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Immune responsiveness in adult blue tits: heritability and effects of nutritional status during ontogeny

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Abstract What is the relative contribution of genetic and various environmental factors to variation in the ability to mount an immune response? We measured antibody responsiveness to diphtheria-tetanus vaccine during the winter in free-ranging blue tits with a known nestling history to investigate (1) if nutritional status during the nestling stage has persistent effects on an individual's immune defence and (2) if immune responsiveness is heritable. There was no correlation between nutritional status during the nestling phase (measured as size-corrected body mass day 14 post-hatch) and antibody responsiveness as an adult. On the other hand, the heritability of responsiveness to diphtheria and tetanus, as estimated by parent-offspring regression, was 0.21±0.51 and 1.21±0.40 SE, respectively. Thus, while there was little evidence that natural variation in antibody responsiveness to these antigens reflected nutritional conditions during early life, responsiveness to at least one of the antigens (tetanus) had a strong genetic component.

Keywords Condition · Ecological immunology · ELISA · Immunocompetence · *Parus caeruleus*

Introduction

What are the causes of variation in immune function in natural populations? We distinguish three types of variation: genetic variation, long-term or permanent environmental variation (i.e. inter-individual variation over and above that due to genetic variation), and intra-individual variation (i.e. short-term environmental variation). Here, we will primarily be concerned with long-term environmental and genetic effects.

An understanding of the environmental sources of variation in immune function is of interest in several ecological contexts. For instance, several studies have documented long-term effects of environmental conditions (e.g. nutrition) during early ontogeny on life history and secondary sexual traits expressed later in life, but the mechanisms that mediate these long-term effects have rarely been pin-pointed (Lindström 1999; Metcalfe and Monaghan 2001). One possibility is that the early environment has persistent effects on physiological functions, such as the immune system (Ohlsson et al. 2002). Compromised immune function would be expected to lead to greater susceptibility to infectious diseases, which clearly could affect the expression of life-history and secondary sexual traits. Thus, if there are such long-term effects on immune function, this could mediate environmental effects on fitness.

The question of whether nutritional status during early life affects immune function in natural populations has recently been investigated in birds, and several studies have now shown a correlation between measures of nutritional status (e.g. body mass) and immune function (cell-mediated responsiveness to PHA) in nestlings (reviewed in Alonso-Alvarez and Tella 2001). However, the extent to which these environmental effects persist into adulthood has not been investigated in natural populations. While studies of humans and laboratory animals have shown that malnutrition during ontogeny may compromise immune function later in life (Myrvik 1988), this effect might be less pronounced in natural populations. One obvious reason is that environmental factors experienced after the nestling stage may obscure any relationship between nutritional conditions during ontogeny and immune responsiveness as an adult. Another reason is natural selection. There is often intense survival selection in juveniles on traits that reflect nutritional conditions during the nestling stage (Alatalo et al. 1990; van Noordwijk 1986; Lindén et al. 1992). If the same holds true for immune responsiveness, the

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correlation between nutritional status and immune function observed in nestlings will be reduced when the birds reach the adult stage.

Besides elucidating the different environmental sources of variation in immune function, it would also be interesting to investigate the relative contribution of (additive) genetic and environmental factors, because this determines the (short-term) evolutionary potential of the immune system (e.g. Falconer and Mackay 1996). There are a number of studies of the quantitative genetics of immune function in domestic and laboratory animals (e.g. Parmentier et al. 1996; Boa-Amponsem et al. 1997). However, because most of these studies concern inbred lines, there are good reasons to assume that these heritability estimates are not representative of natural populations. There are as yet only a few studies that have estimated the heritability of immunological traits in natural populations (Brinkhof et al. 1999; Christe et al. 2000; Roulin et al. 2000; Svensson et al. 2001; Tella et al. 2000).

The main aim of the present study was to investigate the role of nutritional conditions during the nestling phase as a source of variation in immune function in adult blue tits (*Parus caeruleus*). To this end, we measured immune function (primary antibody responsiveness to diphtheria-tetanus vaccine) during the winter in free-ranging birds. We used the responsiveness to these antigens as measures of an individual's ability or propensity to mount a humoral immune response in case of an infection, in the same way as others have used sheep red blood cells (SRBC), Newcastle disease vaccine or KLH (Deerenberg et al. 1997; Nordling et al. 1998; Hasselquist et al. 1999). A part of the data set consisted of birds that had been ringed as nestlings in our study area. This allowed us to test for a correlation between nutritional conditions experienced during the nestling phase (measured as body mass corrected for structural size day 14 post-hatch) and antibody responsiveness as an adult. In addition, we used the data set to estimate the heritability of antibody responsiveness (by means of parent–offspring regression).

Materials and methods

Study species and general methods

The blue tit is a small (ca. 11 g), hole-nesting passerine, which mainly inhabits deciduous woodland. Our study site is Revingehed, 20 km east of Lund, southern Sweden. We used c. 400 nest-boxes, which contained 85–207 breeding pairs during the course of this study (breeding seasons 1998–2001). We caught virtually all breeding adults during incubation or while feeding nestlings. On day 14 after hatching we measured body mass (with a Pesola spring balance to the nearest 0.1 g) and tarsus length (with digital callipers to the nearest 0.1 mm; between observer repeatability: $F_{19,40}$=67.1, P<0.001, R=0.96) of nestlings. Both adults and nestlings were equipped with individually numbered aluminium rings.

In the winter, blue tits roost in holes, for example nest-boxes, in or near the breeding territory. During January–February 1999–2001, we caught blue tits in the nest-boxes during the night (1700–0100 hours). We determined sex and age [birds in their second calendar year (2Y birds) vs birds in their third or later calendar year (3Y+ birds)] according to Svensson (1992), measured tarsus length and body mass, and immunised them with diphtheria-tetanus vaccine (see below). Fourteen days after the visit to a certain part of the study area, we again visited all nest-boxes in that area in an attempt to recapture and take a blood sample (see below) from the immunised birds.

Immunisation and ELISA

Birds were immunised with 100 μl diphtheria-tetanus vaccine (SBL Vaccine, Stockholm, Sweden) intramuscularly in the pectoral muscle. Blood samples (100 μl, taken from the jugular vein) were collected in tubes with EDTA and stored on ice until centrifugation (3,000 rpm for 10 min) later the same day, after which plasma was extracted and stored at −20°C until later analysis.

Antibody titres were analysed by an enzyme-linked immunosorbent assay (ELISA). High binding 96-well plates (Costar) were coated overnight with either diphtheria toxoid or tetanus toxoid (3 μg/ml in 0.15 M carbonate buffer). After three washings (0.01 M PBS with 0.05% Tween 20), wells were blocked with 3% skimmed milk powder in 0.01 M PBS with 0.05% Tween 20 for 2 h at room temperature. After two washings, dilutions of plasma (1:6,000 in PBS/Tween 20 with 1% milk powder) were added in duplicates. The plates were incubated overnight at 4°C. The following day, plates were washed 3 times and thereafter incubated for 1 h at 37°C with rabbit-anti-starling (*Sturnus vulgaris*) antiserum (provided by C. Koch, Statens Serum Institut, Copenhagen, Denmark) diluted 1:1,000. After two additional washings, plates were incubated with a peroxidase-conjugated goat-anti-rabbit antibody diluted 1:2,000 (Cat. A 6154, Sigma) for 30 min at 37°C. Finally, plates were washed twice and colour reactions were achieved by the addition of ABTS and H_2O_2 in citrate buffer [200 μl 0.2 mM ABTS (Cat. A1888, Sigma) and 80 μl 30% H_2O_2 diluted 1:50 in ddH_2O, in 20 ml citrate buffer (pH 4.0)]. Kinetics of colour reactions were measured by reading plates every 30 s for 14 min using a Vmax, MAXline microplate reader (Molecular Devices, Sunnyvale, Calif., USA). Antibody titres were obtained as the slope of the substrate conversion over time in the unit millioptical density/min (mOD/min).

To be able to compare antibody titres of samples run at the same time but on different plates, we ran a serially diluted standard (pooled serum from blue tits immune to diphtheria-tetanus), on all plates. This made it possible to recalculate mOD/min values of individual samples to a common "antibody-titre unit". Antibody-titre values were log-transformed to normalise the distribution of residuals. The samples from the different years were run separately (after each field season). We therefore calculated the residuals from an ANOVA with year as factor and antibody titre as dependent variable. These residuals were used in all the statistical analyses.

The immune response to diphtheria-tetanus vaccine in blue tits

In previous studies of the immune response to diphtheria-tetanus in blue tits, virtually all birds have shown a response to both antigens, and the antibody titre peaks after ca. 14 days (Svensson et al. 1998). The responses to diphtheria and tetanus are generally moderately correlated (r≈0.5; Råberg, unpublished data). Thus, the responses to diphtheria and tetanus involve partly different and independent immunological mechanisms.

Clostridium tetani and *Corynebacterium diphtheriae* presumably do not occur in blue tits; at least we have not found any indication that blue tits in our population have been exposed to these antigens previously, as negative controls (plasma from non-immunised blue tits) are uniformly low (Råberg and Stjernman 2003).

Data set and statistical analyses

During the three seasons, we immunised 202, 123 and 166 birds, respectively. We successfully recaptured and blood-sampled 63% of these. Some of the responses were secondary responses; these are not further considered here. The number of primary responses obtained each year was 99, 48 and 75, a total of 222. This is referred to below as "the full data set". Sixty-nine of these birds had been ringed as nestlings in the study area; these were used to test for a relationship between nutritional status during the nestling phase and antibody responsiveness as an adult. In this analysis, we used body mass corrected for structural size (tarsus length) on day 14 after hatching as a measure of nutritional conditions experienced during the nestling phase. Size-corrected mass is commonly used as a measure of nutritional condition in birds as well as other animals (Brown 1996). Following the recommendation by García-Berthou (2001), we included both body mass and tarsus length, rather than the residuals from a regression of body mass on tarsus, in the models when testing for a correlation between nutritional condition and immune responsiveness.

In 20 cases we had a measure of primary antibody responsiveness for both a parent and its offspring. However, four of these offspring were from the same clutch, so we used the average of their responsiveness in the calculation of heritability estimates. Heritabilities (and their standard errors) were calculated as twice the coefficient (or SE) from a linear regression of offspring values on parent values (Falconer and Mackay 1996).

Sample sizes differ slightly between tests because of missing values for morphological variables or age/gender category. All analyses were performed with Systat 9 (SPSS, Chicago, Ill., USA).

Results

The responses to diphtheria and tetanus were moderately positively correlated (r=0.473, n=222, p<0.001).

Age and gender differences in antibody responsiveness

Using the full data set, we first tested for age and sex differences in ab-responsiveness. There were no interactions between gender and age (ANOVA, diphtheria: $F_{1,208}$=0.275, P=0.60; tetanus: $F_{1,208}$=0.062, P=0.80), and no differences between the sexes (diphtheria: $F_{1,209}$=0.27, P=0.60; tetanus: $F_{1,209}$=1.78, P=0.18), but 2Y birds had stronger responses than 3Y+ birds (diphtheria: $F_{1,209}$= 4.52, P=0.035; tetanus: $F_{1,209}$=8.71, P=0.004).

Nutritional conditions during nestling phase

To test for lasting effects of nutritional conditions during growth on antibody responsiveness, we used a general linear model with the following independent variables: Body mass and tarsus length at day 14 after hatching (i.e. size-corrected body mass), gender, and its interactions with the above variables (to investigate if the sensitivity of the immune system to nutritional conditions differed between the sexes), and the age at which an individual's antibody responsiveness was measured (to control for age-related variation in immune responsiveness; see above).

Table 1 Blue Tit (*Parus caeruleus*). General linear models with antibody responsiveness to diphtheria and tetanus against body mass day 14 after hatching, tarsus length day 14 after hatching, age at measurement of antibody responsiveness (2Y or 3Y+), and gender. Non-significant interactions between body mass, tarsus length and gender are excluded. βs are partial correlation coefficients. Excluding non-significant variables in a stepwise manner did not increase the significance of the remaining variable(s). Also, an analysis with either body mass or tarsus length alone as condition index gave very similar results (not shown)

Antibody responsiveness	β	df	F	P
Diptheria				
Body mass	0.072	1	0.22	0.64
Tarsus	0.067	1	0.18	0.67
Age		1	0.97	0.32
Gender		1	2.01	0.16
Error		63		
Tetanus				
Body mass	−0.058	1	0.14	0.71
Tarsus	0.14	1	0.81	0.37
Age		1	4.48	0.038
Gender		1	0.50	0.48
Error		63		

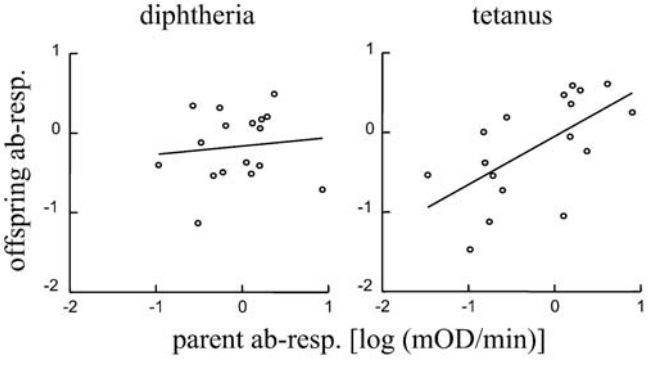

Fig. 1 Blue Tit (*Parus caeruleus*). Parent-offspring regressions for responsiveness to diphtheria and tetanus. For statistics, see text

There was no statistically significant correlation between body mass or tarsus length and antibody responsiveness, and no interactions between these variables and gender (Table 1). The upper 95% confidence limits for the partial correlation between body mass and responsiveness to diphtheria and tetanus were 0.38 and 0.25, respectively.

Heritability

After controlling for the age at which an individual's responsiveness was measured (see above), parent-offspring regressions yielded heritability estimates of 0.21±0.51 (n=17, P=0.69) for diphtheria, and 1.21±0.40 (n=17, P=0.008) for tetanus (Fig. 1). The two heritability estimates are not significantly different from each other (the 95% confidence limits of the difference in slopes are 1.0±1.3).

Discussion

Nestling nutritional status

Studies on a number of bird species have found that cell-mediated responsiveness of chicks is dependent on their nutritional status, and this relationship is often rather strong; the correlation coefficient between body mass and cell-mediated responsiveness in studies of passerine nestlings range between 0.26 and 0.79 (Alonso-Alvarez and Tella 2001). In contrast, we found no evidence that antibody responsiveness of adult blue tits was dependent on the nutritional conditions they experienced as nestlings. The upper 95% confidence limits of the correlation coefficients between body mass as nestling and antibody responsiveness to diphtheria and tetanus as an adult were 0.38 and 0.25, respectively. We cannot exclude the possibility that a larger sample size would allow us to detect a statistically significant correlation between nestling body mass and antibody responsiveness as an adult. Still, the results of the present study indicate that this relationship is considerably weaker than the one between body mass and cell-mediated responsiveness in nestlings. Is this because the effect of nutritional conditions during ontogeny on immune responsiveness fades with time, or because antibody responsiveness (at least to diphtheria-tetanus) is less dependent on nutritional status than cell-mediated responsiveness? To answer this question it would obviously be necessary to measure responsiveness on both nestlings and adults. Unfortunately, measuring antibody responsiveness on nestlings is very demanding logistically because the humoral component of the immune system is not developed until the end of the nestling period (Klasing and Leshchinsky 1998; L. Råberg, unpublished data).

Even if the results of the present study indicate that the contribution of variation in nutritional status as a nestling to variation in primary responsiveness to diphtheria-tetanus during the winter is small, primary antibody responsiveness to these and other antigens can surely be affected by environmental factors. Previous studies of responsiveness to diphtheria-tetanus in blue tits have shown that experimentally induced cold-stress compromises responsiveness (Svensson et al. 1998), while experimental relief from parental duties during nestling feeding enhances responsiveness (Råberg 2002). Similarly, antibody responsiveness to other antigens has been shown to be affected by the level of reproductive effort in birds (Nordling et al. 1998; Hasselquist et al. 1999; Cichon et al. 2001; Saino et al. 2002). Note, however, that all these studies have increased the environmental variation through experimental manipulation. Experiments are useful because they demonstrate the causality of a relationship. However, it is difficult to infer to what extent the manipulated factor contribute to the natural variation in the trait under consideration.

The lack of correlation between nutritional conditions during the nestling stage and antibody responsiveness as an adult is consistent with the results of an analysis of natural selection (survival from winter to the following breeding season) on responsiveness (Råberg and Stjernman 2003). If responsiveness was dependent on some aspect of an individual's condition, one would expect responsiveness to be subject to positive directional selection (cf. Price et al. 1988). However, primary responsiveness to diphtheria was subject to stabilising selection, while there was no significant selection on primary responsiveness to tetanus (see also Råberg and Stjernman 2003).

Heritability

If there was little evidence of environmental variation in antibody responsiveness as a result of nutritional status during ontogeny, the evidence for genetic effects was stronger, at least for responsiveness to tetanus. There are only a few previous studies of the heritability of immune responsiveness in natural population. Svensson et al. (2001) found that the heritability of responsiveness to tetanus in side-blotched lizards was 0.88±0.29, and Roulin et al. (2000) found statistically significant heritability of antibody responsiveness to SRBC in a population of barn owls (*Tyto alba*). Three studies have investigated the heritability of cell-mediated responsiveness (to PHA). In a study of great tit (*P. major*) nestlings, Brinkhof et al. (1999) found a heritability of 0.27 (calculated from their Table 1). In contrast, Christe et al. (2000) and Tella et al. (2000) found that the heritability of this trait in a population of house martins (*Delichon urbica*) and American kestrels (*Falco sparverius*), respectively, was very low and not statistically significant. There are also a few studies that have investigated the heritability of resistance to particular parasites, and these estimates are generally rather high (Möller 1990; Boulinier et al. 1997; Smith et al. 1999). Hence, studies of both responsiveness to non-pathogenic antigens and resistance to particular parasites show that there is often a considerable amount of genetic variation in immune function in natural populations. The studies to date also indicate that antibody responsiveness has a higher heritability than cell-mediated responsiveness. The reason for that requires further investigation.

Concluding remarks

We found no evidence that nutritional conditions experienced as a nestling affected antibody responsiveness as an adult during winter. Indeed, the high heritability of responsiveness to tetanus leaves little room for environmental variation in general. Still, several other studies have shown that antibody as well as cell-mediated responsiveness can be dependent on environmental factors. Clearly, more studies are needed before we can get a general picture of the relative contribution of genetic and various environmental factors to variation in responsive-

ness of different components of the immune system in natural populations.

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