

Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoeological technique

L. B. MARTIN II,*†‡ P. HAN,* J. LEWITTES,* J. R. KUHLMAN,‡
K. C. KLASING§ and M. WIKELSKI*

*Princeton University, Department of Ecology and Evolutionary Biology, Princeton, NJ 08544, ‡The Ohio State University, Department of Psychology, Columbus OH 43210, and §University of California Davis, Department of Animal Science, Davis, CA 95616, USA

Summary

1. Measurements of phytohemagglutinin (PHA)-induced tissue swelling are arguably the most popular surrogates for immunocompetence in wild birds. It is largely unresolved, however, whether the basic assumption underlying these measures is valid, particularly whether more swelling represents a 'better' or 'stronger' cell-mediated immune response.
2. In this study we took a first step towards such validation by characterizing immune cell infiltration over time into the wing-webs (patagia) of PHA-challenged House Sparrows (*Passer domesticus*). Relative to saline-injected wing-webs, PHA-injected wing-webs displayed intensive infiltration of many immune cell types, including basophils, eosinophils, heterophils, lymphocytes, macrophages and thrombocytes. The abundance of most of these cell types changed over the course of the swelling response (6–48 h post-injection). Peak infiltration time varied depending on cell type. At several time points, significant correlations between the numbers of some cell types (particularly heterophils) and the degree of swelling were detected.
3. Together, these data indicate that PHA-induced swelling is related to heightened immune cell activity in House Sparrows, but also that the PHA swelling response in this species is dynamic and involves both innate and adaptive components of the immune system. We thus caution against interpreting larger swellings as 'greater cell-mediated immunocompetence', given the complex nature of this immune response.

Key-words: cell-mediated, DTH, immune, *Passer*, trade-off

Functional Ecology (2006) **20**, 290–299
doi: 10.1111/j.1365-2435.2006.01094.x

Introduction

Over the past decade, the emerging science of immunoeology has made substantial contributions to the fields of sexual selection (Zuk & Johnsen 1998, 2000); life-history evolution (Tella, Scheuerlein, & Ricklefs 2002); environmental toxicology (Smits & Williams 1999); and behaviour (Soler *et al.* 1999). Although several techniques have been used to measure immune function in natural contexts, the phytohemagglutinin (PHA) skin-swelling test has become one of the most popular because of its simplicity (Smits, Bortolotti, & Tella 1999), amenability to field work (Ardia 2005a; Martin *et al.* 2004), and history of use in domestic fowl (Stadecker *et al.* 1977; McCorkle, Olah, & Glick 1980). Phytohemagglutinin-induced immune responses are typically measured *in vivo*

by injecting PHA subcutaneously (ideally dissolved in pyrogen-free saline) and quantifying concomitant swelling at the site of injection over time (Smits *et al.* 1999). In most immunoeological studies, swelling is measured 24 h post-injection (Zuk & Johnsen 1998; Soler *et al.* 1999). Skin swellings at this time are interpreted as indexes of cell-mediated immunocompetence largely based on four observations from domestic animals: (i) leukocytes infiltrate and/or proliferate in injected tissue after challenge (Stadecker *et al.* 1977; McCorkle *et al.* 1980); (ii) swelling responses are blunted in thymectomized birds (Goto *et al.* 1978); (iii) PHA predominantly stimulates T-lymphocytes but not B-lymphocytes *in vitro* (Elgert 1996); and (iv) delayed-type hypersensitivity responses, including PHA swelling, are sometimes correlated with an animal's capacity to control certain types of infection (most often viral: Turk 1967).

Although it is clear from these studies that skin-swelling responses to PHA represent immunological

phenomena, it is not clear what swelling means in a functional sense, and thus how differences in swelling responses should be interpreted. Some studies on wild bird species reported that large swelling responses to PHA were predictive of adult survival probability (Gonzalez *et al.* 1999; Soler *et al.* 1999). For this reason, PHA-induced tissue swelling has been espoused as a reliable surrogate for generic disease resistance (e.g. immuno-competence). Still, many studies have found that PHA-swelling responses are labile and thus might be better characterized as indicators of the general health of individuals (Duffy & Ball 2002; Ardia 2005b) or surrogates for investments in immune activity at certain points in animals' lives (Tella *et al.* 2002; Martin *et al.* 2000). Finally, some immunologists interpret large swelling responses as indications of allergy or unrestrained local inflammatory activity (Elgert 1996), suggesting that more swelling is not always better.

JUST WHAT IS PHA?

Phytohemagglutinin is a compound generated by the red kidney bean (*Phaseolus vulgaris*) that is believed to serve as a defence against herbivory. In humans and other vertebrates, consumption of large volumes of raw kidney beans can cause inflammation of the intestinal tract. Phytohemagglutinin is a large molecule (molecular weight 138 000) with a long history of use in immunology, dating back to its original role as an agglutination agent for vertebrate erythrocytes (Naspitz & Richter 1968). Like other lectins, PHA is mitogenic to many vertebrate cell types including (but not limited to) T lymphocytes (Bonforte *et al.* 1972; Elgert 1996). However, stimulation of T-lymphocyte proliferation by PHA is different from most other antigens. Most importantly, PHA does not require antigen presentation or major histocompatibility complex costimulation by professional antigen-presenting cells (e.g. macrophages) to induce mitogenesis, like most antigens do; as many as 30% of T-cell lines are responsive to PHA, far more than a typical antigen (Elgert 1996). For these reasons, non-ecologists typically do not recognize the PHA-swelling response as solely a T-cell mediated hypersensitivity response. Instead, they typically call it cutaneous basophilic hypersensitivity (Stadecker *et al.* 1977; McCorkle *et al.* 1980).

This name is derived from the two distinct phases that occur during PHA skin swellings in domestic fowl. The first phase involves exudation of plasma from surrounding vascular tissue and edema in the injected region, which occurs within 6–12 h after injection and is driven by local innate cell populations (largely basophils and macrophages) activated by PHA-stimulated CD4+ T-cells (Elgert 1996). The second phase includes an infiltration of additional PHA-sensitive T-lymphocytes and occurs around 24 h post-injection (Stadecker *et al.* 1977; Goto *et al.* 1978; McCorkle *et al.* 1980). From these data, it is clear that activated T-cells are involved in the swelling response; they secrete some of the cytokines that recruit and activate effector

cells. However, other leukocytes (particularly basophils, heterophils and macrophages) actually effect most of the vasodilation, edema and inflammation of tissue; moreover, these cells secrete additional cytokines, which can promote further infiltration by, or proliferation of, additional leukocytes (Stadecker *et al.* 1977; Goto *et al.* 1978; McCorkle *et al.* 1980; Elgert 1996). Even the most commonly cited study identifying the PHA-swelling response as a cell-mediated immune response supports a more complicated immunological cascade underlying swellings than is typically espoused. A few chickens thymectomized early in life (and hence possessing few if any circulating T cells) mounted measurable but reduced swelling responses to PHA relative to controls (Goto *et al.* 1978). Moreover, *in vitro* work indicates that other cell types in addition to T cells must be important mediators of swelling, as lymphocyte proliferation using PHA is often not related to *in vivo* tissue swelling in the same bird (Bayyari *et al.* 1997). Finally, it is clear from genetic studies that both innate and adaptive components of the immune system are involved in the swelling response, and each may be independently regulated (Taylor *et al.* 1987; Cheng & Lamont 1988).

To gain a better understanding of what PHA swellings represent in a functional sense in passerine birds, we investigated the cellular processes underlying the swelling response in the House Sparrow (*Passer domesticus*). Our goals were to (i) describe the cellular infiltration processes involved in the swelling response; and (ii) determine if and when cell infiltration was correlated to swelling. Although our approach cannot indicate whether PHA-induced swellings are predictive of disease resistance, it can provide a more detailed understanding of the currently most favoured technique in immunoeology. Indeed, although it is clear that measures of PHA swelling can be informative of the immunological state of animals in a coarse sense, we expect that more detailed characterizations of this response could provide greater insight into the functional significance of larger or smaller skin swellings.

Materials and methods

STUDY ANIMALS

Thirty birds were caught from the Princeton Shopping Centre, Princeton, NJ, USA (40°21' N, 74°40' W) in late September 2003, when birds had completed post-nuptial or post-juvenile moults. This time of year was chosen because breeding state and moult can affect PHA-induced swelling responses in House Sparrows (Martin *et al.* 2004; Martin 2005; Greenman, Martin, & Hau 2005). At capture, each bird was identified as male or female and adult or juvenile, based on plumage (Summers-Smith 1988), marked with numbered aluminium leg bands, and checked to verify a lack of flight feather or body moult. Birds in moult were released on capture and were not included in the study. After capture, birds were transported to the roof of Guyot

Hall at Princeton University and housed in pairs in cages. These cages were kept in a location where birds would be exposed to ambient temperatures (September mean temperature = 18 °C) and photoperiod (12.25 h light), but sheltered from precipitation and winds. Birds remained in these conditions for 2 days prior to injections. During this adjustment period and throughout the experiment, birds were provided millet, thistle seeds and water for *ad libitum* consumption; they remained in these cages for the duration of the study except for short periods (<30 min) when they were challenged with PHA. After biopsies of injected wing tissue were taken (see below), birds were released back to sites of capture.

IMMUNE CHALLENGE

For PHA challenges, the right wing-web of each bird was injected with 0.1 ml of 1 mg ml⁻¹ PHA-P (Sigma L 9017, St. Louis, MO, USA) dissolved in cell-culture grade (e.g. pyrogen-free) phosphate-buffered saline (PBS: Martin *et al.* 2004; Lee, Martin, & Wikelski 2005). Then as a control the left wing-web of the same bird was injected moments later with 0.1 ml of cell culture-grade PBS. Wing-web swelling was calculated as thickness of the web at the time of biopsy minus thickness prior to injection using a pressure-sensitive spessimeter (Teclock pocket thickness gauge, Model SI-510; Smits *et al.* 1999). The same individual (L.B.M.) performed all injections and swelling measurements between 10.00 and 11.00 h; thickness measures and biopsies were taken 6–48 h later.

HISTOLOGY

To characterize cellular infiltration into injected wing-web tissue, biopsies of injected tissue were taken from birds at 6 ($n = 8$), 12 ($n = 8$), 24 ($n = 8$) or 48 ($n = 6$) h (± 15 min) after injections. This sampling protocol was used because it was clear from other studies in domestic fowl that cellular infiltration varies over time (Stadecker *et al.* 1977; Goto *et al.* 1978). Just prior to each biopsy, each bird was lightly anaesthetized with an isoflurane–air mixture, then placed on its back on a sterile, flat laboratory bench. The right wing was then opened to its full extent and held steady by one researcher (P.H.), and the patagial skin was swabbed with 70% alcohol, which also displaced feathers from the area to be biopsied. A small tissue sample was then taken from the centre of the most inflamed part of the wing-web with a 1.0-mm-diameter tissue corer (FST 18035-01, Fine Science Tools, Foster City, CA, USA) by a second researcher (L.B.M.) applying direct, constant pressure followed by gentle rotation of the corer. Samples were immediately fixed in 10% buffered formalin (Fisher Scientific 23-305510, Pittsburgh, PA, USA). The corer and laboratory bench were cleaned thoroughly with 70% ethyl alcohol, and the process was repeated on the left wing. Birds were then taken to the site of capture and released. The biopsy procedure never caused bleeding at the sampling site. This biopsy procedure is relatively benign, and comparable

in effect to the blood-sampling protocol used by many avian field endocrinologists. Multiple birds have been recaptured by us from the wild several months post-biopsy and showed healed wounds at this time, although some scarring of tissue at the site of biopsy was present.

After tissue collection, samples were sent to Idexx Laboratories (Sacramento, CA, USA) where they were embedded in paraffin, sectioned, stained with haematoxylin and eosin, and mounted on slides following standard procedures. The orientation of these tissue samples (identity of the dorsal vs ventral surface) could not be determined due to the small tissue sample size. Three sections were therefore made randomly through tissue; persons preparing these tissues were blind to the goals of the study. Cell infiltration was quantified in PHA- and saline-injected tissues using NEUROLUCIDA software (ver. 6) on a Nikon Eclipse E800 microscope. Infiltration was characterized by generating a rectangular box (of fixed width, 15 μ m) that traversed the entire section of tissue, then counting all the immune cells inside this box or in contact with the perimeter of the box (at $\times 600$ magnification). To calculate cell densities, absolute cell counts were divided by the area of this box. For each individual bird, all three sections of tissue were counted (for both PHA- and saline-challenged wings), and averages of these three subsamples were used in statistical analyses.

To count cells in fixed tissue samples, leukocytes were differentiated into five categories: lymphocytes; eosinophils or heterophils (morphological differences were not distinct in our samples, and hereafter these cells are referred to as ‘heterophils’); macrophage; basophils; and thrombocytes (Campbell 1995). Lymphocytes were identified as circular cells with large, purple nuclei that filled almost the entire cell. Heterophils were identified by their reddish-staining cytoplasmic granules. Macrophages were identified by their large size and kidney-shaped nucleus. Basophils were identified by their dark purple-staining, circular cytoplasmic granules (small lymphocytes were distinguished from basophils by their lack of deeply staining granules). Finally, thrombocytes were identified as small, purple-staining cells with large, diffuse-staining nuclei and little cytoplasm. Cell counts after histological preparation were conducted by one person blind to the identity of samples (J.R.K.). All methods described in this study were approved by the Princeton University Institutional Animal Care and Use Committee (protocol number 1492) and comply with current US laws regarding animal research.

STATISTICAL ANALYSES

The distributions of variables were tested for normality using one-sample Kolmogorov–Smirnov tests, and variance equality was verified using Levene’s tests. Only the number of macrophages in the infiltration characterization was non-normally distributed, so these data were square-root transformed prior to analysis. Differences in swelling and cellular infiltration over time

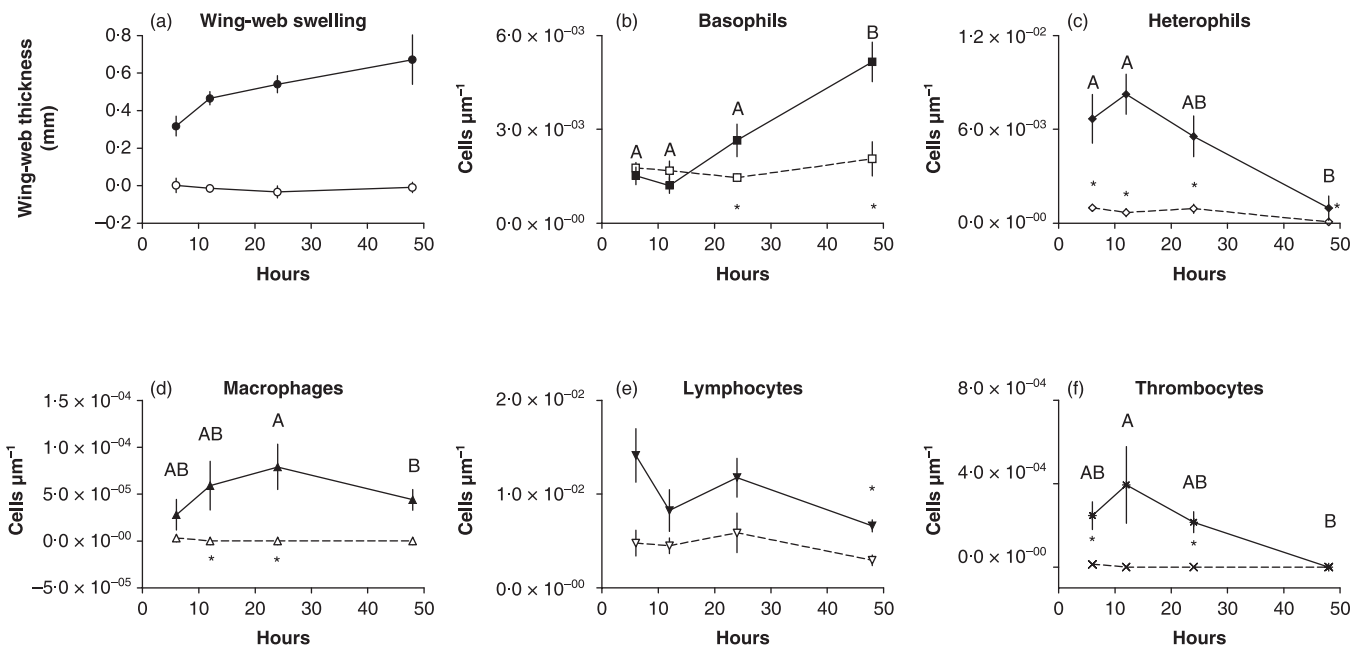


Fig. 1. Comparison of swelling and cellular infiltration into phytohemagglutinin (PHA)-injected (filled) or saline-injected (open) wing-webs of temperate House Sparrows at 6, 12, 24 and 48 h post-injection. Letters indicate significant differences among time points within PHA-challenged birds. Asterisks indicate significant differences between PHA- and saline-injected wings at respective time points.

and between saline- and PHA-injected wings were identified using either ANOVA or independent-sample *t*-tests. Relationships between swelling and cellular infiltrates were identified using Spearman's rank correlation analysis. For all analyses we used SPSS ver. 10 and set $\alpha = 0.05$.

Results

CHARACTERIZATION OF CELLULAR INFILTRATION DURING THE SWELLING RESPONSE

The amount of swelling induced by PHA was different when measured at four distinct times in four distinct groups of House Sparrows (6, 12, 24 and 48 h post-injection: $F_{3,29} = 2.60$, $P = 0.05$; Fig. 1a), but was not affected by the sex or age (juvenile vs adult) of individuals (sex: $F_{1,29} = 0.25$, $P = 0.62$; age: $F_{1,29} = 0.08$, $P = 0.78$). Saline injections induced no significant swelling, as indicated by the lack of difference in wing-web thickness among House Sparrow groups ($F_{3,29} = 0.58$, $P = 0.715$). Wing-web thickness of PHA-injected wings was significantly larger than saline-injected wings in birds from all four time points (6 h: $t_{14} = 4.80$, $P < 0.001$; 12 h: $t_{14} = 12.15$, $P < 0.001$; 24 h: $t_{14} = 9.25$, $P < 0.001$; 48 h: $t_{10} = 5.05$, $P < 0.001$; Fig. 1a).

In PHA-injected wing-webs the number of basophils ($F_{3,29} = 10.25$, $P < 0.001$), heterophils ($F_{3,29} = 3.46$, $P = 0.02$) and macrophages ($F_{3,29} = 3.80$, $P = 0.012$) was different among birds from different time intervals, but neither cell type was affected by sex or age (basophils: sex, $F_{1,29} = 1.33$, $P = 0.26$, age, $F_{1,29} = 0.82$, $P = 0.37$;

heterophils: sex, $F_{1,29} = 0.83$, $P = 0.37$, age, $F_{1,29} = 0.28$, $P = 0.60$; macrophages: sex, $F_{1,29} = 1.49$, $P = 0.23$, age, $F_{1,29} = 3.44$, $P = 0.08$, tendency for more macrophages in adults); Fig. 1(b–d). The number of lymphocytes infiltrating PHA-injected wing-web tissue did not change over time when all birds were included in the statistical comparison ($F_{3,29} = 2.00$, $P = 0.12$). However, the age (but not sex) of birds appeared to affect the number of lymphocytes in samples. When we compared the number of lymphocytes in samples between age classes irrespective of time, we found that juveniles had fewer lymphocytes in tissue post-challenge ($F_{1,28} = 5.74$, $P = 0.02$; one bird was not assigned an age class at capture and was not included in this comparison). Given this age effect, we removed juveniles from the initial comparison and again compared lymphocyte infiltration over time. When only adults were included in the model, lymphocyte numbers did not vary among groups of sparrows ($F_{3,19} = 2.52$, $P = 0.10$; Fig. 1e); we did not have sufficient numbers of juveniles within each group to make the complementary comparison. Thrombocyte numbers also did not vary over time ($F_{1,28} = 2.38$, $P = 0.07$), but again age (but not sex) appeared to influence thrombocyte numbers. As with lymphocytes, thrombocytes were more abundant in adult birds ($F_{1,28} = 4.07$, $P = 0.05$) and, when we compared thrombocyte numbers among time points (adults only), groups of House Sparrows were significantly different ($F_{3,19} = 3.42$, $P = 0.04$; Fig. 1f).

We then compared infiltration of each cell type in PHA- vs saline-injected wings; asterisks in Fig. 1 indicate where differences were detected. Because age affected numbers of lymphocytes and thrombocytes (see above), only adult birds were used in comparisons of these cell types.

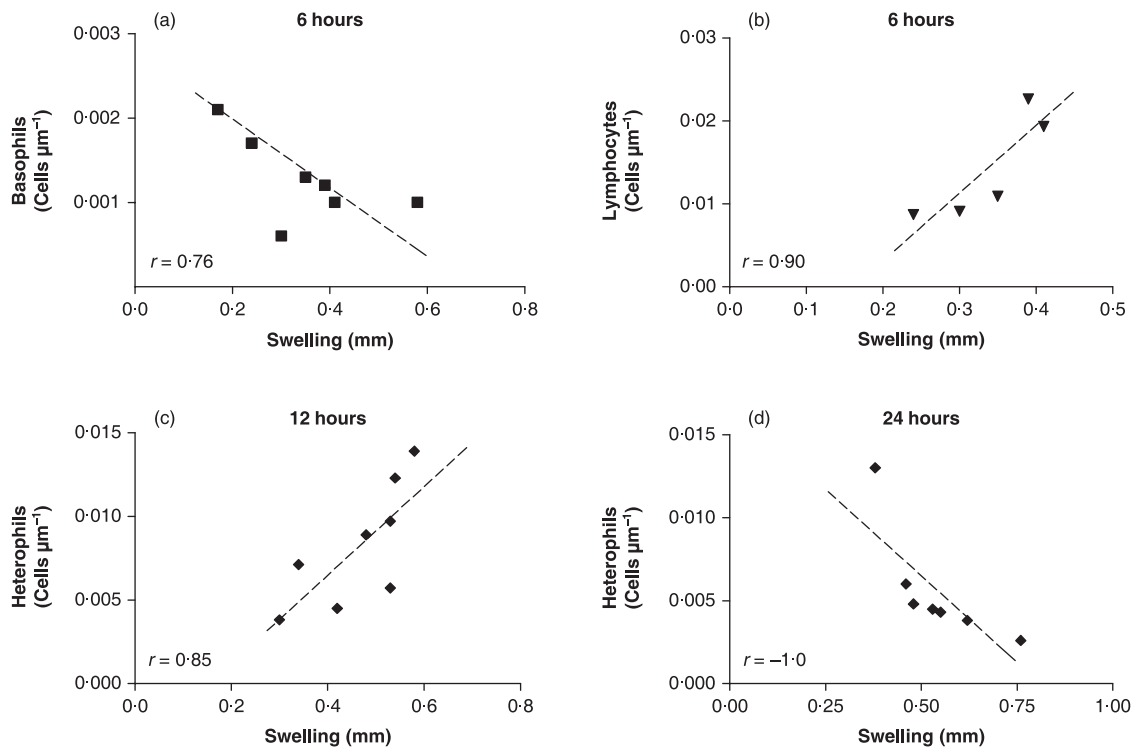


Fig. 2. Significant correlations between wing-web swelling and (a) basophil infiltration at 6 h; (b) lymphocyte infiltration at 6 h; heterophil infiltration at (c) 12; (d) 24 h. Numbers are Spearman's rank correlation coefficients; dashed line is from linear regression.

At 6 h, numbers of heterophils ($t_{12} = 3.13$, $P = 0.01$) and thrombocytes ($t_6 = 2.63$, $P = 0.04$), but not basophils ($t_{12} = -0.67$, $P = 0.51$) or macrophages ($t_{12} = 1.40$, $P = 0.18$) differed between saline- and PHA-injected wings; lymphocytes also tended to be higher in PHA-injected wings, but this difference was not significant ($t_6 = 2.34$, $P = 0.06$). At 12 h, heterophils ($t_{14} = 5.80$, $P < 0.001$) and macrophages ($t_{14} = 3.20$, $P = 0.01$), but not basophils ($t_{14} = -1.19$, $P = 0.26$), thrombocytes ($t_6 = 2.15$, $P = 0.08$) or lymphocytes ($t_6 = 1.58$, $P = 0.17$), differed in numbers between saline- and PHA-injected wings. At 24 h, numbers of heterophils ($t_{12} = 3.47$, $P = 0.005$), macrophages ($t_{12} = 4.63$, $P = 0.01$), basophils ($t_{12} = 2.25$, $P = 0.04$) and thrombocytes ($t_{10} = 4.32$, $P = 0.002$), but not lymphocytes ($t_{10} = 2.00$, $P = 0.07$), differed between saline- and PHA-injected wings. At 48 h, heterophils ($t_8 = 2.45$, $P = 0.04$), basophils ($t_8 = 3.42$, $P = 0.009$) and lymphocytes ($t_6 = 3.85$, $P = 0.008$), but not macrophages ($t_8 = 0.8$, $P = 0.45$), differed between saline- and PHA-injected wings; no thrombocytes were present in saline-injected tissue at this time point.

CORRELATIONS BETWEEN CELLULAR INFILTRATION AND SWELLING

We detected four significant relationships between swelling and infiltration at different time points. At 6 h there was a negative correlation between number of basophils and swelling ($r = -0.762$, $P = 0.03$; Fig. 2a) and a positive correlation between the number of lymphocytes and swelling (when only adults were

included: $r = 0.90$, $P = 0.04$; Fig. 2b). At 12 h there was a significant positive correlation between number of heterophils and swelling ($r = 0.85$, $P = 0.01$; Fig. 2c), but at 24 h there was a significant negative correlation between the same cell type and swelling ($r = -1.0$, $P < 0.001$; Fig. 2d). None of the 16 other correlations was significant.

Discussion

The PHA-swelling technique has routinely been used to quantify vertebrate immune responses in various ecological contexts. From Table 1, it is clear that PHA-induced swelling is traded off with other physiological functions, indicated by the fact that PHA swellings are weaker when other costly activities, such as moult or breeding, are concurrent. Phytohemagglutinin swelling is often positively related to the expression of sexually selected traits, with individuals possessing larger ornaments typically exhibiting larger swellings. Phytohemagglutinin swellings can be markers of individual quality, but can become compromised in highly inbred birds or birds maintained on low-quality diets. Various hormones can influence PHA-swelling responses, most notably testosterone, which has been interpreted as support for the immunocompetence-handicap hypothesis for sexual selection. In addition, there exists inter-population and interspecific variation in PHA-swelling responses indicative of complementarities between animals' life histories and the functioning of their immune systems. Finally, PHA-swelling responses have

Table 1. Variable uses of phytohemagglutinin (*in vivo*) in avian immunoeology

Effects/species	Treatment/trait	Effect on PHA response	Reference
Apparent trade-offs			
<i>Tachycineta bicolor</i>	Increased brood size, high latitude Intermediate latitude	Decreased None	Ardia (2005a)
<i>Falco tinnunculus</i>	Increased brood size (artificial)	Decreased	Fargallo <i>et al.</i> (2002)
<i>Passer domesticus</i>	Induction of breeding state	Decreased	Greenman <i>et al.</i> (2005)
<i>P. domesticus</i>	Moult	Decreased	Martin (2005)
<i>Ficedula hypoleuca</i>	Increased brood size	Decreased	Moreno <i>et al.</i> (1999)
<i>Hirundo rustica</i>	Decreased brood size	Increased	Saino <i>et al.</i> (2002)
<i>Pica pica</i>	Induced clutch replacement	Decreased	Sorci <i>et al.</i> (1997)
<i>Spheniscus magellanicus</i>	Large colony size	Decreased	Tella <i>et al.</i> (2001)
Condition dependence			
<i>Taeniopygia guttata</i>	Aviary-bred <i>vs</i> recent wild-capture	Increased	Ewenson <i>et al.</i> (2001)
<i>T. guttata</i>	Chasing by experimenter	Decreased	Ewenson <i>et al.</i> (2003)
<i>T. guttata</i>	Increasing age	Decreased	Hausmann <i>et al.</i> (2006)
<i>T. bicolor</i>			
<i>Oceanodroma leucorhoa</i>			
<i>T. bicolor</i>	Cold weather	Decreased	Lifjeld <i>et al.</i> (2002)
<i>Spheniscus magellanicus</i>	Males <i>vs</i> females	Decreased	Moreno <i>et al.</i> (2001)
<i>P. domesticus</i>	Night	Increased	Navarro <i>et al.</i> (2003)
	Haemoproteus infection	Decreased	
	Poor body condition	Decreased	
Environmental contamination			
<i>Sialia mexicana</i>	Living in pesticide polluted area	None	Fair <i>et al.</i> (2003)
<i>Myiarchus cinerascens</i>			
<i>Sialia sialis</i>	Pesticide exposure	None	Mayne <i>et al.</i> (2004)
<i>T. bicolor</i>			
<i>T. bicolor</i>	Mine tailings exposure	None	Smits <i>et al.</i> (2000)
Hormone effects			
<i>Junco hyemalis</i>	Testosterone	Decreased	Casto <i>et al.</i> (2001)
	Testosterone (captive birds)	None	
<i>Coturnix chinensis</i>	Leptin	Increased	Lohmus <i>et al.</i> (2004)
<i>P. domesticus</i>	Corticosterone	Decreased	Martin <i>et al.</i> (2005)
<i>Coturnix coturnix</i>	Melatonin	Increased	Moore & Siopes (2003)
<i>S. sialis</i>	Testosterone (in eggs)	Decreased	Navara <i>et al.</i> (2005)
<i>Melospiza melodia</i>	Testosterone	Decreased	Owen-Ashley <i>et al.</i> (2004)
	5 α -dihydrotestosterone	None	
	Dehydroepiandrosterone	None	
Parasitization			
<i>Delichon urbina</i>	Swallow bug (<i>Oeciacus vicarius</i>)	Decreased	Christe <i>et al.</i> (2000)
<i>Coturnix xoturnix japonica</i>	<i>Mycoplasma synoviae</i> or sheep red blood cells	Decreased	Fair <i>et al.</i> (1999)
<i>Gallus gallus</i>	<i>Ascaridia galli</i>	Decreased	Johnsen & Zuk (1999)
<i>Geospiza fuliginosa</i>	Large island size (increased parasite pressure)	Decreased	Lindstrom <i>et al.</i> (2004)
<i>P. pica</i>	Brood parasite (<i>Clamator glandarius</i>)	None	Soler <i>et al.</i> (1999)
Resource availability			
<i>Larus cachimans</i>	Food restriction	Decreased	Alonso-Alvarez & Tella (2001)
<i>Parus caeruleus</i>	Methionine supplementation	Increased	Brommer (2004)
<i>Colinus virginianus</i>	Low protein diet	Decreased	Lochmiller <i>et al.</i> (1993)
<i>H. rustica</i>	Food supplementation	Increased	Saino <i>et al.</i> (1997)
<i>P. pica</i>	Methionine supplementation	Increased	Soler <i>et al.</i> (2003)
Quality indicator			
<i>T. bicolor</i>	Early breeding	Increased	Ardia (2005b)
<i>Delichon urbica</i>	High body condition	Increased	Christe <i>et al.</i> (2001)
<i>Sturnus vulgaris</i>	High song rate and song bout length	Increased	Duffy & Ball (2002)
<i>P. domesticus</i>	Sexual ornament (badge) size, April November	Decreased Increased	Gonzalez <i>et al.</i> (1999)
<i>Luscinia svecica</i>	Siring by extra-pair male	Increased	Johnsen <i>et al.</i> (2000)
<i>Pygoscelis antarctica</i>	Late breeding	Decreased	Moreno <i>et al.</i> (1999)
<i>Melospiza melodia</i>	High inbreeding coefficient	Decreased	Reid <i>et al.</i> (2003)
<i>Oenanthe leucura</i>	Robust sexual behaviour	Increased	Soler <i>et al.</i> (1999)
<i>Larosterna inca</i>	Parental sexual character expression robust	Increased	Velando <i>et al.</i> (2001)
<i>G. gallus</i>	Large sexual ornaments	Increased	Zuk & Johnsen (1998)
<i>G. gallus</i>	Dominant males with large sexual ornaments Subordinate males with large sexual ornaments	Increased Decreased	Zuk & Johnsen (2000)

Table 1. Continued

Effects/species	Treatment/trait	Effect on PHA response	Reference
Heritability			
<i>Parus major</i>	Nest of rearing vs birth	Genetic and environmental	Brinkhof <i>et al.</i> (1999)
<i>Falco sparverius</i>	Nest of rearing vs birth	Environmental over genetic	Tella <i>et al.</i> (2000b)
Species–population variability			
Multiple	Relationships to life-history characters	Increase (with body size)	Tella <i>et al.</i> (2002)
Multiple	Relationships to life-history characters	Increase (with clutch size)	Martin <i>et al.</i> (2000)
<i>P. domesticus</i>	Equatorial vs temperate	Increased (but seasonal)	Martin <i>et al.</i> (2004)
Costs of response			
<i>P. major</i>	Injection on food intake	Increased	Barbosa & Moreno (2004)
<i>P. major</i>	Injection on nestling growth	None	Horak <i>et al.</i> (2000)
<i>P. domesticus</i>	Injection on energy expenditure	Increased	Martin <i>et al.</i> (2003)
<i>P. domesticus</i>	Injection on feather growth (moult)	Decreased	Martin (2005)
<i>D. urbina</i>	Injection on heat-shock proteins	None	Merino <i>et al.</i> (1999)

been shown to have some genetic basis, or at least fixed components, although environmental effects are common and occasionally extensive.

Despite this abundance of functional information, we still know little about what is mechanistically involved in a PHA-induced swelling response. Here we have shown that swellings in House Sparrows are underlain by dynamic cellular processes involving multiple leukocyte populations, much like in domestic fowl (Stadecker *et al.* 1977). Most leukocytes, except heterophils and lymphocytes, increased over time in injected tissue. Heterophils appeared early then disappeared, but lymphocytes were abundant throughout much of the response. This pattern is common to delayed-type hypersensitivity reactions in general; only after local T-cells are stimulated to produce cytokines (for the recruitment of other leukocytes) do most immune cell types appear. One surprising result in House Sparrows compared with domestic fowl was the early arrival of lymphocytes very soon after PHA challenge. This appearance is probably due to rapid infiltration, not proliferation, because avian T cells typically take around 72 h to divide (Stadecker *et al.* 1977). Currently, we are unable to explain why this difference between avian taxa exists. Heterophils, however, arrived within a similar timeframe as in chickens, but disappeared rapidly presumably because they quickly degranulated and died (subsequently causing swelling: Stadecker *et al.* 1977). The decline in lymphocytes over time, on the other hand, may be due to apoptosis of B cells and some T cells that did not receive appropriate signals to promote proliferation. Without these signals, many lymphocytes probably entered terminal chemokine-induced apoptotic pathways (Elgert 1996).

Such conjectures are consistent with the correlation analyses between swelling and infiltration in sparrows that we conducted in this study. In other words, the significant positive relationship between swelling and lymphocytes at 6 h probably represents a high degree of cytokine secretion by these cell types at this time,

leading to increased infiltration and subsequent degranulation of other effector cells (e.g. heterophils, macrophages and basophils) shortly thereafter. The positive relationship between heterophils and swelling at 12 h supports this hypothesis. The negative correlation between heterophils and swelling at 24 h may have occurred because cell numbers decreased as they degranulated and produced inflammation. We were surprised that the only relationship between basophils and the swelling response was a negative one at 6 h. One possible explanation is that local basophils mediate the early swelling response (via degranulation), but are not as active later in the response, which could explain the increase in basophil infiltration over time as well as the absence of correlations with swelling during these time points. Overall, though, for all cell types it remains unclear if they are the cause or consequence of swelling.

(RE)INTERPRETING THE RESULTS OF STUDIES USING PHA IN WILD BIRDS

Our data shed new light on the functional interpretation of PHA-induced wing-web swellings as measures of immunocompetence by characterizing the cellular underpinnings of a PHA response. Measurements of PHA-induced swellings alone can be meaningful indicators of immune activity in ecological contexts (Table 1). We suggest, however, that the practice of injecting PHA just to ‘measure immunocompetence’ should be done with caution. This approach can easily lead to *ad hoc* interpretation of data that might impair progress in understanding the immune system in natural contexts. For instance, both Martin *et al.* (2001) and Tella *et al.* (2002) performed meta-analyses on the PHA-swelling responses of many bird species, both finding relationships – but different ones – between swelling responses and some life-history characters. Could our results here help reconcile inconsistencies between these studies? Mechanistically, differences in PHA-swelling responses among species or populations could be achieved by different groups possessing distinct

leukocyte populations differentially sensitive to PHA. In domestic fowl, numerous classes of macrophages exist, some with the ability to attract lymphocytes to sites of infection, and others with a greater propensity for secreting inflammatory cytokines that induce systemic acute-phase responses (Dietert *et al.* 1991). In the presence of PHA, some macrophages release lymphocyte-activating factor, which causes chemotaxis and proliferation of T-lymphocytes (Meltzer & Oppenheim 1977). Although such immunophysiological differences would not jeopardize the generalizations made by the aforementioned authors (and may even strengthen them), a lack of consideration of the functional interpretation of immune measures themselves potentially ignores more meaningful biological phenomena.

Specifically, it is possible that some birds, particularly those with long developmental periods, may possess macrophage populations (or other effector cells such as basophils) that are more effective in integrating the adaptive arm of the immune system. This difference could ultimately lead to uniqueness in terms of tissue swelling and/or infiltration among species, but such uniqueness may or may not be related to life-history variation. Such differences could occur because the immune system was shaped by the life history of the species; they could arise as a consequence of differential parasite exposure during ontogeny or adult life; or some combination of both. In support of this possibility for House Sparrows, we found that corticosterone, the major avian glucocorticoid, suppressed PHA swelling in one population that lays large clutches over a short breeding season, but the same hormone did not affect swelling in another that lays smaller clutches over a larger number of breeding events (Martin *et al.* 2005). Perhaps this result is a consequence of differential leukocyte profiles maintained in each population. Regardless, this study highlights the importance of taking into account the complex, often redundant nature of the vertebrate immune system, and avoiding simple approaches to measuring 'immunocompetence' (Martin, Hasselquist & Wikelski 2006).

In summary, it is clear from our data that the PHA-induced swelling response in one species of passerine involves changes in multiple immune-cell types over time. Subsequently, PHA-induced swelling does not appear to be an unambiguous index of T-cell-mediated immunity, but rather a multifaceted index of cutaneous immune activity. We expect that part of the differences in swelling responses detected in other studies between species, or among different physiological states within species, may be a consequence of biases of the orientation of the immune system in a cost-benefit context. That is, depending on the physiological state of the animal and/or its life-history orientation, changes in swelling may be mediated by shifts in the predominant leukocyte types in circulation, which may change over the year or over the course of an animal's life (Klasing 2002). In House Sparrows, preliminary data indicate that this possibility may hold for the two populations mentioned

above (L.B.M. and co-workers, unpublished data). In addition, our study supports previous work showing that the one-wing technique is appropriate for characterizing PHA-induced wing-web swelling in birds (Smits *et al.* 1999). Little infiltration occurred in saline-injected wings in our study, so by injecting one wing, researchers can reduce handling time and the possibility of confusion or trauma during field work that may occur when injecting one wing with PHA and the other with saline. Finally, we predict that many of the lasting effects of PHA seen in some studies may be due to induction of both local and systemic immune activity (e.g. acute phase responses) by PHA (Adler *et al.* 2001), and we encourage the study of such possibilities.

Acknowledgements

We thank Monica Pless and Jessica Gilliam for help collecting birds, Laura Spinney for sharing aviary resources, Stephanie Bowers for technical assistance with the microscope, and Steve Dikman for help identifying immune cells. We thank the management of the Princeton Shopping Center for allowing us to work on their property. Kelly Lee, Leah Pyter, Alex Scheuerlein, Brian Trainor, Zachary Weil and an anonymous reviewer provided helpful criticism on earlier versions of the manuscript. A. Courtney DeVries graciously allowed us to use the Nikon microscope. Funding for this work comes from grants to J.L. from the Horton Elmer Fund, the Senior Round Table Fund, and the Princeton Program in Latin American Studies, to L.B.M. from the US-EPA-STAR fellowship program, and to M.W. and K.C.K. from NSF IRCEB-0212587. All experiments were approved by the Princeton University Animal Care Committee and comply with the principles of animal care (Pub. 86-23, NIH) and current US laws.

References

- Adler, K.L., Peng, P.H., Peng, R.K. & Klasing, K.C. (2001) The kinetics of hemopexin and alpha 1-acid glycoprotein levels induced by injection of inflammatory agents in chickens. *Avian Diseases* **45**, 289–296.
- Alonso-Alvarez, C. & Tella, J.L. (2001) Effects of experimental food restriction and body-mass changes on the avian T-cell-mediated immune response. *Canadian Journal of Zoology* **79**, 101–105.
- Ardia, D.R. (2005a) Tree swallows trade off immune function and reproductive effort differently across their range. *Ecology* **86**, 2040–2046.
- Ardia, D.R. (2005b) Individual quality mediates trade-offs between reproductive effort and immune function in tree swallows. *Journal of Animal Ecology* **74**, 517–524.
- Barbosa, A. & Moreno, E. (2004) Cell-mediated immune response affects food intake but not body mass: an experiment with wintering great tits. *Ecoscience* **11**, 305–309.
- Bayyari, G., Huff, W., Rath, N. *et al.* (1997) Effect of the genetic selection of turkeys for increased body weight and egg production on immune and physiological responses. *Poultry Science* **76**, 289–296.
- Bonforte, R., Topilsky, M., Siltzbach, L. & Glade, P. (1972) Phytohemagglutinin skin test: a possible measure of cell-mediated immunity. *Journal of Pediatrics* **81**, 775–780.

- Brinkhof, M.W.G., Heeb, P., Kolliker, M. & Richner, H. (1999) Immunocompetence of nestling great tits in relation to rearing environment and parentage. *Proceedings of the Royal Society of London B – Biological Sciences* **266**, 2315–2322.
- Brommer, J.E. (2004) Immunocompetence and its costs during development: an experimental study in blue tit nestlings. *Proceedings of the Royal Society of London B – Biological Sciences* **271**, S110–S113.
- Campbell, T. (1995) *Avian Hematology and Cytology*, 2nd edn. Iowa State University Press, Ames, IA, USA.
- Casto, J.M., Nolan, V. & Ketterson, E.D. (2001) Steroid hormones and immune function: experimental studies in wild and captive dark-eyed juncos (*Junco hyemalis*). *American Naturalist* **157**, 408–420.
- Cheng, S. & Lamont, S.J. (1988) Genetic analysis of immunocompetence measures in a white leghorn chicken line. *Poultry Science* **70**, 2023–2027.
- 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.
- Christe, P., de Lope, F., Gonzalez, G., Saino, N. & Møller, A.P. (2001) The influence of environmental conditions on immune responses, morphology and recapture probability of nestling house martins (*Delichon urbica*). *Oecologia* **126**, 333–338.
- Dietert, R., Golemboski, K., Bloom, S. & Qureshi, M. (1991) The avian macrophage in cellular immunity. *Avian Cellular Immunology* (ed. J.M. Sharma), pp. 71–96. CRC Press, Boston, MA, USA.
- Duffy, D.L. & Ball, G.F. (2002) Song predicts immunocompetence in male European starlings (*Sturnus vulgaris*). *Proceedings of the Royal Society of London B – Biological Sciences* **269**, 847–852.
- Elgert, K. (1996) *Immunology: Understanding the Immune System*. Wiley & Liss, New York, USA.
- Ewenson, E.L., Zann, R.A. & Flannery, G.R. (2001) Body condition and immune response in wild zebra finches: effects of capture, confinement and captive-rearing. *Naturwissenschaften* **88**, 391–394.
- Ewenson, E., Zann, R. & Flannery, G. (2003) PHA immune response assay in captive zebra finches is modulated by activity prior to testing. *Animal Behaviour* **66**, 797–800.
- Fair, J.M., Hansen, E.S. & Ricklefs, R.E. (1999) Growth, developmental stability and immune response in juvenile Japanese quails (*Coturnix coturnix japonica*). *Proceedings of the Royal Society of London B – Biological Sciences* **266**, 1735–1742.
- Fair, J.M., Myers, O.B. & Ricklefs, R.E. (2003) Immune and growth response of western bluebirds and ash-throated flycatchers to soil contaminants. *Ecological Applications* **13**, 1817–1829.
- Fargallo, J.A., Laaksonen, T., Poyri, V. & Korpimäki, E. (2002) Inter-sexual differences in the immune response of Eurasian kestrel nestlings under food shortage. *Ecology Letters* **5**, 95–101.
- 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*). *Journal of Animal Ecology* **68**, 1225–1234.
- Goto, N., Kodama, H., Okada, K. & Fujimoto, Y. (1978) Suppression of phytohemagglutinin skin response in thymectomized chickens. *Poultry Science* **57**, 246–250.
- Greenman, C.G., Martin, L.B. & Hau, M. (2005) Reproductive state but not testosterone reduces immune function in male house sparrows (*Passer domesticus*). *Physiological and Biochemical Zoology* **78**, 60–68.
- Hausmann, M.F., Winkler, D.W., Huntington, C.E. et al. (2006) Cell-mediated immunosenescence in birds. *Oecologia*, in press.
- Horak, P., Vellau, H., Ots, I. & Møller, A.P. (2000) Growth conditions affect carotenoid-based plumage coloration of great tit nestlings. *Naturwissenschaften* **87**, 460–464.
- Johnsen, A., Andersen, V., Sunding, C. & Lifjeld, J.T. (2000) Female bluethroats enhance offspring immunocompetence through extra-pair copulations. *Nature* **406**, 296–299.
- Johnsen, T.S. & Zuk, M. (1999) Parasites and tradeoffs in the immune response of female red jungle fowl. *Oikos* **86** (3), 487–492.
- Klasing, K.C. (2004) The costs of immunity. *Acta Zoologica Sinica* **50**, 961–969.
- Lee, K.A., Martin, L.B. & Wikelski, M. (2005) Responding to inflammatory challenges is less costly for a successful avian invader, the house sparrow (*Passer domesticus*), than its less invasive congener. *Oecologia* **145**, 244–251.
- Lifjeld, J.T., Dunn, P.O. & Whittingham, L.A. (2002) Short-term fluctuations in cellular immunity of tree swallows feeding nestlings. *Oecologia* **130**, 185–190.
- Lindstrom, K.M., Foufopoulos, J., Parn, H. & Wikelski, M. (2004) Immunological investments reflect parasite abundance in island populations of Darwin's finches. *Proceedings of the Royal Society of London B – Biological Sciences* **271**, 1513–1519.
- Lochmiller, R.L., Vestey, M.R. & Boren, J.C. (1993) Relationship between protein nutritional status and immunocompetence in northern Bobwhite chicks. *Auk* **110**, 503–510.
- Lohmus, M., Olin, M., Sundstrom, L.F., Troedsson, M.H.T., Molitor, T.W. & El Halawani, M. (2004) Leptin increases T-cell immune response in birds. *General and Comparative Endocrinology* **139**, 245–250.
- Martin, L.B. (2005) Trade-offs between molt and immune activity in two populations of House Sparrows (*Passer domesticus*). *Canadian Journal of Zoology* **83**, 780–787.
- Martin, T.E., Martin, P., Olson, C., Heidinger, B. & Fontaine, J. (2000) Parental care and clutch sizes in North and South American birds. *Science* **287**, 1482–1485.
- Martin, L.B., Scheuerlein, A. & Wikelski, M. (2003) Increased immune activity elevates energy expenditure of house sparrows: a link between proximate and ultimate costs of immune activity? *Proceedings of the Royal Society of London B – Biological Sciences* **270**, 153–158.
- Martin, L.B., Pless, M.I., Svoboda, J. & Wikelski, M. (2004) Immune activity in temperate and tropical House Sparrows: a common garden experiment. *Ecology* **85**, 2323–2331.
- Martin, L.B., Gilliam, J., Han, P., Lee, K.A. & Wikelski, M. (2005) Corticosterone suppresses immune function in temperate but not tropical house sparrows (*Passer domesticus*). *General and Comparative Endocrinology* **140**, 126–135.
- Martin, L.B., Hasselquist, D. & Wikelski, M. (2006) Immune investments are linked to pace of life in house sparrows. *Oecologia*, in press.
- Mayne, G.J., Martin, P.A., Bishop, C.A. & Boermans, H.J. (2004) Stress and immune responses of nestling tree swallows (*Tachycineta bicolor*) and eastern bluebirds (*Sialia sialis*) exposed to nonpersistent pesticides and p,p'-dichlorodiphenyldichloroethylene in apple orchards of southern Ontario, Canada. *Environmental Toxicology and Chemistry* **23**, 2930–2940.
- McCorkle, F., Olah, I. & Glick, B. (1980) The morphology of the phytohemagglutinin-induced cell response in the chicken's wattle. *Poultry Science* **59**, 616–623.
- Meltzer, M. & Oppenheim, J. (1977) Bidirectional amplification of macrophage–lymphocyte interactions: enhanced lymphocyte activation factor production by activated adherent mouse peritoneal cells. *Journal of Immunology* **118**, 77–82.
- Merino, S., Martinez, J., Møller, A.P. et al. (1999) Phytohaemagglutinin injection assay and physiological stress in nestling house martins. *Animal Behaviour* **58**, 219–222.

- Moore, C.B. & Siopes, T.D. (2003) Melatonin enhances cellular and humoral immune responses in the Japanese quail (*Coturnix coturnix japonica*) via an opiate mechanism. *General and Comparative Endocrinology* **131**, 258–263.
- Moreno, J., Sanz, J.J. & Arriero, E. (1999) Reproductive effort and T-lymphocyte cell-mediated immunocompetence in female pied flycatchers *Ficedula hypoleuca*. *Proceedings of The Royal Society of London B – Biological Sciences* **266**, 1105–1109.
- Moreno, J., Sanz, J.J., Merino, S. & Arriero, E. (2001) Daily energy expenditure and cell-mediated immunity in pied flycatchers while feeding nestlings: interaction with moult. *Oecologia* **129**, 492–497.
- Naspitz, C.K. & Richter, M. (1968) The action of phytohemagglutinin *in vivo* and *in vitro*: a review. *Progress in Allergy* **12**, 1–85.
- Navara, K.J., Hill, G.E. & Mendonça, M.T. (2005) Variable effects of yolk androgens on growth, survival, and immunity in eastern bluebird nestlings. *Physiological and Biochemical Zoology* **78**, 570–578.
- 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.
- Owen-Ashley, N.T., Hasselquist, D. & Wingfield, J.C. (2004) Androgens and the immunocompetence handicap hypothesis: unraveling direct and indirect pathways of immunosuppression in song sparrows. *American Naturalist* **164**, 490–505.
- Reid, J.M., Arcese, P. & Keller, L.F. (2003) Inbreeding depresses immune response in song sparrows (*Melospiza melodia*): direct and inter-generational effects. *Proceedings of The Royal Society of London B – Biological Sciences* **270**, 2151–2157.
- Saino, N., Calza, S. & Møller, A.P. (1997) Immunocompetence of nestling barn swallows in relation to brood size and parental effort. *Journal of Animal Ecology* **66**, 827–836.
- Saino, N., Ferrari, R.P., Romano, M., Ambrosini, R. & Møller, A.P. (2002) Ectoparasites and reproductive trade-offs in the barn swallow (*Hirundo rustica*). *Oecologia* **133**, 139–145.
- Smits, J.E. & Williams, T. (1999) Validation of immunotoxicology techniques in passerine chicks exposed to oil sands tailing water. *Ecotoxicology and Environmental Safety* **44**, 105–112.
- Smits, J.E., Bortolotti, G.R. & Tella, J.L. (1999) Simplifying the phytohemagglutinin skin-testing technique in studies of avian immunocompetence. *Functional Ecology* **13**, 567–572.
- Smits, J.E., Wayland, M.E., Miller, M.J., Liber, K. & Trudeau, S. (2000) Reproductive, immune, and physiological end points in tree swallows on reclaimed oil sands mine sites. *Environmental Toxicology and Chemistry* **19**, 2951–2960.
- Soler, M., Martin-Vivaldi, M., Marin, J. & Møller, A.P. (1999) Weight lifting and health status in the black wheatear. *Behavioral Ecology* **10**, 281–286.
- Soler, J.J., de Neve, L., Perez-Contreras, T., Soler, M. & Sorci, G. (2003) Trade-off between immunocompetence and growth in magpies: an experimental study. *Proceedings of The Royal Society of London B – Biological Sciences* **270**, 241–248.
- Sorci, G., Soler, J.J. & Møller, A.P. (1997) Reduced immunocompetence of nestlings in replacement clutches of the European magpie (*Pica pica*). *Proceedings of the Royal Society of London B – Biological Sciences* **264**, 1593–1598.
- Stadecker, M., Lukic, M., Dvorak, A. & Leskowitz, S. (1977) The cutaneous basophil response to phytohemagglutinin in chickens. *Journal of Immunology* **118**, 1564–1568.
- Summers-Smith, J.D. (1988) *The Sparrows: A Study of the Genus Passer*. T. & AD Poyser, Staffordshire, UK.
- Taylor, R.L.J., Cotter, P.F., Wing, T.L. & Briles, W.E. (1987) Major histocompatibility B complex and sex effects on the phytohemagglutinin wattle response. *Animal Genetics* **18**, 343–350.
- Tella, J.L., Bortolotti, G.R., Dawson, R.D. & Forero, M.G. (2000a) The T-cell-mediated immune response and return rate of fledgling American kestrels are positively correlated with parental clutch size. *Proceedings of the Royal Society of London B – Biological Sciences* **267**, 891–895.
- Tella, J.L., Bortolotti, G.R., Forero, M.G. & Dawson, R.D. (2000b) Environmental and genetic variation in T-cell-mediated immune response of fledgling American kestrels. *Oecologia* **123**, 453–459.
- Tella, J.L., Forero, M.G., Bertelotti, M., Donazar, J.A., Blanco, G. & Ceballos, O. (2001) Offspring body condition and immunocompetence are negatively affected by high breeding densities in a colonial seabird: a multiscale approach. *Proceedings of the Royal Society of London B – Biological Sciences* **268**, 1455–1461.
- Tella, J.L., Scheuerlein, A. & Ricklefs, R.E. (2002) Is cell-mediated immunity related to the evolution of life history strategies of birds? *Proceedings of the Royal Society of London B – Biological Sciences* **269**, 1059–1066.
- Turk, J.L. (1967) *Delayed Hypersensitivity*. John Wiley and Sons, New York, USA.
- Velando, A., Lessells, C.M. & Marquez, J.C. (2001) The function of female and male ornaments in the Inca Tern: evidence for links between ornament expression and both adult condition and reproductive performance. *Journal of Avian Biology* **32**, 311–318.
- Zuk, M. & Johnsen, T.S. (1998) Seasonal changes in the relationship between ornamentation and immune response in red jungle fowl. *Proceedings of the Royal Society of London B – Biological Sciences* **265**, 1631–1635.
- Zuk, M. & Johnsen, T.S. (2000) Social environment and immunity in male red jungle fowl. *Behavioral Ecology* **11**, 146–153.

Received 6 September 2005; revised 6 December 2005;
accepted 8 December 2005

Editor: C. Fox