

Growth, developmental stability and immune response in juvenile Japanese quails (*Coturnix coturnix japonica*)

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Stresses are environmental factors which restrict growth or cause a potentially adverse change in an organism. The exposure of developing organisms to environmental stresses may have several physiological consequences including a decrease in immunocompetence. However, mounting an immune response against a foreign antigen may in itself constitute a cost for developing organisms. This cost has potentially long-term consequences for adult function and fitness. This study examines the growth and developmental stability of Japanese quail chicks challenged by three non-pathogenic antigens: sheep red blood cells, which assess T-cell-dependent immune responses, and *Mycoplasma synoviae* and Newcastle disease virus, which assess T-cell-independent responses. Increases in both body mass and wing length were significantly reduced in antigen-challenged birds compared to control birds. Fluctuating asymmetry (FA) in the masses of primary feathers increased from the innermost (1) to the outermost (10) position on the wing. In addition, antigen challenge by *M. synoviae* and sheep red blood cells was associated with an increase in FA. The cell-mediated response measured by reaction to phytohaemagglutinin was significantly depressed in *M. synoviae*-challenged birds. White blood cell counts, except for monocytes, were elevated in response to all three antigen treatments. Total plasma protein and haematocrit also differed between treatments but exhibited no clear relationship to antigen challenge. Immune responses clearly impose a stress on developing chicks. Additional research will be required to determine the long-term consequences of developmental stress and assess the selective forces that influence the strength of the immune responses of chicks.

Keywords: Japanese quail; developmental stability; ELISA; fluctuating asymmetry; immunocompetence; phytohaemagglutinin

1. INTRODUCTION

Stress can be considered any environmental factor which restricts the growth and reproduction of an organism or population or causes a potentially adverse change in an organism (Lincoln *et al.* 1998). The exposure of developing organisms to environmental stresses may have several physiological consequences including a decrease in immunocompetence. However, an immune response to foreign antigens may also impose a cost on developing organisms and result in decreased growth, perhaps with long-term consequences for fitness (Gebhardt-Henrich & Richner 1998). Immune responses to non-pathogenic antigens, such as vaccines, have been shown to impair growth performance in domestic poultry in one study (Klasing *et al.* 1987).

Although intraspecific variation in growth has numerous sources (Gebhardt-Henrich & Richner 1998), no study has investigated how immunological challenges by different types of antigens affect growth. Immune responses have two kinds of costs associated with (i) maintaining a functioning immune system and

(ii) mounting a specific or non-specific immune response. Growth responses to non-pathogenic antigen challenges may be mediated through the reallocation of limited resources, although this may impose only a small energetic cost (Klasing 1998). Alternatively, the effects of mounting an immune response may be exerted through systemic physiological controls on metabolic and developmental pathways.

A common method of quantifying humoral responses is to measure antibody production following the introduction of a non-pathogenic antigen. Peak antibody titres are usually reached in 5–15 days (Nelson *et al.* 1995). Antigen challenge is a potentially useful tool for investigating the effects of various stresses in the environment on an organism's performance. However, the costs of mounting immune responses could be an important confounding factor in such studies, particularly those involving growing organisms with immature and developing immune systems.

The growth of nestling birds is commonly used as an index of environmental and genetic stresses (e.g. Richner *et al.* 1989; Eeva & Lehikoinen 1996). In addition to body mass and the lengths of feathers and appendages, asymmetrical development of bilateral characters may

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also reveal the effects of stress on development (Swaddle & Witter 1994). Presumably, the growth of each one of a pair of bilateral characters is influenced by the same genetic and environmental factors. Accordingly, small errors in development affecting either one of a pair of characters independently cause random deviations from perfect symmetry (fluctuating asymmetry (FA)) and, thus, provide a combined measure of innate developmental stability and stress on the growing individual (Teather 1996; Møller & Swaddle 1997).

Variance in the length of a bilateral character has several components, only one of which includes FA. These components may be quantified by analysis of variance using an approach outlined by Palmer & Strobeck (1986). The effects of interest are the sides (right versus left), individuals and repeated measurements of the same character. The mean square for the side \times individual interaction includes measurement error and non-directional asymmetry, which consists of anti-symmetry and FA. Anti-symmetry is distinguished from FA by producing a platykurtic or bimodal distribution, rather than a normal distribution, of the right-left difference around a mean of zero (Palmer & Strobeck 1986). Thus, anti-symmetry, which represents the exaggerated development of one side or the other, can be detected by testing the left-right difference for normality.

Numerous studies have used FA as an indicator of developmental stability, which presumably reflects the influences of both environmental and genetic stresses on development. Most such studies have addressed the relationship between the asymmetry of adult characters and genetic or environmental perturbations (e.g. Teather 1996), presuming that environmental and/or genetic stresses during ontogeny may interfere with the precise regulation of developmental processes and, thereby, result in an increase in FA (Clarke 1993). The relationship between growth, FA and stress imposed by challenges to the immune system has not been investigated.

Additional measures of stress in studies of laboratory and natural populations of animals include the circulating levels of protein in the blood, which are thought to vary in relation to the total protein reserves in an animal (Allison 1955). Total plasma protein levels may also become elevated when dietary protein intake increases (Leveille & Sauberlich 1961) and, thus, indicate improved physiological condition in wild birds (Dawson & Bortolotti 1997a). Total plasma protein can vary with sex and breeding stage and typically declines in nestlings as the breeding season progresses (Dawson & Bortolotti 1997a). Diseased animals or those in poor condition are known to have reduced haematocrits (Dawson & Bortolotti 1997b), although a concrete relationship with condition is still questionable. However, nothing is known of the relationship between either the packed cell volume (haematocrit) or total plasma protein, on the one hand, and immunological and growth parameters, on the other. Because an immune response may cause changes in both packed cell volume and total plasma protein, more research is needed to validate both haematocrit and plasma protein as indicators of stress and condition.

The objectives of this study were to (i) determine the effects of three commonly used, non-pathogenic antigens

on growth and developmental stability, (ii) establish the relationship between the responsiveness of the immune system and several haematological parameters, growth and FA, and (iii) determine the developmental stability of primary feathers in juvenile Japanese quails under controlled conditions.

Japanese quails (*Coturnix coturnix japonica*) are frequently used as indicators in avian toxicity tests (Romijn *et al.* 1995) and were therefore a logical choice for this study. We focused on chicks because they represent a vulnerable stage of the life cycle and offer the potential for competition between growth and immune responses. In addition, because the immune system continues to develop during the postnatal growth period, variations in its rate of maturation could produce substantial differences in immune responses between individuals. Given such variation, negative correlations between immune responsiveness and growth could result from the allocation of limited resources between growth and immune system function or from interference between the immune system and physiological controls over growth and development. Positive correlations between immune responsiveness and growth could result from variations in the overall condition of chicks that affect both the immune system and growth processes.

2. METHODS

(a) *Animal care and antigen treatment*

Unsexed quail were obtained at 27 days of age from the World Bird Sanctuary, St Louis, Missouri and acclimated to laboratory surroundings for one week before immunizations and measurements were begun. Five quails were housed in each of six cages measuring 61 cm \times 56 cm \times 81 cm. The temperature in the animal room was maintained at 23 °C. Food and water were available *ad libitum*. Quails were fed pullet food containing around 18% crude protein, which is adequate for normal growth at this age. Fluorescent lights provided a photoperiod of 16 L:8 D. All protocols were approved by the University of Missouri-St Louis Animal Care and Use Committee.

The T-cell-dependent response was measured following injection of sheep red blood cells (SRBCs) (Sigma Co., St Louis, MO, USA). The T-cell-independent response to antigens was measured following vaccination with chemically inactivated *Mycoplasma synoviae* or killed Newcastle disease virus (NDV) (Fort Dodge Animal Health, Fort Dodge, IA, USA). Birds were randomly inoculated with either 0.075 ml of 10% SRBC solution in phosphate-buffered solution (PBS) ($n=8$), 0.075 ml of *M. synoviae* ($n=7$) or NDV ($n=7$) or a dummy vaccine ($n=8$) containing no antigen but the same amount of oil medium. All chicks were injected intraperitoneally (i.p.) at 34 days of age. Their feathers were out of their sheath but were not fully grown. Birds were weighed and measured and *ca.* 80 μ l of blood was drawn from the wing vein on day 34 and then again on days 37, 39, 41, 44 and 49 (0, 3, 5, 7, 10 and 15 days post-vaccination).

(b) *Fluctuating asymmetry and growth*

Tarsus length was measured to the nearest 0.01 mm using calipers. The length of the right wing was measured with a ruler to the nearest millimetre using the flattened-wing method (Svensson 1984). All of the birds were weighed on a triple-beam balance to the nearest 0.1 g. After day 54 the birds were euthanized and sexed by dissection.

Each wing was removed and air-dried for several weeks at room temperature and ten primary feathers were pulled and weighed on a top-loading analytical balance to the nearest 0.001 g. Feathers could be cleanly removed from the wing and were found to have no tearing or damage from other birds. To estimate measurement error, each feather was weighed twice, with the weighing cycle running from feather 1 to feather 10 on each side and then repeated for each feather (individuals 1–5 were not remeasured). In addition, secondary feathers 7 through to 3 were removed, dried and weighed in the same fashion, except that individual feathers were not remeasured.

(c) *Antibody response*

Blood (ca. 80 µl) was collected from the brachial vein of the wing into both heparinized and non-heparinized microcapillary tubes, which were kept at room temperature. The heparinized tubes were spun for 10 min in a microcapillary centrifuge within 1 h of collection. The non-heparinized tubes were centrifuged 12–15 h after collection. All samples were maintained at 4 °C until analysis. Antibody responses to *M. synoviae* and NDV were measured by a monoclonal antibody-blocking ELISA (Svanova Biotech, Sweden) (Czifra *et al.* 1996). Optical density (OD) was determined with an EL-312 Bio-Tek Instruments microtitre plate photometer.

Serum samples were heat inactivated at 56 °C for 30 min and agglutination of antibodies to SRBC antigens in sera was serially titrated. Antibody titres were determined with modified microtitre techniques and the scoring system of Wegmann & Smithies (1966).

(d) *Cell-mediated response and non-specific immunity*

Cell-mediated immunity was measured using the dermal phytohaemagglutinin (PHA) reaction in the wing web. PHA (Sigma Chemical Co., St Louis, MO) injected for a localized *in vivo* inflammatory response in birds has long been used to measure cell-mediated immunity (Stadecker *et al.* 1977; Lamont & Smyth 1984). Birds were inoculated intradermally in the wing web on day 50 with either 0.05 ml (1.0 mg ml⁻¹) PHA in PBS (right side) or 0.05 ml PBS only (left side). The amount of swelling in the wing web 24 h after inoculation was measured by a micrometer to the nearest 0.001 mm. A PHA index was computed as the width of the PHA-inoculated wing web minus the width of the opposite wing web, standardized for wing thickness:

$$\text{PHA index} = \frac{\text{post-PHA} - \text{post-PBS}}{(\text{pre-PHA} + \text{pre-PBS})/2}$$

White blood cells (WBCs), classified according to Dein (1984), were counted on blood smears stained with Wright–Giemsa stain. Five randomly selected fields were examined using ×400 magnification (Zeiss Axioskop microscope) for leucocyte counts. The reliability of this sampling design has been previously assessed by Apanius (1991), who determined that 82% of the observed variation could be attributed to differences between birds and 18% to sampling error. Granulocytes were double-checked for identification using ×1000 magnification and basophils were omitted because of their relative rarity. The results were analysed as total WBCs minus thrombocytes or grouped as lymphocytes, granulocytes, eosinophils, heterophils and monocytes.

(e) *Haematology*

Haematocrits of blood collected in heparinized capillary tubes were determined with a microhaematocrit reader. Total plasma protein was estimated using a refractometer (Westover Model RHC-200). Although it has been suggested that the refractometric method may be unreliable in determining plasma protein concentrations in pigeons (Lumeij & De Bruijne 1985), refractometers are commonly used in clinical practice and have been shown to be reliable for mammalian plasma (Schalm *et al.* 1975) and American kestrels (Dawson & Bortolotti 1997a).

(f) *Data analysis*

The Statistical Analysis System (SAS Institute, Inc. 1987) was used for all statistical analyses and assumptions for parametric statistics were examined. Growth variables (body weight and wing length) were compared between antigen treatments using repeated measures ANOVA. The means for each treatment were compared with Duncan's multiple range test. Because of the relatively short period during which the birds were measured, the growth curves were incomplete and, thus, growth curve variables were omitted from the analysis. All data not normally distributed or having unequal variances were compared with Kruskal–Wallis non-parametric tests.

Other variables measured at each bleeding, such as haematocrits and WBC counts, were analysed for antigen and sex differences using repeated measures ANOVA. We used correlation analysis to test for associations between haematocrit and plasma protein for each age.

The analysis of FA included the determination of measurement error by comparing the duplicated measurements of each feather. The pattern of variation in the mass of feathers between individuals and between right and left wings was quantified for each primary feather by a two-way, mixed-model ANOVA according to Palmer & Strobeck (1986), using repeated measurements of each feather on each side to estimate measurement error. Three statistical tests were used in this analysis: (i) $F([\text{bird} \times \text{wing}]/\text{error})$ estimates the magnitude of non-directional asymmetry (anti-symmetry and FA), (ii) $F(\text{bird}/[\text{bird} \times \text{wing}])$ estimates the magnitude of size variation among individual birds, and (iii) $F(\text{wing}/[\text{bird} \times \text{wing}])$ estimates the magnitude of directional asymmetry. ANOVAs were performed on individuals 6–30, which had repeated measurements. Anti-symmetry is expressed as platykurtosis in the distribution of left–right differences (Palmer & Strobeck 1986). Accordingly, we tested the normality of the left–right distributions for each feather.

We calculated a variance component (V_{ij}) for each feather (i) of each individual (j) as the square of the normalized difference of the left and right sides,

$$V_{ij} = \left[\frac{2(R - L)}{R + L} \right]^2,$$

which was tested for individual and feather effects by ANOVA. An FA index was then calculated for each individual as the square root of the mean of the variance components across feathers, which provides a measure of FA expressed as a decimal fraction of the average feather mass. Thus,

$$FA = \sqrt{\frac{\sum_{i=1}^N V_{ij}}{N}}.$$

Table 1. Duncan's multiple range test results (\bar{x}) for the variables mass, wing length, FA index, PHA response, haematocrit and total plasma protein for the three antigen treatments and the control

(Different letters denote groups that were significantly different from each other. Root mean square error values: mass, 11.6; wing length, 3.6; FA index, 0.078; PHA response, 0.34; haematocrit, 0.034; total plasma protein, 2.13.)

variable	SRBC	<i>M. synoviae</i>	NDV	control
mass	125.8b	123.4bc	120.4c	138.7a
wing length (mm)	105.0b	103.8c	102.0d	106.5a
FA index	0.118ab	0.154a	0.065b	0.067b
PHA response	1.47ab	1.16b	1.64a	1.28ab
haematocrit	0.42b	0.44a	0.42b	0.44a
total plasma protein (g dl ⁻¹)	3.46b	5.05a	5.25a	4.70a

This index was then analysed for each bird in a two-way ANOVA in which the effects were treatment and sex. We also determined whether FA was correlated with PHA skin response, haematocrit, plasma protein and growth in analyses of covariance with sex entered as an effect.

3. RESULTS

All quail (17 females and 13 males, evenly distributed between the antigens) survived until the end of the experiment (day 54). Females weighed significantly more than males over all the ages represented in the study (ANOVA, $F_{1,41}=60.26$ and $p=0.0001$). The mean masses at the end of the experiment (age 54 days) were 158.1 g (s.d. = 24.1 g) for females and 132.5 g (s.d. = 30.4 g) for males. The quail began mating and laying eggs by day 51, although Japanese quail are generally not considered to be fully grown until 60 days (Marsh 1976).

(a) Antibody response

The antibody response to NDV generally increased over time through to the last day of the experiment, day 17 after injection. At the same time, the variance in the ELISA OD reading between individuals increased, suggesting individual differences in antibody response. The response differed significantly with respect to days following immunization (Kruskal–Wallis test, $\chi_6^2 = 27.10$ and $p=0.0001$) and sex ($\chi_1^2 = 3.86$ and $p=0.049$). For most birds the antibody response to *M. synoviae* peaked on day 7 post-immunization. However, the response varied only marginally between dates post-immunization (Kruskal–Wallis test, $\chi_6^2 = 11.4$ and $p=0.078$) and sex ($\chi_1^2 = 3.00$ and $p=0.083$). The haemagglutination titres (expressed as the log of the highest titre giving visible agglutination) peaked ten days after immunization and did not differ significantly between the sexes ($F_{1,39}=3.21$ and $p=0.08$).

(b) Cell-mediated and non-specific immunity

The antigen treatments influenced the skin response to PHA ($F_{3,25}=3.2$ and $p=0.039$) (table 1). Challenge with *M. synoviae* significantly reduced the PHA response compared to NDV challenge. The PHA responses of the control and SRBC-challenged birds were intermediate.

Table 2. Duncan's multiple range test results (\bar{x}) for the main WBC categories (average number of cells per five random fields) at seven days post-immunization for the three antigen treatments and control

(Different letters denote groups that were significantly different from each other.)

WBC category	SRBC	<i>M. synoviae</i>	NDV	control
WBCs	104.7bc	186.7a	117.3b	71.1c
lymphocytes	12.5ab	19.7a	21.1a	5.4b
eosinophils	3.5b	7.7a	7.9a	3.0b
heterophils	12.7bc	21.2a	14.7b	8.8c
monocytes	24.6a	31.0a	25.4a	21.8a
thrombocytes	42.7b	87.7a	48.9b	35.9b

The response to PHA did not differ significantly between the sexes ($F_{1,25}=3.5$ and $p=0.073$). The PHA response was not correlated with FA, wing length or haematocrit (p -values, 0.27–0.98). However, the PHA response was positively correlated with total plasma protein ($r^2=0.28$, $F_{2,20}=3.87$ and $p=0.038$). The PHA index was unrelated to mass ($r^2=0.002$, $F_{1,28}=0.08$ and $p=0.78$).

Counts of the lymphocytes and granulocytes, as well as the total number of WBCs in all categories, had unequal variances between ages (Bartlett's test (Montgomery 1997), $\chi_4^2 = 15.6$ and $p=0.0035$, $\chi_4^2 = 24.0$ and $p=0.001$ and $\chi_4^2 = 9.9$ and $p=0.042$, respectively). Therefore, variation in WBC counts with respect to age and individuals was analysed by Kruskal–Wallis tests. The sexes did not differ with respect to the blood cell categories. In an analysis of all the blood samples taken after immunization (days 3–18), the counts for WBCs differed between ages with a decreasing trend ($\chi_4^2 = 31.39$ and $p=0.0001$) and between antigen treatments ($\chi_3^2 = 7.6$ and $p=0.05$). Granulocytes also differed over time, but showed no consistent temporal trends ($\chi_4^2 = 18.2$ and $p=0.001$) and did not differ between treatments ($\chi_3^2 = 6.9$ and $p=0.07$). Lymphocytes did not vary over time ($\chi_4^2 = 8.6$ and $p=0.07$) or between treatments ($\chi_3^2 = 2.3$ and $p=0.51$). Eosinophils differed over time ($\chi_4^2 = 17.2$ and $p=0.0018$) and between treatments ($\chi_3^2 = 20.3$ and $p=0.0001$). For monocytes, the variances were homogeneous between days and treatments (Bartlett's test, $\chi_4^2 = 3.4$ and $p=0.33$). They differed with respect to sex ($F_{1,130}=2.82$ and $p=0.027$) but did not differ between treatments ($F_{3,130}=1.15$ and $p=0.33$). Multiple comparison tests for treatment effects for each day after immunization showed no differences between treatments except on day 7. The results for this day revealed elevated counts of lymphocytes, eosinophils, heterophils and thrombocytes, in particular for the *M. synoviae* and NDV treatments (table 2). Thus, each of the treatments elicited a transient increase in WBCs, peaking at approximately one week after immunization. Control birds had the lowest WBC counts for every category for every day.

(c) Growth

At the start of the experiment neither mass ($F_{3,26}=0.98$ and $p=0.42$) nor wing length ($F_{3,26}=1.05$ and $p=0.39$) differed between the antigen treatments. Over the experiment as a whole, mass differed significantly between

antigen treatments ($F_{3,148}=15.1$ and $p=0.0001$). The significant time \times treatment interaction ($F_{17,148}=1.75$ and $p=0.04$) revealed that control birds grew more rapidly than birds in the antigen treatment groups (figure 1). Although the control birds were the heaviest individuals at the end of the experiment (age 54 days), the control and treatment groups did not differ significantly ($F_{3,25}=0.33$ and $p=0.81$) because mass differences evident during the second week post-injection had been made up. The maximum immune response for each bird and each assay was calculated as the maximum haemagglutination titre or the inverse of the minimum ELISA OD. The maximum immune response was not correlated with mass across the three treatments ($F_{1,20}=0.14$ and $p=0.71$).

Wing length was significantly affected by immunological challenge ($F_{3,147}=43.0$ and $p=0.0001$) and exhibited a significant date \times treatment interaction ($F_{17,147}=1.70$ and $p=0.048$). The mean wing length differed between all antigen treatments (Duncan's multiple range test) with control birds having the longest mean wing lengths and NDV- and *M. synoviae*-challenged birds the shortest (table 1). The mass of the tenth primary feather did not differ significantly between treatments (right side $F_{3,25}=0.83$ and $p=0.49$, and left side $F_{3,25}=0.54$ and $p=0.66$). The masses of the right and left tenth primaries also did not differ between sexes ($F_{1,25}=0.83$ and $p=0.37$, and $F_{1,25}=0.78$ and $p=0.39$, respectively). The right tenth primary was significantly positively correlated with wing length ($F_{1,28}=49.01$ and $p=0.0001$) and bird mass ($F_{1,28}=15.18$ and $p=0.0006$).

(d) Fluctuating asymmetry

The measurement error for both absolute and normalized repeated measurements was extremely small. The mean measurement error for the 250 repeated feather masses, obtained by calculating the square root of the mean of the squared difference of the first and second measurements, was 0.00036 g. The mean feather mass varied between 0.015 g for primary 1 and 0.044 g for primary 10. Normalized by feather mass, the mean right and left differences in all feathers ($n=300$) was -0.0049 (i.e. -0.5%) and the standard deviation of the distribution was 0.130. These results are consistent with an absence of directional asymmetry and a combined FA and anti-symmetry of around 13% of the mean weight of the feather. The data normalized by feather mass were approximately normally distributed (Wilk-Shapiro test, $W=0.684$). Thus, the non-directional asymmetry component of variance consists principally of FA. The results of ANOVAs of the masses of each primary feather uniformly showed no directional asymmetry, except for feather 6, moderate variation between individuals and strong FA (table 3). The results were similar for all feathers combined in a single ANOVA model. Non-directional asymmetry and variation between birds were significant ($F_{24,49}=7.9$ and $p=0.0001$, and $F_{24,24}=4.18$ and $p=0.0004$, respectively). Directional asymmetry was not significant ($F_{1,24}=0.22$ and $p=0.64$).

The variance components (V_{ij}) differed significantly between individuals ($F_{29,38}=5.5$ and $p=0.0001$) and feathers ($F_{9,38}=5.7$ and $p=0.0001$). Multiple comparisons showed that the variance components of feather 1 greatly

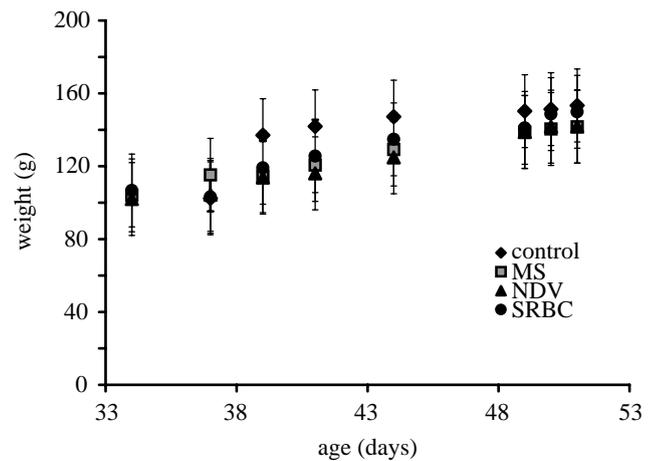


Figure 1. Juvenile Japanese quail growth from day 33 to day 50 for the four antigen treatments.

exceeded those of the other nine feathers (Duncan's multiple range test). There was a general but not consistent trend for decreasing FA index with increasing feather number (table 3). Most of the FA indices for individual birds were less than 10% of the mean feather mass and the range was 0.03–0.33. The FA indices differed significantly between sexes ($F_{1,7}=5.7$ and $p=0.02$), with males being more asymmetrical and between the three experimental groups and the control group ($F_{3,7}=3.1$ and $p=0.05$). Duncan's multiple range test indicated that the *M. synoviae* and SRBC groups had significantly higher FA than the NDV and control groups (table 1). The FA index was correlated with mass ($r=0.38$ and $p=0.04$), but not with wing length ($r=0.16$ and $p=0.39$), haematocrit ($r=0.27$ and $p=0.14$) or total plasma protein ($r=0.16$ and $p=0.47$). The mean secondary feather mass varied between 0.0061 g for secondary 1 and 0.0118 g for secondary 3. Normalized by feather mass, the mean right and left differences in all feathers ($n=150$) was 0.0066 (i.e. -0.7%) and the standard deviation of the distribution was 0.322. The masses of the secondary feathers did not exhibit significant directional asymmetry ($F_{1,29}=0.05$ and $p=0.83$) but did differ significantly between individuals ($F_{29,29}=2.45$ and $p=0.009$). We could not assess non-directional FA statistically because feathers were not remeasured. The variance components (V_{ij}) did not vary significantly between either individuals ($F_{29,33}=1.34$ and $p=0.14$) or feathers ($F_{4,33}=1.54$ and $p=0.20$). The FA index did not differ with respect to antigen treatment ($F_{3,25}=0.48$ and $p=0.70$) or sex ($F_{1,25}=0.00$ and $p=0.99$). The FA indices for primary and secondary feathers were not correlated ($r^2=0.07$ and $p=0.15$). The FA indices for secondary feathers were also not correlated with the normalized maximum immune response for each bird ($F_{1,2}=2.94$ and $p=0.23$).

(e) Haematology

The haematocrit values for females (41.2 ± 0.04 , range 33–51% and $n=116$) and males (45.9 ± 0.05 , range 31–58% and $n=87$) differed significantly ($F_{1,161}=58.79$ and $p=0.0001$). A significant time \times sex interaction ($F_{6,161}=7.71$ and $p=0.0001$) indicated that the haematocrit increased at a different rate for each sex. For the control birds, the female haematocrits

Table 3. *Analysis of variance results for individual primary feathers*

(Bird \times wing/error estimates non-directional asymmetry or FA, bird/bird \times wing (as the error term) estimates size variation between individuals and wing/bird \times wing estimates directional asymmetry. The table contains *F*-values with (significance). The FA index for each feather is presented on the bottom row.)

	feather									
	1	2	3	4	5	6	7	8	9	10
bird \times wing/error	813.62	900.00	390.54	215.87	158.33	31.58	183.46	257.19	60.77	230.36
non-directional FA	(0.0001)	(0.0001)	(0.0001)	(0.0001)	(0.0001)	(0.0001)	(0.0001)	(0.0001)	(0.0001)	(0.0001)
bird/bird \times wing	1.78	2.18	2.47	5.40	5.36	4.66	7.97	2.00	3.77	7.75
variation between birds	(0.0825)	(0.0313)	(0.0153)	(0.0001)	(0.0001)	(0.0002)	(0.0001)	(0.0483)	(0.0009)	(0.0001)
wing/bird \times wing	0.80	0.03	0.01	0.67	0.16	3.05	0.39	0.40	0.13	0.42
directional asymmetry	(0.3810)	(0.8690)	(0.9435)	(0.4221)	(0.6946)	(0.0935)	(0.5369)	(0.5340)	(0.7171)	(0.5240)
FA index	1.0	0.14	0.12	0.12	0.12	0.10	0.07	0.14	0.09	0.05

increased more slowly than males. Haematocrit values differed significantly between treatments ($F_{3,167}=3.75$ and $p=0.012$) with the control and *M. synoviae* groups being higher than the NDV and SRBC groups (Duncan's multiple range test, table 1).

Total plasma protein did not differ significantly between females (4.26–2.4 g dl⁻¹, range 0.6–12 and $n=97$) and males (4.89–2.3 g dl⁻¹, range 0.5–12.5 and $n=73$) ($F_{1,134}=3.28$ and $p=0.07$). The absence of a significant time \times sex interaction indicates that changes in plasma protein with respect to age were parallel in males and females. There was a significant time effect for plasma protein ($F_{6,134}=3.22$ and $p=0.005$) with the plasma decreasing around five to eight days post-immunization and then increasing. The control birds showed a similar trend. The plasma protein values differed significantly between the treatments ($F_{3,134}=6.04$ and $p=0.0007$) owing to reduced values for individuals challenged by SRBCs (Duncan's multiple range test, table 1). Haematocrit and total plasma proteins were not significantly correlated at any age ($r^2 < 0.266$).

4. DISCUSSION

This study has showed that non-pathogenic antigen challenges reduce weight and wing length and increase FA in primary feathers in juvenile Japanese quails. These results are consistent with those of Klasing *et al.* (1987), who found that experimental immunization of chicks of domestic poultry reduced their subsequent growth rates shortly after hatching. Apparently, mounting an immune response imposes a cost expressed in terms of impaired development. Thus, immune responses may be regarded as stresses in growing chicks. Therefore, the costs of mounting immune responses may be critical, not only during periods of stress, for example in conjunction with poor nutrition, but also during development. Considering the relatively small increase in cell proliferation and immunoglobulin production, the costs of mounting an immune response are unlikely to result from the reallocation of limited energy, protein or other nutrients.

T-cell-dependent (SRBC) and T-cell-independent (NDV and *M. synoviae*) responses may result in different growth and physiological responses. Haematocrits were significantly lower in SRBC- and NDV-challenged birds, while total plasma protein was reduced only in SRBC-challenged

birds. However, there was no consistency between haematological variables and the type of antigen challenge. Responses to PHA differed between *M. synoviae*- and NDV-treated young, with control and SRBC birds being intermediate. That is, immunization with the various antigens affected the cell-mediated response of the birds differently.

Increases in mass and the length of the wing were lowest in NDV- and *M. synoviae*-challenged (T-cell-independent) birds. In this study, the effect of antigen challenge on an increase in mass was apparent within five days post-injection and appeared to persist for one week thereafter. Between ten and 15 days post-injection, the masses of chicks in the different treatments converged towards the same adult mass (figure 1). By 20 days post-injection (age 54 days) there were no significant differences in either body mass or wing length between the control and antigen treatments. Thus, the depression of growth associated with immune responses appears to be transient. This is consistent with the high degree of flexibility of growth and maturation in Japanese quail chicks in response to food restriction and the ability of chicks to restore normal growth following realimentation (Schew & Ricklefs 1998).

A fairly clear pattern was apparent in the variation in WBC counts over time. Each treatment elicited an increase in total WBCs, peaking one week after immunization. Differences between treatments for all blood cell categories were evident only on day 6 post-immunization. Another clear pattern was that birds with T-cell-independent vaccinations all had higher blood cell counts for all cell categories on day 6. Control birds had the lowest mean blood cell count for all categories except monocytes. In this case, vaccination with T-cell-independent antigens caused a WBC proliferation which peaked at day 6 post-immunization and returned to normal by day 9 post-immunization.

A remarkable result of this study was the substantial increase in the FA index for the primary feathers in response to *M. synoviae* and SRBC challenge. Although the FA index was correlated with body mass over all treatments, NDV-challenged chicks showed no increase in FA in spite of suffering the greatest reduction in growth of any of the antigen treatments. This suggests that immune responses may interfere with growth and development in highly specific ways, possibly involving disruptions of hormonal and other controls over growth. FA was highest for the inner primary feathers and

decreased towards the distal feathers. Indeed the left-right differences for the inner primaries were so great as to obscure differences between individuals in the weights of the feathers. On the whole, the FA indices for individual feathers were correlated within individuals, suggesting that developmental controls influence sets of feathers in concert. Another interesting finding was that the FA index of the secondary feathers of the wing greatly exceeded that of the primaries but did not exhibit significant differences with respect to antigen treatment. These feathers are of similar length to the primaries and elongate during the same portion of the development period as the primaries (R. E. Ricklefs, unpublished data). However, the mean mass of each secondary feather is much smaller than the average primary feather. The secondary feathers had a higher measurement error, as weights were not repeated. Thus, the randomness of asymmetry is enough to mask any correlations (Whitlock 1998).

It is clear that exposure to antigens creates stress on an organism resulting from the immune response itself, over and above any direct effects of the antigen or its pathogen bearer on physiology. However, these effects can be rather different depending on which part of the immune system is activated by each antigen. This immunological stress may cause hormonal and metabolism changes that depress growth and metabolism. Although Japanese quail chicks appeared to recover from transient immunological stress during the growth period, other types of birds with less flexible development programmes may suffer permanent changes in structure and function (Desai & Hales 1997; Gebhardt-Henrich & Richner 1998). Svensson *et al.* (1998) have shown with blue tits (*Parus caeruleus*) some support for the idea that there may be a trade-off between immunocompetence and energetically costly activities such as thermoregulation and reproduction. Studies have not yet addressed the long-term effects of immune responses, particularly during the development period, on adult function. This step is necessary to determine the physiological and fitness consequences of immune responsiveness more generally. Furthermore, other kinds of stresses incurred by individuals in wild populations of animals may add to and compound the costs and long-term effects of an immunological challenge early in development.

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