to tetanus toxoid was positively related to plasma bactericidal activity among broods, indicating that broods with higher mean humoral immunocompetence also had enhanced mean innate immunity. However, this relationship should be interpreted with caution as bactericidal activity was by far the largest contributor to canonical variate 1, suggesting that this response is not strongly correlated with antibody and PHA responses among broods. We also found that nestling humoral immunity was negatively related to mean PHA response among broods. Thus,

Table 1 MANOVA summary statistics for PHA response, bactericidal activity, and antibody response to tetanus toxoid immunization in nestling house wrens.

					Standardized canonical coefficients		
Source	Variance accounted	d.f.	*F*	*P*	PHA response	Bactericidal activity	Tetanus response
Canonical variate 1	71.36%	36, 101.18	5.89	<0.0001*	−0.1309	2.2112	0.6499
Canonical variate 2	25.85%	22, 70	3.16	0.0001*	0.7977	0.1743	−1.1093
Canonical variate 3	2.79%	10, 36	0.99	0.4661	1.0042	−0.5738	0.6881

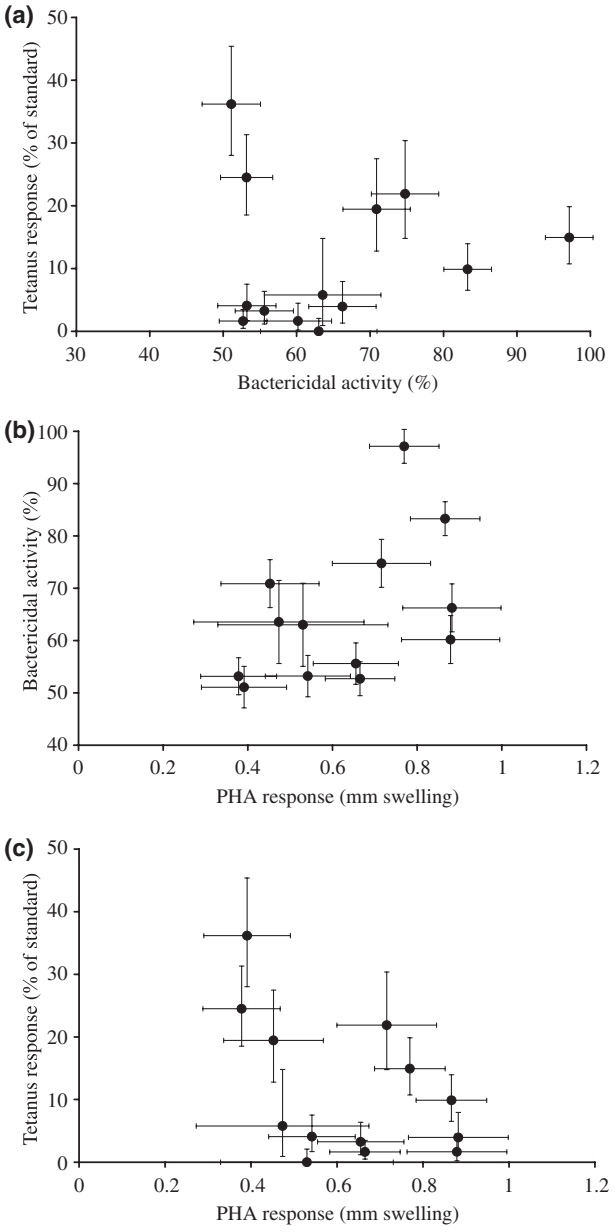

Fig. 1 Bivariate least squares means by brood for (a) bacterial killing capacity of plasma and tetanus toxoid-specific antibody production, (b) phytohaemagglutinin (PHA) response and bacterial killing capacity of plasma and (c) PHA response and tetanus toxoid-specific antibody production in nestling house wrens. Error bars represent standard errors of means.

broods mounting more robust mean humoral immune responses also expressed weaker mean cutaneous immune activity, suggesting that some broods may be biased towards one type of response over another. These results may reflect differential immune investment resulting in an immunological bias among broods mediated by offspring genotype, maternal effects, or nest environment. We found no evidence that measures of cutaneous immune activity, humoral immunity, or plasma bactericidal activity are either positively or negatively correlated among individual house wren nestlings within broods. Thus, both among broods and within broods, our data do not support the assumption that all components of the immune system are positively correlated. These results are consistent with a previous study that found limited evidence for covariation among immunological parameters in barn owls (*Tyto alba*; Roulin *et al.*, 2007).

The significant effect of nest of origin suggests that there may a heritable component to humoral immunity and cutaneous immune cell activity. Selection studies in poultry have demonstrated the heritability of immune responsiveness by producing divergent lines of high- and low-responding individuals to PHA challenge (e.g. Sundaresan *et al.*, 2005) and antibody-stimulating antigens (e.g. Shivakumar & Kumar, 2005). Studies of free-living species indicate great variability in immunological heritability, but the balance of the evidence suggests that humoral responsiveness may have higher heritability than cutaneous immune cell activity in response to PHA stimulation (Cucco *et al.*, 2006). Although a few studies have found significant additive genetic variance in PHA response (Brinkhof *et al.*, 1999; Ardia & Rice, 2006; Cichoń *et al.*, 2006), the majority have not (Christe *et al.*, 2000; Tella *et al.*, 2000; Soler *et al.*, 2003; Kilpimaa *et al.*, 2005; Cucco *et al.*, 2006; Roulin *et al.*, 2007). Ardia & Rice (2006) found that heritability of PHA responsiveness varies geographically, suggesting that under some conditions environmental and maternal effects may have a greater influence on immunological phenotype. Nutritional studies indicate that diet is a significant factor influencing immune responsiveness (Tengerdy *et al.*, 1990; Alonso-Alvarez & Tella, 2001). Humoral immunity (e.g. Roulin *et al.*, 2000; Sivaraman *et al.*, 2005), on the other hand, may be less influenced by nutritional status (Råberg *et al.*, 2003) and more dependent on the particular antigen used to assess responsiveness (Råberg *et al.*, 2003; Kilpimaa *et al.*, 2005). Only a cross-fostering experiment (e.g. Soler *et al.*, 2003) would allow us to investigate the influence of environmental vs. genetic and maternal effects on nestling immunity in this system.

In conclusion, our results are consistent with the hypothesis that different broods (i.e. families) are biased to invest differentially in the branches of their immune system. Further study is needed to characterize the roles of maternal, genetic, and environmental effects on the expression of this physiological bias.

Acknowledgments

We thank Dr Steven A. Juliano for extensive statistical and editorial advice, Ebony Murrell for help with graphics, Karen M. Thompson for editorial help, the 2006 Wren Crew for field assistance, and the ParkLands

Foundation and Butler-Sears families for access to their properties. This research protocol was approved by the Illinois State University animal care committee (IACUC #17-2003). Our research was supported by NSF grant IBN-0316580 to CFT, LAV and SKS. AMF was partially supported by NSF GK-12 grant 0086354 and a Phi Sigma (Beta Lambda Chapter) Weigel Research Grant. JLG was partially supported by a postdoctoral fellowship from the Program of Excellence in Neuroscience and Behavior at Illinois State University.

References

Adamo, S.A. 2004. How should behavioural ecologists interpret measurements of immunity? *Anim. Behav.* **68**: 1443–1449.

Alonso-Alvarez, C. & Tella, J.L. 2001. Effects of experimental food restriction and body-mass changes on the avian T-cell-mediated immune response. *Can. J. Zool.* **79**: 101–105.

Ardia, D.R. & Rice, E.B. 2006. Variation in heritability of immune function in the tree swallow. *Evol. Ecol.* **20**: 491–500.

Blount, J.D., Houston, D.C., Møller, A.P. & Wright, J. 2003. Do individual branches of immune defence correlate? A comparative case study of scavenging and non-scavenging birds. *Oikos* **102**: 340–350.

Braude, S., Tang-Martinez, Z. & Taylor, G.T. 1999. Stress, testosterone, and the immunoredistribution hypothesis. *Behav. Ecol.* **10**: 345–350.

Brinkhof, M.W.G., Heeb, P., Kölliker, M. & Richner, H. 1999. Immunocompetence of nestling great tits in relation to rearing environment and parentage. *Proc. R. Soc. Lond. B* **266**: 2315–2322.

Christe, P., Møller, A.P., Saino, N. & De Lope, F. 2000. Genetic and environmental components of phenotypic variation in immune response and body size of a colonial bird, *Delichon urbica* (the house martin). *Heredity* **85**: 75–83.

Cichoń, M., Sendecka, J. & Gustafsson, L. 2006. Genetic and environmental variation in immune response of collared flycatcher nestlings. *J. Evol. Biol.* **19**: 1701–1706.

Cucco, M., Malacarne, G., Ottonelli, R. & Patrone, M. 2006. Repeatability of cell-mediated and innate immunity, and other fitness-related traits, in the grey partridge. *Can. J. Zool.* **84**: 72–79.

Dhabhar, F.S. 1998. Stress-induced enhancement of cell-mediated immunity. *Ann. N. Y. Acad. Sci.* **840**: 359–372.

Dhabhar, F.S. & McEwen, B.S. 1996. Stress-induced enhancement of antigen-specific cell-mediated immunity. *J. Immunol.* **156**: 2608–2615.

Dhabhar, F.S., Miller, A.H., McEwen, B.S. & Spencer, R.L. 1995. Effects of stress on immune cell distribution – dynamics and hormonal mechanisms. *J. Immunol.* **154**: 5511–5527.

Eckerle, K.P. & Thompson, C.F. 2006. Mate choice in house wrens: nest cavities trump male characteristics. *Behaviour* **143**: 253–271.

Fairbrother, A., Smits, J. & Grasman, K.A. 2004. Avian immunotoxicology. *J. Tox. Env. Health B: Cri. Rev.* **7**: 105–137.

Faivre, B., Preault, M., Salvadori, F., Thery, M., Gaillard, M. & Cezilly, F. 2003. Bill colour and immunocompetence in the European blackbird. *Anim. Behav.* **65**: 1125–1131.

Gonzalez, G., Sorci, G., Møller, A.P., Ninni, P., Haussy, C. & De Lope, F. 1999. Immunocompetence and condition-dependent sexual advertisement in male house sparrows (*Passer domesticus*). *J. Anim. Ecol.* **68**: 1225–1234.

Kean, R.P., Cahaner, A., Freeman, A.E. & Lamont, S.J. 1994. Direct and correlated responses to multitrait, divergent selection for immunocompetence. *Poult. Sci.* **73**: 18–32.

Kilpimaa, J., Van de Casteele, T., Jokinen, I., Mappes, J. & Alatalo, R.V. 2005. Genetic and environmental variation in antibody and T-cell mediated responses in the great tit. *Evolution* **59**: 2483–2489.

Martin, L.B. II, Han, P., Lewittes, J., Kuhlman, J.R., Klasing, K.C. & Wikelski, M. 2006a. Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. *Funct. Ecol.* **20**: 290–299.

Martin, L.B. II, Weil, Z.M., Kuhlman, J.R. & Nelson, R.J. 2006b. Trade-offs within the immune systems of female white-footed mice, *Peromyscus leucopus*. *Funct. Ecol.* **20**: 630–636.

Martin, L.B. II, Weil, Z.M. & Nelson, R.J. 2006c. Refining approaches and diversifying directions in ecoimmunology. *Integr. Comp. Biol.* **46**: 1030–1039.

Matson, K.D., Tieleman, B.I. & Klasing, K.C. 2006. Capture stress and the bactericidal competence of blood and plasma in five species of tropical birds. *Physiol. Biochem. Zool.* **79**: 556–564.

McCorkle, G., Olah, I. & Glick, B. 1980. The morphology of the phytohemagglutinin-induced cell response in the chicken's wattle. *Poult. Sci.* **59**: 616–623.

Møller, A.P., Merino, S., Brown, C.R. & Robertson, R.J. 2001. Immune defense and host sociality: a comparative study of swallows and martins. *Am. Nat.* **158**: 136–145.

Navarro, C., Marzal, A., de Lope, F. & Møller, A.P. 2003. Dynamics of an immune response in house sparrows *Passer domesticus* in relation to time of day, body condition and blood parasite infection. *Oikos* **101**: 291–298.

Råberg, L., Stjernman, M. & Hasselquist, D. 2003. Immune responsiveness in adult blue tits: heritability and effects of nutritional status during ontogeny. *Oecologia* **136**: 360–364.

Roulin, A., Jungi, T.W., Pfister, H. & Dijkstra, C. 2000. Female barn owls (*Tyto alba*) advertise good genes. *Proc. R. Soc. Lond. B* **267**: 937–941.

Roulin, A., Christe, P., Dijkstra, C., Ducrest, A.L. & Jungi, T.W. 2007. Origin-related, environmental, sex, and age determinants of immunocompetence, susceptibility to ectoparasites, and disease symptoms in the barn owl. *Biol. J. Linn. Soc.* **90**: 703–718.

SAS Institute, Inc. 2002. *SAS Version 9.1*. SAS Institute Inc., Cary, NC.

Scheiner, S. 2001. MANOVA: multiple response variables and multispecies interactions. In: *Design and Analysis of Ecological Experiments*, 2nd edn. (S. M. Scheiner & J. Gurevitch, eds), pp. 99–115. Oxford University Press, New York, USA.

Shivakumar, B.M. & Kumar, S. 2005. Influence of divergent selection for response to sheep RBC on immunoresponsiveness to Newcastle disease vaccine in white leghorn chickens. *Indian J. Anim. Sci.* **75**: 1365–1367.

Sivaraman, G.K., Kumar, S., Saxena, V.K., Singh, N.S. & Shivakumar, B.M. 2005. Genetics of immunocompetent traits in a synthetic broiler dam line. *Brit. Poultry Sci.* **46**: 169–174.

Smits, J.E., Bortolotti, G.R. & Tella, J.L. 1999. Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. *Funct. Ecol.* **13**: 567–572.

Soler, J.J., Moreno, J. & Potti, J. 2003. Environmental, genetic and maternal components of immunocompetence of nestling

pied flycatchers from a cross-fostering study. *Evol. Ecol. Res.* **5**: 259–272.

Sundaresan, N.R., Ahmed, K.A., Saxena, V.K., Sastry, K.V.H., Saxena, M., Pramod, A.B., Nath, M., Singh, K.B., Rasool, T.J., DevRoy, A.K. & Singh, R. 2005. Differential expression of inducible nitric oxide synthase and cytokine rnRNA in chicken lines divergent for cutaneous hypersensitivity response. *Vet. Immunol. Immunopath.* **108**: 373–385.

Tella, J.L., Bortolotti, G.R., Forero, M.G. & Dawson, R.D. 2000. Environmental and genetic variation in T-cell-mediated immune response of fledgling American kestrels. *Oecologia* **123**: 453–459.

Tengerdy, R.P., Lacetera, N.G. & Nockels, C.F. 1990. Effect of beta-carotene on disease protection and humoral immunity in chickens. *Avian Dis.* **34**: 848–854.

Viney, M.E., Riley, E.M. & Buchanan, K.L. 2005. Optimal immune responses: immunocompetence revisited. *Trends Ecol. Evol.* **20**: 665–669.

Received 15 November 2007; revised 7 January 2008; accepted 8 January 2008