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Environmental and genetic variation in T-cell-mediated immune response of fledgling American kestrels

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Abstract We investigated genetic and environmental components of variance in avian T-cell-mediated immune response (CMI) through a cross-fostering experiment conducted on wild American kestrels (*Falco sparverius*). CMI was evaluated in vivo by an experimental challenge with phytohaemagglutinin, a T-cell mitogen, injected intradermally in fledglings. Additionally, we assessed two measures of nutritional condition (body mass and circulating plasma proteins) which could influence the variance components of CMI. A two-way nested ANOVA indicated that CMI of fledgling kestrels was explained more by the nest where the bird was reared (33% of the explained variance) than by the nest of origin (12%). Body mass was explained equally by familial and environmental components, while plasma proteins were only related to the rearing environment. CMI of fledglings was not related to their circulating plasma proteins, but was positively correlated with their body mass. Fledgling body mass seemed to be influenced by pre-hatching or post-hatching maternal effects prior to manipulation since resemblance in body mass of sibships at the age of manipulation was high ($h^2 \leq 0.58$), and body mass at this age predicted body mass at fledging. Therefore, pre-manipulation parental effects on body mass, such as investment in egg size, could have inflated the familial effects on body mass of fledglings and then on its correlated CMI. When controlling for body mass, most of the variation in CMI of fledglings was explained by the nest where the bird was reared (36.6%), while the variance explained by the nest of origin (4%) was not significant. This means that environmental influences are major determinants of offspring CMI. The low proportion of variance explained by the familial component may have been due to the high correlation of CMI to fitness.

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Introduction

Conditions experienced during early life are known to affect a number of long-term fitness components in vertebrates (Bernardo 1996; Lindström 1999; Potti 1999). Many phenotypic traits of adults are influenced by conditions experienced during development, which result from the combination of genetic, environmental, and maternal components (Falconer 1989). Determining the relative contribution of these components to a trait is important for a better understanding of the trait's potential susceptibility to natural selection (Mousseau and Roff 1987). Birds have been extensively used for disentangling the components of phenotypic variance for traits such as body size, body mass, and physiological variables of fledglings (e.g., Potti and Merino 1994; Merilä 1996, 1997; Potti 1999; Potti et al. 1999), which are usually thought to influence their future survival and reproductive prospects (e.g., Both et al. 1999; Moreno et al. 1999a; Potti 1999).

More recently, immunocompetence, i.e., the ability of an organism to mount a successful immune response to an infection, has been considered as a valuable measure of viability in birds. Recent studies have shown relationships between T-cell-mediated immune response (CMI) of fledglings and conditions experienced during growth at the nest (Saino et al. 1997a, 1998; Sorci et al. 1997a; Christe et al. 1998; Soler et al. 1999a). Since T-lymphocytes are fundamental components of the immune system, and a wide array of pathogens and parasites are known to affect survival of their avian hosts, the ability to mount a CMI may influence the survival prospects of birds. In fact, a positive correlation between immunocompetence and survival has been demonstrated recently both for nestling (Christe et al. 1998) and adult birds (Saino et al. 1997b; Soler et al. 1999b). Saino et al. (1997a) showed through a cross-fostering experiment

that the CMI of fledgling barn swallows (*Hirundo rustica*) was related to rearing conditions such as diet quality, brood size and parental effort, but also to the nest where chicks were hatched. These results suggested the existence of maternal and/or additive genetic components of variance for this measure of immunocompetence, but its relative contribution versus environmental effects were not determined. Although work done on poultry has shown some genetic control of the immune system (e.g., Taylor et al. 1987; Cheng and Lamont 1988), as far as we are aware the genetic component of variation in immunocompetence has never been studied in a wild bird population.

Here, we addressed the estimation of environmental and genetic components of variance on avian CMI, one of the three main branches of the immune system (Pastoret et al. 1998), using a free-ranging population of American kestrels (*Falco sparverius*). We conducted a cross-fostering experiment to partition the variance in immune response into familial and environmental components. Because resemblance between relatives under natural conditions may arise from a variety of non-genetic causes (Falconer 1989), switching chicks between nests allows one to break the correlation between genetic and environmental effects (e.g., Smith and Wettermark 1995; Merila 1996, 1997; Potti et al. 1999). We measured T-cell-mediated immunocompetence through the phytohaemagglutinin (PHA) skin-testing technique (Goto et al. 1978; Smits et al. 1999). It is based on the injection of a mitogen (PHA) under the skin of birds, which produces a prominent perivascular accumulation of T-lymphocytes followed by macrophage infiltration (Goto et al. 1978; McCorkle at al. 1980). This technique has been routinely used to evaluate in vivo thymus-dependent immune function in poultry (e.g., Tsiagbe et al. 1987; Cheng and Lamont 1988). More recently, the intensity of skin swelling as a response to PHA injection has been proven to correlate with a number of components of fitness in free-living birds (Saino et al. 1997a, 1998; Sorci et al. 1997a; Christe et al. 1998; Moreno et al. 1998, 1999b; Gonzalez et al. 1999a, b; Merino et al. 1999; Soler et al. 1999a, b; Zuk and Johnsen 1999). Additionally, since T-cell-mediated immunocompetence is known to be correlated with dietary protein intake and body mass (e.g., Lochmiller et al. 1993; Saino et al 1997a), we also measured the body mass and circulating plasma proteins of fledglings to assess their potential influence on variance components of the immune response.

Materials and methods

Cross-fostering experiment

The study was conducted in 1997 in the boreal forest of northcentral Saskatchewan, Canada, in the vicinity of Besnard Lake (55°N, 106°W), where a population of American kestrels breeding in nest-boxes has been studied for several years (e.g., Bortolotti and Iko 1992; Wiebe and Bortolotti 1994,1995; Dawson and Bortolotti 1997). We determined nesting chronology by repeated visits to all boxes with an interval of 2 days, starting 2 weeks before the first pair laid eggs. Once the first egg was laid, we waited until the clutch was complete to visit again to record clutch size. We revisited each nest at the predicted time of hatching to determine accurately hatching date and hence nestling age. We manipulated nests when the oldest chick was 5 days old, which assured us all chicks had hatched, and avoided disturbance during the hatching span which could have provoked stress and even the death of hatchlings (Wiebe and Bortolotti 1994). Nestlings were individually marked with waterproof markers, weighed (to the nearest gram), ranked by size, and then two of them were randomly transferred between nests (n=44) matched by hatching date and clutch size. The remaining nestlings not transferred between nests were removed and transported in the same manner, and for the same length of time, as their siblings, and then put back in their nests to control for potential effects of handling stress. Each nestling was banded at 12 days old, and weighed again. Twenty-two unmanipulated nests were used as a control. As a result of this design, all nests remained with the original number of young hatched, so that breeding chronology, breeding effort of adults, and intra-nest competition of nestlings could be held constant [see Potti et al. (1999) for a similar approach]. Although broods were created where approximately the same numbers of nestlings from two different families were raised together in the same nest, mortality during the nestling stage due to starvation and predation resulted in unbalanced sample sizes.

Immunocompetence and nutritional condition

We sampled fledglings at 22 days old, just prior to nest departure (American kestrels usually begin to fledge at 25 days old in our population; G. R. Bortolotti and R. D. Dawson, unpublished data). To evaluate CMI, we injected 50 µl of 1 mg/ml PHA-P (Sigma) in phosphate buffered saline (PBS) intradermally in a marked site of the right wing web. The left wing web was injected with an equal quantity of PBS as a control. We measured the thickness of the marked sited in each wing web with a micrometer (nearest 0.001 mm) 4 times just prior to and 24 h (±16 min) after injection. As repeatability of measurements on individuals was high (r=0.99; Smits et al. 1999), the mean was used for statistical analysis. The CMI was the difference in wing-web swelling between the mitogen-injected and control sites 24 h after injection (Smits et al. 1999). We also recorded body mass and length of the tenth primary (to the nearest mm) of 22-day-old birds. As an index of body condition we calculated the residuals from a linear regression of body mass on the cube length of the tenth primary (r=0.44, $F_{1,140}$=15.15, P<0.001). In addition, just prior to the mitogen injection we took about 0.2 ml blood from the brachial vein, kept it in a cooler, and centrifuged it within 6 h after collection. Total plasma protein concentration was then estimated using a refractometer (Model 10400 A, American Optical Corporation, Keene, N.H.) [see Dawson and Bortolotti (1997) for more details].

Statistical analyses

Genetic and environmental effects on offspring CMI, body mass, and plasma proteins were estimated following a widely used approach (e.g., Merilä 1996; Potti et al. 1999). This consists on a two-factor nested ANOVA where the main effects were duplicate (a pair of nests matched by clutch size and hatching date) and nest of origin (nested within duplicates). The term "duplicate" accounts for any differences between pairs of nests. Within duplicates, the variation due to nest of origin estimates variation attributable to half of the additive genetic variance transmission (1/2 V_A), but also includes a quarter of the dominance variance (1/4 V_D) and any pre-manipulation parental effects (V_P) if present. The term nest of rearing estimates the effects of the common environment (V_{EC}), and error variance equals random environmental variations (V_{EI}) plus 1/2 V_A+ 3/4 V_D. Analyses were run with PROC GLM in SAS

(SAS Institute 1988), using type III sums of squares due to unequal family sizes. Both duplicate and nest of origin were considered random effects, and therefore appropriate sums of squares and *F*-statistics were calculated by using RANDOM in SAS (SAS Institute 1988).

To assess whether CMI was related to variables that could obscure the above analyses, we previously performed generalized linear models (GLM) using the GLIM package (Crawley 1993). For each fledgling, the explanatory variables were laying date, clutch size, ranked hierarchy within brood at 5 days old (rank 1 to the largest nestling, and so on), and sex, size (tenth primary length), mass, body condition, and presence or absence of mortality in the nest at 22 days old. Results showed that CMI was only related to body mass and condition, and to clutch size (Tella et al. 2000). On the other hand, plasma proteins of fledglings only varied with sampling date (Dawson and Bortolotti 1997). Therefore, since in our experiment duplicates were matched by clutch size and hatching date, only body mass needed to be controlled for when dealing with CMI.

To investigate whether pre-manipulation parental effects on offspring body mass at 5 days old could have affected CMI at fledging, we calculated the intraclass *r* which estimates the fraction of total phenotypic variance attributable to factors causing resemblance between sibs of the same family. Variance components were obtained by one-way ANOVA (Lessells and Boag 1987), and the SE of the intraclass *r* following Becker (1984). Heritability is estimated as twice the intraclass *r* of chick measurements (Falconer 1989). These analyses were performed with SPSS (Norusis 1991). All statistics are two-tailed.

Results

We first assessed potential effects of our cross-fostering experiment. There were no differences between control and experimental nests in the number of chicks that died between hatching and fledging (respective means±SD: 0.77±1.02, n=22, and 0.48±1.11, n=44; t-test=–1.0, P=0.30). Average body mass of fledglings per nest was slightly higher in control (127.7±6.66 g, n=22) than in experimental nests (122.9±7.62 g, n=42); however, these differences were not significant when controlling for clutch size and brood size ($F_{1,56}$=0.90, P=0.41). Total plasma proteins did not differ between control (3.14±0.27 g/dl, n=22) and experimental nests (3.17±0.27 g/dl, n=42; t-test=0.48, P=0.63). Because of logistic constraints, we were unable to measure CMI in control nests. However, CMI was unlikely to be affected by our experiment given the above results.

The two-factor nested ANOVAs, performed on data from experimental nests, showed that variation in CMI was significantly explained both by the nest where the bird was reared (33% of the variance) and by the nest of origin (12% of the variance) (Table 1). Body mass at fledging was equally explained by these two factors (20% and 18%, respectively), although the effect of nest of rearing only approached significance (Table 1). A nearly identical result was obtained for body condition (Table 1). However, all measured variation in circulating plasma proteins was explained by the nest where the bird was reared (37%), not by the nest of origin (0%) (Table 1).

The CMI of fledglings was not related to their circulating plasma proteins (r=–0.003, n=131, P=0.97); however, fledglings with higher body mass had larger CMI responses (r=0.25, n=131, P=0.003; Fig. 1). Fledgling body mass seemed to be influenced by genetic and/or common environmental effects prior to manipulation, since the intraclass correlation coefficient for body mass among 5-day-old sibships was highly significant (r=0.29, $F_{31,130}$=2.67, n_0=4.05, SE=0.06, P<0.001). This set an upper limit of heritability (h^2) to 0.58 (SE=0.12). Furthermore, body mass at 5 days (day of manipulation) predicted body mass at 12 days (r=0.83, n=131, P<0.001), and at 22 days (r=0.32, n=131, P<0.001) when CMI was measured. Therefore, pre-manipulation parental effects on body mass could have inflated the variation in CMI attributable to the nest of origin (Table 1). To control for this possibility, we performed a nested ANOVA on the residuals from a linear regression of CMI on body mass (r=0.25, $F_{1,129}$=8.96, P<0.003). The results (Table 2) indicated that variation in CMI was then explained by the nest where the bird was reared (36.6%), and the variance explained by nest of origin (4%) was not significant. This means that, when

Table 1 Results from two-way nested ANOVAs of T-cell-mediated immune response (CMI), body mass, body condition, and plasma proteins of fledglings in cross-fostered broods of American kestrels. Data are analyzed in relation to rearing environment and family of origin (nested within rearing). *df* are: origin, 30; rearing, 29; residual, 71; model, 59

Trait	Source of variation	SS	MS	*F*	*P*	r^2 (%)
CMI	Origin	14.89	0.49	1.63	0.048	11.99
	Rearing	36.29	1.25	2.52	0.007	32.98
	Residual	21.66	0.30			
	Model	51.18	0.87	2.84	0.0001	70.25
Body mass	Origin	4051.91	112.37	1.64	0.034	19.97
	Rearing	6383.20	182.38	1.62	0.077	18.12
	Residual	5708.67	68.78			
	Model	10594.43	149.22	2.17	0.0004	64.98
Body condition	Origin	3864.52	107.65	1.74	0.019	16.96
	Rearing	6112.69	174.65	1.63	0.075	19.64
	Residual	5117.69	61.66			
	Model	10039.58	141.40	2.29	0.0002	66.24
Plasma proteins	Origin	3.07	0.08	1.07	0.397	0.0
	Rearing	10.62	0.30	3.56	0.0001	37.22
	Residual	6.64	0.08			
	Model	13.40	0.19	2.36	0.0001	66.85

Table 2 Results from the two-way nested ANOVA of CMI after controlling for fledgling body mass in cross-fostered broods of fledgling American kestrels. *df* as in Table 1

Source of variation	SS	MS	*F*	*P*	r^2 (%)
Origin	12.069	0.402	1.34	0.155	4.23
Rearing	34.562	1.192	2.96	0.002	36.58
Residual	21.262	0.299			
Model	46.853	0.794	2.65	0.0001	68.78

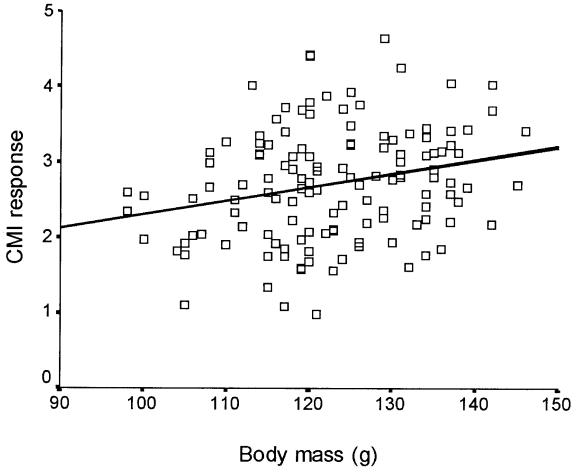

Fig. 1 Significant positive relationship between T-cell-mediated immune response (CMI) and body mass of 131 fledgling American kestrels. The regression equation was CMI=0.494+0.08 (SE=0.006)×body mass

controlling for body mass, there was a very small, non-significant resemblance between the CMI of genetically related sibs when reared in different environments.

Discussion

Some genetic components of the immune system have been identified by studying poultry in captivity (e.g., Lamont et al. 1987; Taylor et al. 1987; Cheng and Lamont 1988), but these results may not necessarily be applicable to wild species. First, domestic fowl have been strongly selected for genetic resistance to disease to increase production efficiency (Gavora and Spencer 1983; Warner et al. 1987). Furthermore, heritability estimates of most traits are usually higher when measured in the laboratory as compared with estimates from the field (Sorci et al. 1997b). This discrepancy has important evolutionary implications, since the response to selection depends on both genetic and environmental sources of variation (Falconer 1989).

Parent-offspring regressions suggested heritable susceptibility to ectoparasites in two populations of wild birds (Møller 1990; Boulinier et al. 1997), and hence maternal and/or additive genetic components on some undetermined components of the immune system were suspected to play a role in this. More specifically, Saino et al. (1997a) found evidence for the existence of an unmeasured amount of familial effects on CMI of barn swallows. Our first results suggested that there was a significant familial component in such a measure of the immune system, accounting for 11% of the variance, while the environmental component was threefold higher. However, nutritional aspects may have masked these first estimates of variability on cellular immunocompetence. Relationships between nutrition, development of immune system organs, and immunocompetence are well known for some organisms (Gershwin et al. 1985). More concretely, studies conducted both in the laboratory (Glick et al. 1983; Lochmiller et al. 1993; Birkhead et al. 1999) and in the wild (Saino et al. 1997a) showed higher CMI for nestlings with supplemented dietary proteins. We found that all measurable variance in circulating plasma proteins of fledgling kestrels was attributable to rearing environment; however, plasma proteins were not related to CMI of fledglings. This may have been due to the fact that total plasma proteins do not only reflect dietary proteins (Butler 1971), or the possibility that the availability of proteins in the kestrel diet (primarily small mammals) is at a threshold above which there is no significant increase in CMI (see Lochmiller et al. 1993). Whatever the explanation, this nutritional measure is unlikely to have biased our estimates of CMI variance. However, body mass of fledglings seems to have inflated the familial component of variance on CMI.

As is the case for other wild avian species (Saino et al. 1997a; Sorci et al. 1997a; Christe et al. 1998; Merino et al. 1999; Soler et al. 1999a), body mass, and hence body condition, of fledgling kestrels was positively correlated with their CMI. Given such covariance, any biases in the estimation of variance components for body mass could have biased the estimations for CMI. We found that variation in fledgling body mass was almost equally explained by familial and environmental components, while in other avian species most of the variance has usually been explained by rearing conditions (Gebhardt-Henrich and Noordwijt 1991; Price 1991; Smith and Wettermark 1995; Merilä 1996). The fact that there was a significant resemblance between full sibs at 5 days old in body mass, and that their body mass at that age predicted body mass after swapping the chicks, suggest that pre-hatching or early post-hatching common environmental/maternal effects were present and inflated the familial effects on fledgling body mass and on the correlated CMI response. Environmental and maternal effects acting prior to manipulation are indistinguishable from additive genetic effects in cross-fostering experiments (Merilä 1996; Merilä et al. 1999). In birds, it is known that physiologically active compounds derived from maternal diets are incorporated into eggs and subsequently enhance immunocompetence of neonates (Graczyk et al. 1994; Smith et al. 1994; Haq et al. 1996; Pastoret et al. 1998). On the other hand, both nutritional status and diet of females before laying affect egg size (Potti 1993; Selman and Houston 1996; Ramsay and

Houston 1998), and hence the body mass of hatchlings even until they achieve asymptotic mass (Styrsky et al. 1999). Moreover, variation in steroids of maternal origin in the eggs is associated with size differences among the offspring hatching from these eggs (Schwabl 1996). American kestrels pair assortatively with respect to condition (Bortolotti and Iko 1992), males feed mates during egg-formation (Balgooyen 1976), and females with better pre-laying body condition lay larger eggs (Wiebe and Bortolotti 1995). Accordingly, both parents may contribute to a non-genetic, familial component of variation in body mass early in the life of chicks, which persists until fledging and inflates the true familial estimations of CMI. Therefore, we believe our estimates for sources of variation in CMI were more reliable when controlling for body mass, and so most variation in T-cell proliferation was explained by environmental components during growth. But, being conservative by accepting our first results, most of the variance is still attributable to environmental effects, 33% corresponding to rearing conditions at the nest plus an unknown fraction of the familial component (12%) corresponding to pre-manipulation parental effects.

Although it is not clear whether additive genetic variance is more likely to be expressed under stressful or benign conditions (Hoffman and Parsons 1991), heritabilities of a number of traits in wild bird populations have been found to be lower in poor than in good environments (e.g., Gebhardt-Henrich and Noordwijk 1991; Merilä 1997; but see Merilä et al. 1999). Putting the year we performed the cross-fostering experiment within the context of a 10-year data set, it gave a normal average from the standpoint of reproductive success and body mass of fledglings (G. R. Bortolotti, unpublished data). Larger genetic variance may be expressed in very good years, but it is unlikely that would overcome the large environmental component we found in this study. Low heritability of a trait is usually related to its positive contribution to fitness, as strong directional and constant selection makes the depletion of genetic variation likely, with all remaining variation being environmentally determined (Mousseau and Roff 1987; Roff 1997). Such an interpretation is consistent with recent findings linking CMI to several measures of fitness (Saino et al. 1997a, 1998; Sorci et al. 1997a; Christe et al. 1998; Moreno et al. 1998, 1999b; Gonzalez et al. 1999a; Merino et al. 1999; Soler et al. 1999a, b; Zuk and Johnsen 1999). Moreover, offspring of three avian species (including American kestrels) with higher CMIs have a greater chance of surviving or being recruited to the breeding population than offspring with poor responses (Christe et al. 1998; Gonzalez et al. 1999b; Tella et al. 2000). Therefore, directional selection for increased immunity may have exhausted genetic variation for immunocompetence. However, Merilä et al. (1999) give evidence for weak directional selection for nestling condition, a fitness trait, allowing significant additive genetic variation to persist. In this sense, Merilä and Sheldon (1999), through a profound review, have recently reversed the current view that fitness traits (under strong directional selection) should have lower levels of genetic variability than non-fitness traits (under weak stabilizing selection). The low heritability of fitness components does not necessarily mean that there is no additive genetic variation in these traits, but that this variation may be masked by higher environmental variances than in non-fitness traits (Merilä and Sheldon 1999; see also Price and Schluter 1991). On the other hand, there is the unexplored possibility that autoimmunity, as a cost of increased immune activity (Råberg et al. 1998; Svensson et al. 1998), may induce stabilizing selection to some extent and thus the maintenance of significant heritable variation in nestling immunocompetence.

Overall immunocompetence cannot be assessed by using a single challenge technique (Owens and Wilson 1999). Therefore, it should be emphasized that the T-cell proliferative response represents only one of the three major facets of the avian immune response, the other two being humoral, or antibody-mediated immunity, and non-specific cell-mediated defense mechanisms, and that a full-scale estimate of genetic and environmental components of the avian immune system would be desirable. However, our study has shown that at least one of these components, which in turn is of considerable interest in behavioral and evolutionary ecology (see references above), has a largely environmental component, while the genetic contribution, although it appears to be small, requires further attention. Whether or not additive genetic variance of cell-mediated immunity varies among contrasted environments (Merilä et al. 1999), and how selection acts on that, are open questions which require further research.

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