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GENETIC AND ENVIRONMENTAL VARIATION IN ANTIBODY AND T-CELL MEDIATED RESPONSES IN THE GREAT TIT

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Abstract.—Host parasite coevolution assumes pathogen specific genetic variation in host immune defense. Also, if immune function plays a role in the evolution of life history, allocation to immune function should be heritable. We conducted a cross-fostering experiment to test the relative importance of genetic and environmental sources of variation in T-cell mediated inflammatory response and antigen specific antibody responses in the great tits *Parus major*. Cell mediated response was measured during the nestling period and antibody response against two novel antigens was measured in two-month-old juveniles raised in a laboratory. We found no effect of nest of origin, but a strong effect of rearing environment on cell mediated response. In contrast, we found a large effect of nest of origin on antibody response to both, diphtheria and tetanus antigens suggesting genetic variation. In a model where responses to both antigens were analyzed simultaneously, we found a significant origin-by-antigen interaction, suggesting that genetic variation in antibody responses is specific to particular antigens. Large genetic variation in antibody responses found in this study suggests that host immune defense may evolve and specificity of genetic variation in antibody responses suggests that host defense may be pathogen specific as models of host-parasite coevolution suggest. Our results also suggest that different immune traits are to some degree independent and outcome of the interactions between immune function and the environment may depend on the particular immune trait measured.

Key words.—Diphtheria, heritability, immune function, specificity, tetanus.

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Immune defense has become a central research topic in evolutionary ecology (Sheldon and Verhulst 1996; Westneat and Birkhead 1998; Mallon et al. 2003; Schmid-Hempel 2003; Schmid-Hempel and Ebert 2003) because immune function reflects an important aspect of an individual's ability to cope with parasites and diseases. Immune function has been hypothesized to affect residual reproductive value in the context of life-history theory (Sheldon and Verhulst 1996) and sexual advertisement in the context of sexual selection theory (Westneat and Birkhead 1998). Models of parasite mediated sexual selection suggest that male ornamentation has developed to signal a male's ability to resist pathogens. (Hamilton 1980; Hamilton and Zuk 1982; Schmid-Hempel 2003; Schmid-Hempel and Ebert 2003). These models assume that there is genetic variation in host immune defence and this variation is specific to particular parasite types. Also, if immune function can shape the evolution of host life history (Sheldon and Verhulst 1996, Zuk and Stoehr 2002), there should be genetic variation in allocation to immune function.

In birds, knowledge of the genetic control of immune function originates primarily from poultry studies. Selection studies on domestic chickens suggest that a higher general immune responsiveness can be selected (see Bacon 1992) but many immune traits are partly independent and selecting on one immune trait may not necessarily modify other immune traits (Pinard-van der Laan 2002). Moreover, there may be trade-offs between different immune traits and resistance to different pathogens (Gross et al. 1980; Kreukniet et al. 1992; Bumstead and Millard 1992; Wakelin and Blackwell 1993). In wild birds genetic control of immune function is not well understood. Cross-fostering experiments have revealed both the effect of rearing environment and the effect of nest of origin (Saino et al. 1997; Brinkhof et al. 1999; Soler et al. 2003) or only environmental variation in cell mediated response (Christe et al. 2000; Tella et al. 2000). Very few studies have demonstrated an effect of parentage in antibody responses in natural populations of vertebrates (Roulin et al. 2000; Svensson et al. 2001; Råberg et al. 2003). In invertebrates there is some empirical evidence of the pathogen specific genetic variation in host immune defense that is assumed by theories of host parasite coevolution. In *Daphnia* strong host clone by parasite genotype interactions on both host survival and parasite fecundity have been shown (Carius et al. 2001; Decaestecker et al. 2003). However, there are no studies on specificity of immune function in a wild vertebrate population.

We studied genetic and environmental variation in both antibody responses and T-lymphocyte mediated inflammatory response in juvenile great tits. A cross-fostering experiment was conducted to separate the effects of nest of origin (including both genetic and possible maternal effects) and rearing environment to the extent possible. We measured antibody responses against two novel antigens. To partition variation in immune responsiveness into broad sense additive genetic (including early maternal) and environmental variance components for general immune responsiveness and for specificity of immune responsiveness, we used an approach based on the statistical analysis of genotype × environment interactions (Lynch and Walsh 1998; Fry 1992).

METHODS

Study Species and Study Area

The great tit (*Parus major*) is a small, monogamous, sexually dimorphic, hole-breeding passerine bird of deciduous, mixed, and conifereous forests. The study was conducted during the 2000 breeding season in a nestbox population in Konnevesi, Finland (62°37′ N, 26°20′ E). Nestboxes were

regularly checked to determine the start of egg laying, hatching date, and the number of eggs and nestlings.

Cross-Fostering Experiment

To separate common environmental from prehatching maternal and genetic causes of resemblance, a partial cross-fostering experiment was conducted using first clutches only. Half of the two-day-old nestlings were swapped between pairs of nests with the same hatching date. When the number of nestlings was uneven in one of the nests, a number below one-half was swapped. The average number of nestlings brought to each nest of the 28 broods raised in the laboratory was 4.82 (range 4–6). Hatching date was defined as the day on which at least half of the eggs had hatched. Claws of fostered nestlings were painted to distinguish original from fostered nestlings, before they were banded with individually numbered aluminium rings at the age of eight days. When nestlings were 13 days old, randomly chosen fostered and original nestlings, three of each, and both parents were brought to the laboratory. Remaining nestlings were placed in extra nests containing nestlings of the same age. In the laboratory nestlings and their parents were housed in 1 m $\times$ 1 m $\times$ 1 m cages in visual isolation from other families. Families were supplied with food (mealworms, ant pupae, and a mixture of juvenile dog food [Purina trademark], vitamin-enriched tallow, calcium, peanuts, oat grains, and wheat grains) and vitamin-enriched water ad libitum. Two weeks after fledging several broods and their mothers were put together in five large indoor aviaries, matching fledgling ages to reduce the impact of age-related competition among broods. Birds were captured and kept with the permission of the Central Finland Regional Environmental Centre (permission no. 0900 L0312/254) and the Animal Care Committee of the University of Jyväskylä (permission no. 20/29.5.00).

Measurement of T-Cell Mediated Response

T-cell mediated inflammatory response of 13-day-old nestlings was assayed using a phytohemagglutinin (PHA) skin test. The PHA skin test has been used as a standard method to assess cell mediated immunity in poultry (Goto et al. 1978; McCorkle et al. 1980). PHA is a mitogen that stimulates T-lymphocytes to proliferate without previous immunization. The T-cells proliferating release cytokines that recruit granulocytes and other nonspecific mechanisms of the innate immune response. Nestlings were injected intradermally in the center of the right wing web with 0.2 mg of PHA (Sigma, L-8754) in 0.04 ml PBS (phosphate buffered saline pH 7.4). The left wing (control) was injected with 0.04 ml PBS only. Thickness of the injection site was measured with an accuracy of 0.01 mm using a spessimeter just prior and 24 hours postinjection. The swelling of the wing web was calculated as the difference in thickness prior to and after the injection. The cell mediated response was expressed as wing web index (the difference in swelling between the PHA-injected wing and the control wing).

Measurement of Antibody Responses

When juveniles were six weeks old, their tarsus length (to the nearest 0.01 mm) and body weight (to the nearest 0.1 g) was measured. Body condition was calculated as the residual of a regression of body weight on tarsus length. By puncturing the branchial vein 120–150 μl blood samples were collected in microcapillary tubes. Tubes were centrifuged for 5 min at 14,000 rpm to measure the hematocrit and to separate plasma for evaluation of preimmune levels of antibodies. Plasma was stored at $-30°$. Blood sampling birds were injected in the pectoral muscle with 100 μl of diphtheria-tetanus vaccine (Finnish National Public Health Institute, diphtheria toxoid 38 limit of flocculation (Lf) and tetanus toxoid 10 Lf, mixed with adjuvant aluminium phosphate at 1.0 mg ml^{-1}). This vaccine contains two antigens novel to the birds. Blood samples were taken twelve days postinjection to determine the final antibody levels.

Specific antibodies against diphtheria and tetanus were measured using enzyme-linked immunosorbent assay (ELISA). ELISA-plates (Cliniplate EB, Thermolabsystems, Helsinki, Finland) were first coated with antigens (diphtheria or tetanus toxoid, National Public Health Institute, Helsinki). Samples and standards were added into the wells and incubated for three hours at room temperature. After washing the plates, alkaline phosphatase conjugated anti-chicken IgG antibody (A-9171, Sigma Chemical, St. Louis, MO) was added to the wells and incubated overnight at 4°C. Finally, alkaline phosphatase substrate pNPP (p-nitrophenyl phosphate, Sigma 104 phosphatase substrate, Sigma Chemical) in 1 M diethanol amine buffer (1 $mgml^{-1}$) was applied. The absorbance of the plates was read in an ELISA reader at 405 nm. The samples, standards and conjugated antibodies were diluted in 1% BSA (bovine serum albumin, Fraction V, Roche Diagnostics GmbH, Mannheim, Germany) prepared in PBS. One percent BSA-PBS was also used for masking the wells before applying the samples. After each incubation step, the plates were washed three times with PBS 0.05% Tween 20. The assay was calibrated with a series of diluted standard samples applied on every plate. As a standard we used a pooled plasma from all immunized individuals measured. An arbitrary concentration of 10^6 Units ml^{-1} (Uml^{-1}) was given to the standard, and concentration of samples in each assay was expressed as Uml^{-1}.

Because antichicken IgG antibody (Sigma A-9171) has not been used earlier in determination of great tit immunoglobulins, the methodology was validated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and ELISA. Cross-reactivity of Ab with plasma immunoglobulins of great tit was first established in ELISA. Reactivity of the antibody was confirmed using PAGE followed with western blotting and immunostaining with the antibody. Native PAGE showed staining of both IgG and IgM. Under reducing conditions only the light chains were stained.

Statistical Analyses

Estimation of variance components

General linear mixed models (Verbeke and Molenberghs 2000) were used to model variation in immune responses as

a function of several random factors. We used the restricted maximum likelihood (REML) method rather than analysis of variance to obtain unbiased estimates of variance components and used likelihood ratio test statistics to test if variances differ significantly from zero and to simplify models (Littell et al. 1996; Lynch and Walsh 1998; Verbeke and Molenberghs 2000). Unless stated otherwise, estimation of negative variances was allowed to obtain unbiased estimation (Lynch and Walsh 1998).

In a first step, separate univariate analyses were conducted for each of the three different antibody responses with random factors representing pair of nests (Pair), nest of origin (Origin, nested within Pair), and rearing environment (Rearing, nested within Pair), In a second step we analyzed responses to the two antigens simultaneously to model specificity of immune responses using an approach similar to the statistical analysis of genotype × environment interactions (Lynch and Walsh 1998; Fry 1992). For the latter analysis, immune response (against diphtheria or tetanus) was modeled as a function of a fixed continuous variable indicating antigen (Antigen), and random variables Origin × Antigen and Rearing × Antigen. By setting the values of Antigen to −1 (tetanus) and 1 (diphtheria), variation in immune response was split into additive components that have a clear biological meaning (for another application of this idea, see Van Dongen 1999): a random intercept, indicating variation in average immune responsiveness (Origin or Rearing), and a random slope, indicating variation in specificity of immune responsiveness (Origin × Antigen and Rearing × Antigen). Random variables Individual, Pair, and Pair × Antigen were modeled to take into account pseudoreplication within individuals and to model the nested design. Models assuming homogeneous variances were compared with models allowing variances to differ between antigens using Akaike's Information Criterion (AIC): the model with the lowest AIC value is considered to be the best compromise between model complexity and fit (Littell et al. 1996). To investigate whether significant interactions Origin × Antigen and/or Rearing × Antigen could be an artifact of the choice of scale (Lynch and Walsh 1998), analyses of log-transformed and untransformed data were compared.

Heritability, additive genetic coefficient of variation and genetic correlation

Heritabilities, genetic coefficients of variation, and genetic correlations were calculated from the obtained variance estimates. For full sibling analysis, the between-family variance equals the sum of half the additive genetic variance, one quarter of the dominance variance, and variance due to maternal effects (Lynch and Walsh 1998). Assuming that dominance variance and maternal effects are negligible, the heritability (h^2) can be estimated as twice the between-family variance divided by the total variance (Lynch and Walsh 1998). The genetic coefficient of variation (CV_g) is calculated as the square root of twice the between-family variance divided by the trait mean and multiplied by 100 (Lynch and Walsh 1998; Houle 1992). In the presence of a significant interaction between Origin and Antigen, the additive genetic correlation can be calculated in the same way as a genetic correlation between the same character expressed in different environments where environment, in this case antigen, is modeled as a discrete variable (Lynch and Walsh 1998; Fry 1992):

$$r_g = \frac{\sigma^2_{\text{Origin}}}{\sigma^2_{\text{Origin}} + \sigma^2_{\text{Origin}\times\text{Antigen}}},$$

where σ^2_{Origin} equals the within-family covariance between responses to both antigens and $\sigma^2_{\text{Origin}\times\text{Antigen}}$ equals the interaction variance due to variation among families in the difference between responses to the two antigens. This formula is correct, provided among-family variances do not differ between environments (Fry 1992).

Calculation of confidence intervals and significance

We used the bias-corrected bootstrap percentile method to determine confidence intervals and significance levels of h^2, CV_g, and r_g (Lynch and Walsh 1998; Manly 1991; Aastveit 1990). Pairs of nests or nests of origin were resampled depending on whether or not rearing environment significantly contributed to variation in responses. Each of 1000 samples was obtained by randomly drawing subjects with replacement as many times as there were subjects in the original sample.

All analyses were conducted with SAS version 8.01 (SAS Institute, Cary, NC). Models were analyzed using PROC MIXED (see Littell et al. 1996).

Results

For 28 nests of origin six fledglings were kept in aviaries. Some fledglings died before antibody responses were measured such that 26 nests of origin contributed at least four juveniles to the data (four fledglings: $n = 7$ broods; five fledglings: $n = 13$ broods; six fledglings: $n = 6$ broods). Two nests of origin contributed one and three juveniles, respectively. The pair of nests containing the nest of origin with only one observation and its paired nest were dropped to obtain a similar data set for univariate and multivariate analyses ($n = 26$ nests in 13 pairs). As a dependent variable we used the postinjection antibody level rather than the difference between final and initial antibody levels, but all results presented were similar for both measures.

The wing web index and both antibody responses deviated significantly from normality (Shapiro Wilk's test: wing web index: $W = 0.97$, $P < 0.0001$; diphtheria: $W = 0.79$, $P < 0.001$; tetanus: $W = 0.83$, $P < 0.0001$). After log-transformation deviations from normality were nonsignificant (diphtheria: $W = 0.99$, $P = 0.45$; tetanus: $W = 0.99$, $P = 0.45$) or significant but small (wing web index: $W = 0.97$, $P < 0.0001$) as indicated by a W-statistic value larger than 0.95. Therefore, we conducted analyses using log-transformed data.

For the cell mediated response there was a significant effect of rearing environment but no significant effect of nest of origin (Table 1). In responses to both antigens, diphtheria and tetanus toxoid, there was a significant effect of nest of origin but no significant effect of rearing environment (Table 1).

There was significant origin-antigen interaction but not rearing-antigen interaction in antibody responsiveness (Table

TABLE 1. Univariate mixed model analysis of log-transformed immune responsiveness (see statistical analysis in Methods). Wing web index is a measure of T-cell mediated response. Terms significant in a simplified model are in bold.

Source	Estimate	ΔL	*P*
Wing web index			
Pair	−0.0102	0.4	0.53
Origin	0.0035	0.7	0.40
Rearing	**0.0607**	72.81	<0.0001
Residual	**0.0793**		
Diphtheria			
Pair	−0.1489	3.2	0.07
Origin	**0.2405**	28.7	<0.0001
Rearing	0.0250	0.6	0.44
Residual	**0.3610**		
Tetanus			
Pair	0.0188	0.1	0.75
Origin	**0.1019**	11.5	<0.001
Rearing	0.0126	0.2	0.65
Residual	**0.3735**		

TABLE 2. Multivariate mixed model analysis of log-transformed immune responsiveness (see statistical analysis in methods). Terms significant in a simplified model are in bold.

Source	Estimate	ΔL	*P*
Model without covariance between slope and intercept			
Individual	**0.1384**	15.4	<0.0001
Pair	−0.0409	0.7	0.40
Pair × Antigen	−0.0457	1.2	0.27
Origin	**0.1240**	—	<0.016*
Origin × Antigen	**0.0541**	17.1	<0.0001
Rearing	0.0198	0.8	0.37
Rearing × Antigen	0.0007	0.0	1.00
Residual	**0.2268**		
Model with covariance between slope and intercept			
Cov(Intercept, Slope)	0.0327	2.1	0.15

* Significance determined from bootstrapped data.

2). This is illustrated in Figure 1: the difference in immune responsiveness against tetanus and diphtheria varies more strongly among nests of origin than among common rearing environments. This suggests that genetic variation in antibody responsiveness is specific to particular antigens. There was no significant covariance between random intercepts and slopes in the log-transformed data, but the opposite was true for untransformed data. This suggests that the covariance was mostly due to a scale effect. Importantly, for log-transformed data Origin × Antigen was significant in the model allowing random intercepts and random slopes to covary. The origin-antigen interaction could thus not be explained as an additive effect on a log-scale. The data showed a better fit to a model assuming homogeneous variances (AIC = 518.0) than to a model allowing antigens to have unequal origin-related and residual variances (AIC = 522.1) and a model allowing antigens to have unequal residual variances (AIC = 520.0)

For estimates of h^2, CV_g, and r_g, we only report results for untransformed data, but results for log-transformed data were qualitatively similar. Heritabilities and genetic coefficients of variation were large and significant for tetanus and diphtheria, but small and nonsignificant for cell mediated immune responsiveness (Table 3). The genetic correlation between immune responses for both antigens was positive but nonsignificant.

DISCUSSION

We found a large effect of nest of origin on antibody response for both novel antigens studied: origin-related variation was significant both for general immune responsiveness (average response to two antigens) and for specificity of immune responsiveness (significant origin by antigen interaction). Hence, our results provide evidence that genetic variation in antibody response is specific to particular antigens. However, variation in cell mediated response was strongly affected by rearing environment but not by nest of origin.

The interpretation of our results depends on several factors. First, separating additive genetic effects from prehatching maternal effects is impossible with our experimental design. For example maternal antibodies can both improve the strength of offspring immune responses and affect the diversity of immune repertoire of the offspring, although the physiological mechanisms behind these effects are not fully understood (see Grindstaff et al. 2003). Hence, our results represent broad sense heritabilities including nongenetic maternal effects. Neither can we rule out the possibility that the antibody response to tetanus did not affect the response to diphtheria and vice versa (Graham 2002). However, because concomitant infections are common in nature our design is biologically relevant. Further studies are needed to investigate the effects of single antigen versus multiple antigen injections. Contrasting results for cell mediated response and antibody responses in our study could be due to the fact that antibody responses were measured at a later life stage. Also, selection after the nestling period could have reduced rearing-environment related variation through the removal of low-condition individuals from the dataset. However, mortality of 17% after fledging in our study can be considered small or moderate. Moreover, previous field studies suggest that cellular immune responses are often positively correlated with condition both in nestlings and in fully grown birds (Saino et al. 1997; Birkhead et al. 1999; Lifjeld et al. 2002), whereas antibody responses may be less constrained by condition (Råberg et al. 2003). Our estimates of origin-related variation for antibody response could be biased upward due to the reduced environmental variation in laboratory conditions or due to reduced mortality in the laboratory compared to mortality in the field (Riska et al. 1989). However, laboratory experiments often provide reasonable estimates of both magnitude and significance of heritabilities in nature (Weigensberg and Roff 1996; Hoffmann 2000).

Even though estimates of heritabilities and additive genetic coefficients of variation differed between antigens in the univariate analyses, their confidence intervals overlapped considerably (Table 3). Thus, the magnitude of additive genetic variation for both antigens was comparable. Compared with heritabilities for other traits in several organisms our estimates for antibody responses are large (Weigensberg and Roff 1996; Hoffmann 2000). The genetic correlation between responses against both antigens was moderate compared with genetic correlations between other traits (Roff 1996). Lack

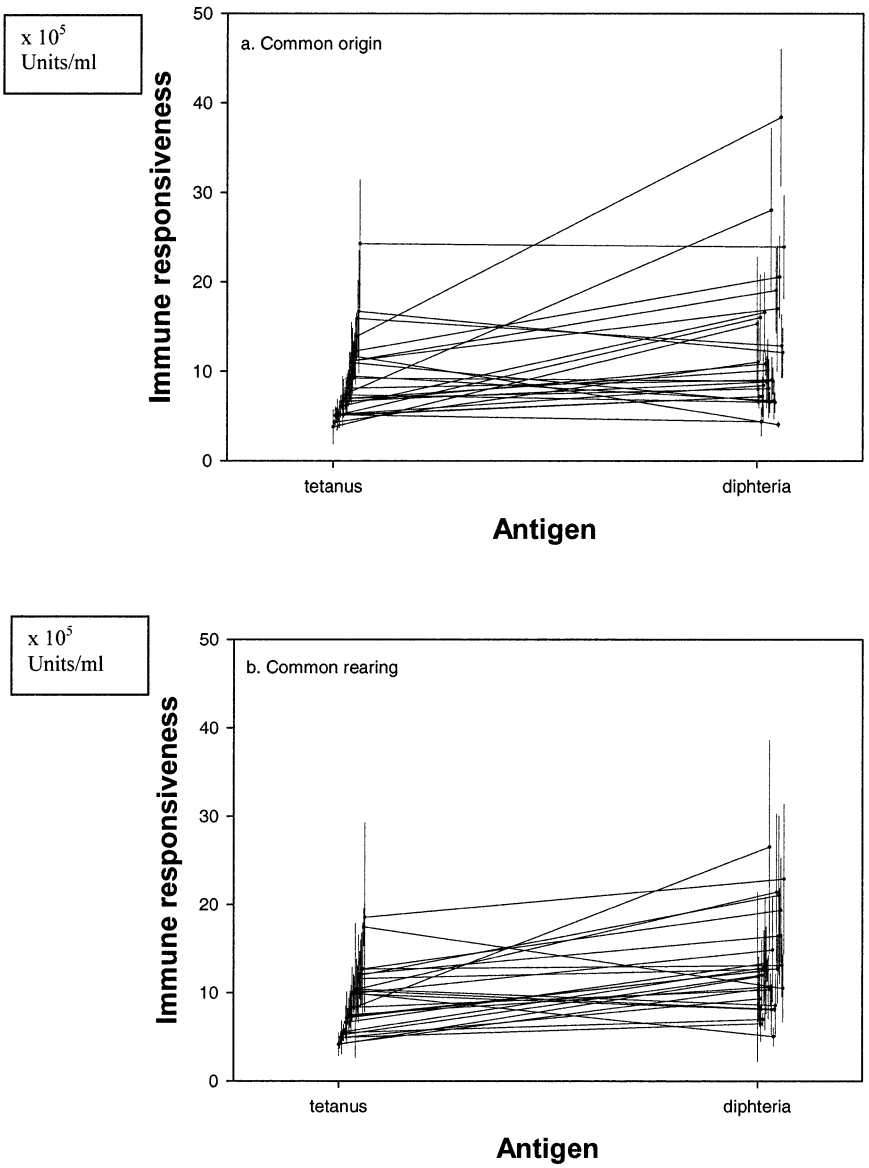

FIG. 1. Untransformed immune responsiveness against tetanus and diphtheria. Lines connect means and standard errors for (a) nests of origin and (b) rearing environments. Nests are ranked according to mean responsiveness for tetanus.

TABLE 3. Heritabilities, additive genetic coefficient of variation, and genetic correlation for untransformed immune responsiveness. For the T-cell mediated response (wing web index) pairs of nests ($n = 22$) were resampled and parameters were estimated from a model containing Origin and Rearing as random factors. For diphtheria and tetanus nests of origin ($n = 26$) were resampled and parameters were calculated from a model containing Origin as a random factor.

Model	Parameter	Estimate	Bootstrap CI	Bootstrap *P*
Wing web index	mean ± SD	62.92 ± 22.30		
	h^2	0.07	−0.02; 0.19	=0.15
	CV_a	9.26	0; 15.35*	=0.15
Diphtheria	mean ± SD	13.17 ± 11.25		
	h^2	0.79	0.27; 1.09	<0.006
	CV_a	76.00	33.44; 91.10	<0.009
Tetanus	mean ± SD	9.21 ± 6.82		
	h^2	0.56	0.10; 0.75	<0.010
	CV_a	56.19	18.40; 69.00	<0.012
Diphtheria–Tetanus	*r*	0.38	−0.21; 0.77	=0.157

* Negative variance estimates were set to zero.

of significance is certainly due in part to the small sample size; it is well known that the power for detecting significant genetic correlations is low (Lynch and Walsh 1998). The significant genetic variation for specificity suggests that antibody response to the two antigens is at least partially under independent genetic control. Hence, the quality of host immune function may not only depend on host genotype but also on the genotype of parasites at the given time. We do not know what fitness consequences genetic variation in antibody responses against tetanus and diphtheria antigens have. Nevertheless, large genetic variation in antibody responses found in this study suggests that host immune defense may evolve and specificity of genetic variation in antibody responses suggest that host defense may be pathogen specific as models of host-parasite coevolution suggest.

Variation in cell mediated response was strongly affected by the rearing environment but not by nest of origin, in contrast to antibody response. Our results suggest that nestling PHA response is a plastic trait that is environmentally determined and probably reflects nutritional state affected by the quantity and the quality of food provisioned by the parents. Low additive genetic variation in cell mediated response may also imply that this trait is under strong directional selection. PHA response is often positively correlated with survival in birds (Moller and Saino 2004).

To conclude, our results on antibody responses provide evidence of pathogen specific genetic variation in immune function assumed by models of host parasite coevolution and models of parasite mediated sexual selection. Our results also suggest that different immune traits are partly independent and the outcome of the interactions between immunity and the environment may depend on the particular immune trait measured. Our results stress the complexity of the vertebrate immune system and the importance of considering which immune traits to measure in the immunoecological studies.

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