

Environmental, genetic and maternal components of immunocompetence of nestling pied flycatchers from a cross-fostering study

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ABSTRACT

Genetic, environmental and maternal effects can all affect the phenotypic expression of a trait. Through cross-fostering experiments in nesting birds, variation due to genetic and environmental effects can be partitioned and identified as being due to nest of origin and nest of rearing in a two-factor nested analysis of variance, but both can be confounded with maternal effects if their phenotypic influences depend upon the environment in which nestlings develop. In this article, we adopt a widely applied two-factor nested design using data for T-cell-mediated immune responses of nestling pied flycatchers (*Ficedula hypoleuca*) from a cross-fostering experiment. We use maternal immune responses and differences between immune responses of mothers as covariables that accounted for a significant proportion of variance in nestling immunocompetence. This suggests that those variables can also be used in multiple regression analyses to explore genetic and environmental effects on nestling traits. Finally, we use differences among nestlings (siblings, non-siblings and reared or not in the same nest) while using trait values of mothers and differences between mothers to control for genetic and environmental effects, respectively, to explore possible maternal effects affecting nestling immune responses. Our results suggest a strong environmental effect on nestling immune response, which includes a significant maternal effect.

Keywords: environmental factors, *Ficedula hypoleuca*, genetic factors, immune response, maternal effects, pied flycatcher.

INTRODUCTION

The post-fledging survival of birds is a crucial determinant of the reproductive success of their parents (Clutton-Brock, 1988) and is known to be affected by conditions experienced during nestling development (e.g. Stark and Ricklefs, 1998; Lindström, 1999). The state of health of fledglings may also influence survival probabilities, either through its effects on offspring size and condition (Moreno *et al.*, 1997) or directly through pathogen-derived

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mortality. Furthermore, unhealthy fledglings may be unable to both fight disease and expend energy in costly foraging activities (Weathers and Sullivan, 1989). There is experimental evidence for a link between nestling condition and the ability of the immune system to resist pathogen attacks (Merino *et al.*, 1996; Saino *et al.*, 1997; Christe *et al.*, 1998). Thus, immunocompetence is of central importance in evolutionary ecology (Sheldon and Verhulst, 1996).

Genetic, environmental and maternal effects can all affect the phenotypic expression of a trait (Falconer, 1989). However, it is often assumed that most natural variation in nestling body condition is environmentally determined, with no or only a weak additive genetic component (Alatalo *et al.*, 1990; Schluter and Gustafsson, 1993; Moreno *et al.*, 1997; but see Merilä, 1996; Sheldon, 1997; Merilä *et al.*, 1999; Gosler and Harper, 2000). Nestling immunocompetence may be a consequence of the quantity and quality of the resources apportioned to nestlings by parents (Chandra and Newberne, 1977; Gershwin *et al.*, 1985; Esparza *et al.*, 1996; J.J. Soler *et al.*, 1999), but laying date (Sorci *et al.*, 1997b), brood size (Saino *et al.*, 1997; Tella *et al.*, 2000a) and parasitization intensity (Christe *et al.*, 1998; Merino *et al.*, 2000) are additional environmental factors that affect nestling immune response. Recently, strong evidence was presented for maternal effects on the probability of nestlings avoiding infections, both through allocation of particular antibodies to eggs (Heeb *et al.*, 1999) and indirectly through allocation of other substances with health-related functions to the eggs (i.e. carotenoids, testosterone; Haq *et al.*, 1996; Gil *et al.*, 1999; Sheldon, 2000). However, an additive genetic component of the immune system has not been detected in some natural populations (Christe *et al.*, 2000; Tella *et al.*, 2000a,b; but see Saino *et al.*, 1997; Brinkhof *et al.*, 1999). The difficulty of detecting genetic influences on immunocompetence in natural populations is somewhat surprising, because work done on poultry has shown significant genetic control of the immune system (e.g. Taylor *et al.*, 1987; Cheng *et al.*, 1991; Kean *et al.*, 1994). Since it is known that heritability estimates are affected by environmental conditions (Falconer, 1989), and usually are higher when measured in the laboratory than in wild populations (Sorci *et al.*, 1997a), statements about the relative importance of the genetic component of immunocompetence should be made with caution.

In this study, we used data from a cross-fostering experiment performed in a pied-flycatcher (*Ficedula hypoleuca*) population to explore the genetic, environmental and maternal effects on the nestling immune response by testing some new predictions for all three factors and using differences, or contrasts, between different kinds of nestlings and mothers (see below). Since covariance between one parent and their offspring reflects one-half of the additive genetic variance of the parents (similar to the one parent-offspring regression method to estimate additive genetic variance; see Falconer, 1989), the immune response of parents can be used to explore genetic influences of nestling immune response. On the other hand, it is known that the adult immune response is related to physical condition and reproductive effort (Deerenberg *et al.*, 1997; Nordling *et al.*, 1998; Moreno *et al.*, 1999). Moreover, Råberg *et al.* (2000) found that an experimental vaccination reduced parental effort measured as feeding rate, and thus adult immunocompetence is probably related to parental quality. If that were the case, differences in immune response between experimental parents should be related to differences in the environment (parental quality) in which siblings develop. Then, differences between siblings reared in different nests should be positively related to differences in immune response between real and foster parents. If that were the case (see Results), differences between parents could be used to

indicate differences between environments (cross-fostered nests) and to explore the environmental influence of nestling immune response.

We used a common statistical approach, two-factor nested analysis of variance (ANOVA), to partition variance in nestling immune response into the hypothetical environmental and genetic (although both of them may be confounded by maternal effects; see Sheldon, 2000, for a discussion) components (e.g. Merilä, 1996; Potti *et al.*, 1999; Cadee, 2000; Christe *et al.*, 2000; Kunz and Ekman, 2000; Tella *et al.*, 2000a,b). The main prediction in previous articles using cross-fostering experiments (e.g. Potti *et al.*, 1999; Kunz and Ekman, 2000; Tella *et al.*, 2000a,b) was that factors like nest of origin (genetic influence) and nest of rearing (environmental influence) should explain a significant proportion of variance in the nestling trait. Covariation among full siblings is equal to half of the additive genetic variance plus a quarter of dominance variance plus common maternal effect if present ($\frac{1}{2}V_A + \frac{1}{4}V_D + V_M$) (Falconer, 1989). Then, using a two-factor nested ANOVA, in which the main effects are nest of origin (nested within nest of rearing) and nest of rearing, variation due to nest of origin is assumed to estimate variation attributable to half of the additive genetic variance (but also include a quarter of dominance variance and any premanipulation maternal effects if present), while variation explained by nest of rearing estimates the effect of a common environment (V_E) (e.g. Merilä, 1996).

Theoretical basis for the use of differences between nestling traits from cross-fostered nests to explore environmental, genetic and maternal effects on nestling traits

Exploring genetic and environmental effects on nestling traits

The basic assumptions are that all full siblings share the genetic component ($\frac{1}{2}V_A$), while covariance of the two types of nestlings (full siblings and foster siblings) reared in the same nest should be related to the influence of the environmental component (V_E). Thus: (i) differences between siblings reared in different nests should reflect environmental differences, but not genetic differences, between cross-fostered nests; (ii) differences between full and foster siblings reared in the same nest should reflect genetic differences, but not environmental differences, between cross-fostered nestlings; and (iii) differences between non-siblings reared in different nests should account for both genetic and environmental differences (see Fig. 1). The use of these differences allows us to estimate the relative importance of each of the hypothetical factors affecting immunocompetence. If environmental influence is the main factor explaining nestling immunocompetence, it could be predicted that differences between foster and full siblings reared in the same nest should be smaller than differences between full siblings reared in different nests.

Exploring maternal effects on nestling traits

In birds, it is known that active compounds derived from maternal diet are incorporated into eggs and subsequently enhance the immunocompetence of nestlings (Haq *et al.*, 1996; Heeb *et al.*, 1999). Moreover, a well-developed immune system requires a large amount of resources (e.g. Lochmiller and Deerenberg, 2000) that should be provided by parents, resources that otherwise could be used in developing other traits (see reviews by Lochmiller and Deerenberg, 2000; Norris and Evans, 2000). Therefore, it could be predicted that maternal allocation of substances to the eggs, related to the future quality of the immune system of their offspring, should be adjusted not only for the availability of those

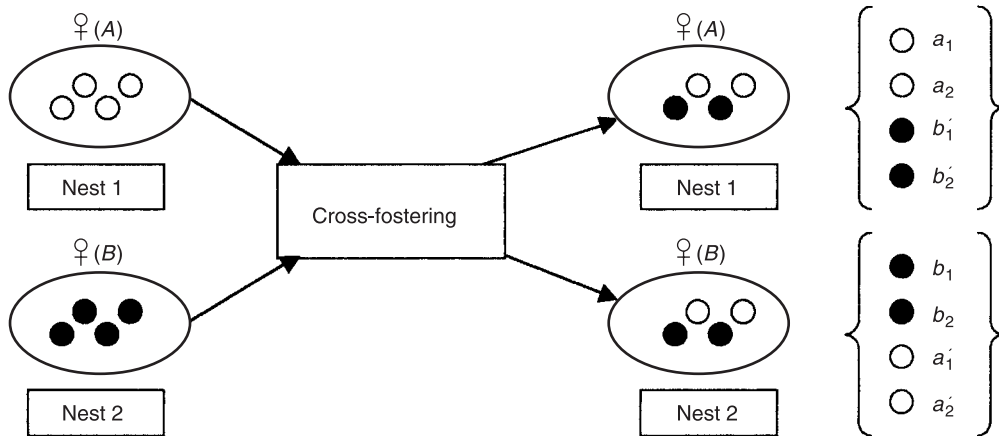


Fig. 1. Schematic representation of the cross-fostering experiments indicating the different kinds of mothers and nestlings used in the study. A and B = the immune response of females from nests 1 and 2, respectively; a_n and b_n = the immune response of nestlings from nests 1 and 2, respectively; a_n' and b_n' = the immune response of cross-fostered nestlings from nests 1 and 2, respectively; $(a_1 + a_2)/2 = a$ = the immune response of nestlings from nest 1 and reared in nest 1; $(a_1' + a_2')/2 = a'$ = the immune response of nestlings from nest 1 but reared in nest 2; $(b_1 + b_2)/2 = b$ = the immune response of nestlings from nest 2 and reared in nest 2; $(b_1' + b_2')/2 = b'$ = the immune response of nestlings from nest 2 but reared in nest 1.

substances in the females, but also for the quantity and quality of resources that parents are able to provide (Gil *et al.*, 1999; Sheldon, 2000).

In cross-fostering experiments, covariation among siblings reflects $\frac{1}{2}V_A + \frac{1}{4}V_D + V_M$ (Merilä, 1996; Merilä *et al.*, 1999). Thus, if we were able to control for genetic relatedness simply by using differences among siblings reared in different nests, such differences between nestlings would be related to differences in environmental conditions. However, if adjustment of maternal effects to a target environment (nest of hatching) exists (as shown above), it could be confounded by the environmental effect; it should then help to explain the variance of differences in immune response (IR) between siblings reared in different nests due to the misadjustment of parental investment (i.e. related to differences in the environment where nestlings develop) suffered by one of the sibling groups. If such an adjustment in maternal effects exists in our cross-fostering experiments, it should be detected by comparing mean values of a foster-nestling trait with the development of this trait in natural nestlings in the same nest (for which hypothetical maternal adjustment to later investment in resource provisioning should be close to the optimum value) after controlling for the effect of differences in environment for which the maternal factor was hypothetically adjusted. Those residuals should be explained by differences between full siblings reared in different nests after controlling for the effect of being reared in different nests.

In other words, if some nestlings (a') were moved from nest A to another nest (B) in a much better environment (i.e. $IR_{(\text{real mother})} - IR_{(\text{adoptive mother})} < 0$), they should develop a better immune response (in nest B) than that of their siblings (a) reared in the original, but worse, nest (A). This is not only because of the environmental effect on the immune response, but also because development of the nestling immune response to the hypothetical level

(adjusted by the mother by addition of substances) would not be constrained by parental investment in reproduction (i.e. for siblings reared in different nests: $IR_{(\text{natural nestling}(a))} - IR_{(\text{experimental nestling}(a'))} < 0$). In addition, if adjustment of maternal effects exists, the mother of offspring from the high-quality nest (*B*) would have included more compounds in her eggs – subsequently enhancing the nestling immune response – than the other mother at the low-quality nest (*A*).

Thus, if we now take into account the immune response of foster and real siblings (*b'* and *a*, respectively) at the low-quality nest (*A*), two possibilities arise depending on whether or not maternal effects are constrained by the amount of parental investment. First, we would expect the immune response of foster siblings (*b'*) to be higher (i.e. for foster siblings reared in the same nest: $IR_{(\text{natural nestling})} - IR_{(\text{experimental nestling})} < 0$) than that of natural siblings (*a*) if maternal or genetic effects enhance the nestling immune response (see Fig. 1); therefore, its development would not be constrained by the amount of parental investment related to the level of maternal substances in the eggs. However, this hypothetical result does not imply the existence of maternal effects because genetic effects also predict that offspring from parents of high genetic quality should have higher genetic quality than those from parents of low genetic quality (offspring and mother share $\frac{1}{2}V_A$ of the parents; see above).

If we controlled these differences for differences in environment for which maternal effects are supposed to be adjusted (i.e. differences between mothers), a positive relationship between those residuals and the others described above (differences between siblings reared in different environments controlled for environmental effects) would suggest a maternal effect because differences between siblings would not be related to genetic differences. Thus, second, if the development of the nestling immune response, enhanced by maternal effects, is constrained by the amount of parental investment, a negative misadjustment between future parental investment and maternal effects could result in a negative effect on the immune response developed by offspring. If this negative effect of misadjustment between maternal effects and environment occurs in our cross-fostering experiment, experimental nestlings from good-quality nests (*B*), but reared in a worse nest (*A*), could even develop less of an immune response than nestlings hatched and reared in the worse nest (*A*) but from which adjustment between maternal effects and parental investment was close to the optimum value [i.e. for foster siblings reared in nest *A* ($a - b'$): $IR_{(\text{natural nestling})} - IR_{(\text{experimental nestling})} > 0$]. Then, if our results show a negative effect of a misadjustment between maternal and parental effects in nestling immune response, it cannot be explained by differences in genetic quality, and we can conclude that maternal effects are important in determining that nestling trait.

Our aim in this study was to detect maternal effects on the T-cell-mediated immune response of nestling pied flycatchers in a cross-fostering experiment using the logic presented above.

MATERIALS AND METHODS

Study area

The study was conducted in 1997 in an oak (*Quercus pyrenaica*) forest near la Hiruela, central Spain (41°4'N, 3°27'W), provided with 150 nest boxes. Pied flycatchers were the predominant species occupying nest boxes in the area, having been intensively studied since 1984 (Potti and Montalvo, 1991; Potti, 1993; Potti and Merino, 1994). Laying date, clutch size and hatching date were checked during daily visits.

Cross-fostering experiment

Nests were assigned at random to two treatments. In experimental nests, half of the chicks were exchanged 1 day after hatching with chicks of another nest with the same hatching date and clutch size. For each two experimental nests, one was kept as a control. In control nests, half of the chicks were briefly removed and put back in the nest again. Adopted chicks were marked by head down clipping and painting their toe nails until the age of 13 days, when they were ringed.

Sampling procedure

Females were captured on day 12 (day 1 being the day of hatching of the majority of the clutch), weighed, measured and subjected to the immunization procedure (see below) before release. They were recaptured the next day (approximately 24 h after the first capture), weighed and measured following the immunization protocol.

On day 13, they were also weighed to the nearest 0.1 g with a Pesola spring balance and their tarsus was measured with a digital calliper to the nearest 0.05 mm. Chicks were subjected to the immunization procedure on days 12 and 13. A phytohaemagglutinin-P (PHA) injection assay (Cheng and Lamont, 1988) was used to evaluate the *in vivo* T-cell-mediated immune response of nestlings. Birds were injected intradermally in the right external wing web with 0.05 ml of 5 mg · ml⁻¹ phytohaemagglutinin-P (Sigma Chemical Co.) in phosphate-buffered solution (PBS). The external web in the other wing (control) was injected with 0.05 ml of PBS (Tsiagbe *et al.*, 1987). The thickness of each wing web was measured with a digital pressure-sensitive micrometer (to the nearest 0.01 mm) at the injection site before and 24 h after the injection. The T-cell-mediated immune response or wing web index was estimated as the change in thickness of the right wing web from the day of injection until the following day minus the change in thickness of the left wing web during the same period (Lochmiller *et al.*, 1993). The same researcher took three measurements of each web on each occasion, and the mean was used in subsequent analyses. These measurements allowed us to calculate the repeatability of wing web thickness. The injectable solutions were maintained in the field in a portable ice-box at a temperature of around 5°C. There was a highly significant difference between adult and nestling responses [0.20 ± 0.21 ($n = 27$) vs 0.45 ± 0.12 ($n = 35$), $t = 5.99$, $P < 0.001$], indicating strong age-dependent changes in T-cell-mediated immunocompetence. However, since adult immune response was used as the covariate or in multiple regression analyses, standardization of nestling and adult immune response was not necessary. The results, therefore, did not vary when using standardized data to a mean of zero and a standard deviation of 1 before the analyses.

Statistical analyses

Two-factor nested analyses of variance were performed using StatSoft (1998), module 'Variance Components', in which factor was considered a random factor and the variance components were estimated using the type III estimation of mean squares method.

To avoid pseudoreplication, when using differences between different kinds of nestlings, we used mean values. Frequency distributions for all variables used in the analyses did not differ significantly from a normal distribution (Kolmogorov-Smirnov test for continuous variables, $P > 0.15$). Thus, we used parametric statistical tests following Sokal and Rohlf

(1995). We used multiple regression analyses to explore relationships between differences in nestling immune response (between sibling and non-sibling, reared or not in the same nest) and differences between foster mothers (i.e. environmental influence) and real mothers (i.e. genetic influence). Because of the interrelation of the independent variables and, therefore, the possible associated problem of multicollinearity, we provide information on tolerance ($1 - R^2$) between independent variables. However, since redundancy ($1 - \text{tolerance}$) was less than 0.6 in all cases, there was no problem with multicollinearity (i.e. an ill-conditioned matrix) in our statistical tests (StatSoft, 1998).

All tests were two-tailed and the results are reported as the mean \pm standard deviation.

RESULTS

The repeatability of wing web measurements was high and significant (Table 1). We therefore used average values in the subsequent analyses. To assess whether moving hatchlings between nests had any effect on fledgling tarsus length, mass, condition and immunocompetence, the averaged measurements of full-sibs reared apart were compared between nests. There were no significant differences in the average measurements of full-sibs reared in their own nests and in other nests for any variable (paired *t*-tests: $n = 20$, $P > 0.10$ in all cases). The fact that nestlings from different nests competed in the broods included in the cross-fostering experiment could also have affected their measures of condition and, thus, immunocompetence. However, average measurements did not differ significantly between cross-fostered ($n = 17$) and control ($n = 13$) broods for any variable (*t*-tests: $P > 0.10$ in all cases). The experimental manipulation could also have affected nestling mortality. An average of 1.5 ± 1.5 ($n = 35$) chicks died in experimental nests between hatching and nestling age of 13 days, apparently due to starvation. However, for those nests in which at least one chick fledged, there were no significant differences between control and experimental nests in the number of chicks that died: 1.7 ± 1.3 ($n = 13$) versus 3.0 ± 1.3 ($n = 24$), respectively ($F_{1,36} = 1.0$, $P = 0.32$). Thus we disregarded the effects of moving chicks in subsequent analyses.

There was no significant correlation between females from the same pair of cross-fostering nests in body mass ($R = 0.315$, $n = 18$, $P = 0.20$), tarsus length ($R = 0.332$, $n = 20$, $P = 0.15$) or immune response ($R = 0.259$, $n = 11$, $P = 0.44$). Thus, although we matched experimental nests according to laying date and clutch size, it did not result in pairs of nests

Table 1. Repeatabilities derived from intraclass correlation coefficients estimated from the among-individual and within-individual variance components in one-way analyses of variance

Measurement	d.f.	<i>F</i>	<i>P</i>	<i>r</i>
Nestling left wing web, day 12	156,470	27.43	< 0.001	0.90
Nestling left wing web, day 13	148,446	54.70	< 0.001	0.95
Nestling right wing web, day 12	156,470	21.79	< 0.001	0.87
Nestling right wing web, day 13	148,446	14.60	< 0.001	0.82
Female left wing web, day 12	33,101	11.04	< 0.001	0.77
Female left wing web, day 13	26,80	14.67	< 0.001	0.82
Female right wing web, day 12	33,101	13.71	< 0.001	0.81
Female right wing web, day 13	26,80	58.28	< 0.001	0.95

with similar females. Moreover, differences in immune response between adult females of the same cross-fostering experiment were explained by differences in their body condition (multiple $R = 0.676$, $F_{2,9} = 3.79$, $P = 0.064$; body mass: partial $r = 0.665$, $P = 0.026$; tarsus length: partial $r = 0.669$, $P = 0.024$).

Environment and differences in adult female immune response

We found that differences in the immune response of siblings reared in different nests, which should reflect environmental differences, were explained by differences in immune response among real and foster mothers (adjusted $R^2 = 0.47$, $F_{1,8} = 8.9$, $P = 0.017$; Fig. 2a), whereas the immune response of real mothers did not explain any additional significant variance. Moreover, when trying to explain variation in immune response between genetically unrelated nestlings that were reared in different nests, differences in immune response between mothers at the two nests did not explain such variation (adjusted $R^2 = -0.13$, $F_{1,7} = 0.09$, $P = 0.78$; Fig. 2b). Thus, the use of differences between females as a variable related to differences in environmental conditions is justified.

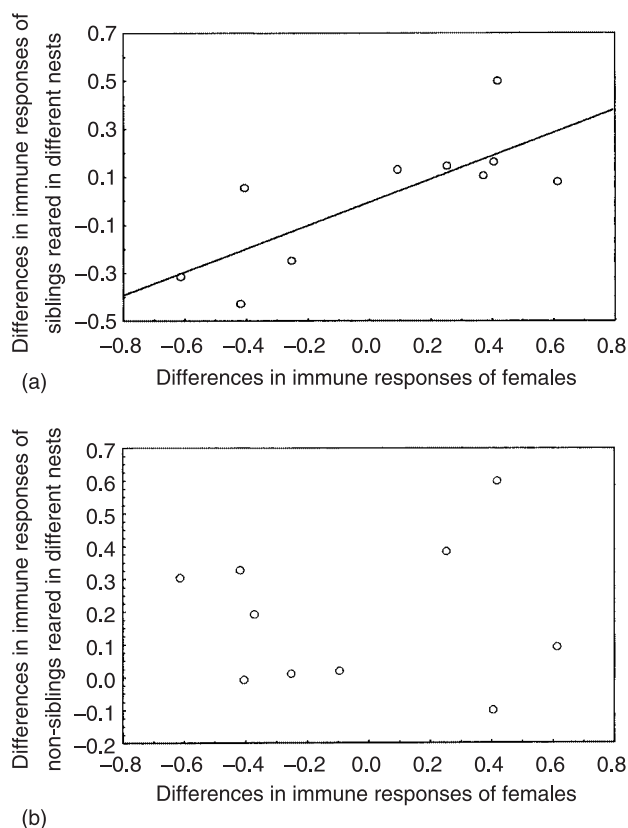


Fig. 2. Relationships between differences in the T-cell-mediated immune responses of pairs of mothers and differences in the T-cell-mediated immune response of (a) siblings reared in different nests (regression line = $y = 0.484x - 0.03$) and (b) non-siblings reared in different nests.

Genetic and environmental effect of nestling immune response using different methods

Two-factor nested ANOVA

Nest of rearing, but not nest of origin, explained a significant proportion of the variance in nestling immune response (Table 2) and, although much variance remained to be explained (variance explained by the error term = 59.6%), the environmental component explained more than three times (31.7%) the variance explained by the genetic component (8.7%). Thus, in accord with previous studies, our results show that environment is the main influence on nestling immune response.

Using differences between different kinds of nestlings

Comparisons of unsigned differences among different kinds of nestlings revealed that differences due to environmental variation (differences between siblings reared in different nests) were slightly greater than those related to genetic variation and variation in maternal effects (differences between natural and foster nestlings reared in the same nest), but not significantly so (nested ANOVA, pair of nests nested in groups of nestlings: $F_{1,14} = 1.30$, $P = 0.27$; Fig. 3). Moreover, we found that the immune response of foster siblings (nestlings that were not reared in their nest of hatching) is explained to a significant extent by differences in the immune response of real and foster mothers (adjusted $R^2 = 0.52$, $F_{1,9} = 12.01$, $P = 0.007$), but the immune response of the real mother did not explain a significant proportion of the variance in nestling immune response, neither alone (adjusted $R^2 = 0.04$, $F_{1,9} = 1.37$, $P = 0.27$) nor in a multiple regression together with differences between mothers as independent factors (multiple regression: adjusted $R^2 = 0.57$, $F_{6,8} = 7.62$, $P = 0.014$; immune response of real mother, partial $R = 0.44$, $P = 0.20$; differences in immune response between females, partial $R = 0.78$, $P = 0.008$; tolerance among independent variables = 0.45). Thus, taking all these results into account, we conclude that environmental effects are probably the most important in explaining immune response.

Table 2. Results of two-factor (random) nested ANOVA and ANCOVA (nest of origin nested within nest of rearing) to test the effects of rearing environment and family of origin on measures of T-cell-mediated immunocompetence of pied flycatcher offspring in nests used in the cross-fostering experiment. Percentage of variance (% variance) explained by each factor and results from the two-factor nested ANCOVA, with immune response of genetic mother and differences between real and foster mother as covariables (cov.), are also shown

Factors	MS	d.f. effect	Error	d.f. error	<i>F</i>	<i>P</i>	% variance
Nest of rearing	0.084	15	0.031	16.2	2.74	0.026	31.65
Nest of origin (nested)	0.031	16	0.023	40.0	1.32	0.23	8.73
<i>Including level of immune response of real mother and differences between real and foster mother</i>							
Real mother (cov.)	0.116	1	0.039	19.2	2.96	0.10	
Differences between mothers (cov.)	0.198	1	0.050	14.2	3.94	0.067	
Nest of rearing	0.058	11	0.027	7.8	2.16	0.14	25.56
Nest of origin (nested)	0.027	8	0.023	27.0	1.16	0.36	5.14

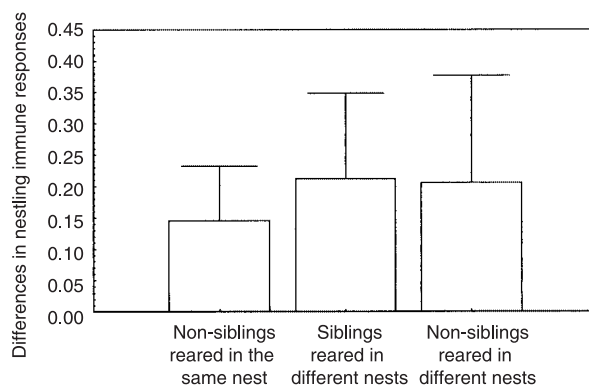


Fig. 3. Differences in nestling T-cell-mediated immune response between different categories of nestlings (mean \pm standard deviation).

Exploring the influence of maternal effects on nestling immune response

In a multiple regression analysis, we found that signed differences between natural (a) and experimental (a') siblings reared in different nests (A and B , respectively) were explained by signed differences in immune response between real (from nest A) and adoptive mothers (from nest B), and by differences between natural (a) and foster siblings (b') (reared in nest A) (adjusted $R^2 = 0.73$, $F_{2,6} = 11.97$, $P = 0.008$; partial correlation coefficients: difference between females, $R = 0.84$, $P = 0.008$; difference between natural and foster siblings reared in the same nest, $R = 0.76$, $P = 0.029$; tolerance among independent variables = 0.997). Therefore, when nestlings were moved to a nest of higher parental quality, they demonstrated a more marked immune response than their real siblings reared by their natural parents, but in their original nests foster nestlings showed a greater immune response than natural nestlings, when both relationships were controlled for environmental differences.

Moreover, the intercepts of the regression lines did not differ from zero (differences between females as independent variable, intercept = -0.003 , $P = 0.96$; adding differences between real and foster siblings reared in the same nest as independent variable, intercept = 0.002 , $P = 0.96$). This leads to the prediction that if the cross-fostered nests are from females of similar quality, the immune response of real and foster siblings reared in the same nest should not differ.

DISCUSSION

Our results, which are in line with those of previous articles (Brinkhof *et al.*, 1999; Christe *et al.*, 2000; Tella *et al.*, 2000a,b), suggest that the environment is the main effect explaining nestling immune response. This is exactly what is predicted for traits under strong selection pressure (Mousseau and Roff, 1987; Roff, 1997). Thus, since the T-cell-mediated immune response is related to fitness (e.g. González *et al.*, 1999; M. Soler *et al.*, 1999; Christe *et al.*, 2001), it should be under strong selective pressure and should, therefore, show low additive genetic variance.

We used differences in immune response between real and adoptive mothers to estimate differences in environment between pairs of nests, mainly because they explain differences

in immune response between siblings reared in different nest (Fig. 2), which should be related to differences in environmental conditions (e.g. Merilä, 1996; Potti *et al.*, 1999). Moreover, when introducing both the immune response of real mothers and differences in immune response between mothers in the two-factors nested ANOVA, the variation explained by nest of rearing decreased drastically. Moreover, the immune response of mothers (genetic factor) and differences in immune response between mothers (environmental factor) became the variables that best explained variation in nestling immune response (Table 2). Therefore, these results suggest that, by using the immune response of real and adoptive parents in cross-fostered nests, it would be possible to estimate the effects of genetic and environmental factors on nestling immune response in a multiple regression analysis, although such estimations could be confounded by maternal effects (see below).

Differences among nestlings as a tool to explore maternal effects

In cross-fostering experiments, it is assumed that differences between nestlings reared in the same nest not only correspond to genetic differences, but also to any pre-manipulation maternal effects (e.g. Merilä, 1996; Merilä *et al.*, 1999). Moreover, maternal effects, which are not possible to estimate with a two-factor nested ANOVA (Falconer, 1989), can also be confounded within the variance explained by nest of rearing if the phenotypic expression of any pre-manipulation maternal effect differs depending on the environment in which nestlings develop (e.g. Mousseau and Fox, 1998; Wolf *et al.*, 1998).

In the Introduction, we suggested that if this adjustment in maternal effects existed in our cross-fostering experiments, it would be detected by looking at the positive relationship between differences among siblings reared in different nests, after controlling for differences between mothers for the same trait, and differences between siblings and non-siblings reared in the same nest, after controlling for differences of mothers' traits. Our results are in accordance with that scenario and suggest, therefore, maternal effects on nestling immune response. We found that, on average, when a nestling is taken from one nest and put in another of better quality (i.e. adult female with larger immune response), it develops a stronger immune response than its siblings reared in their original nest. However, those siblings reared in their original poor nests developed a weaker immune response than foster siblings, which came from the nest of higher quality. Since that relationship was controlled for differences in immune response between mothers (in a multiple regression analysis), the results suggest a maternal effect on nestling immune response. Differences between mothers are closely related to differences in environment in which nestlings develop, and differences between siblings are not due to genetic but to environmental and differential effects of maternal components.

Moreover, we did find support for the hypothesis of a genetic and maternal influence on nestling immune response being constrained by the level of parental investment, since nestlings that hatched and were reared in good quality nests showed a stronger immune response than their siblings reared in nests of lower quality or foster siblings reared together with them. The use of maternal trait values for dissection of the environmental, genetic and maternal factors affecting any phenotypic trait has precedents (e.g. Travis *et al.*, 1987; Emerson *et al.*, 1988), and it is claimed that its utility rests upon measuring trait values at comparable life stages of mother and offspring. However, since the method described above is related to estimates of correlation coefficients between contrasts (differences) and does not include comparisons between adults and offspring, our analytical approach is valid.

Thus, an advantage of the use of differences between different kinds of nestlings from cross-fostering experiments, compared to the used of two-factor nested analyses of variance, is the possibility of detecting significant maternal effects in nestling traits. Here we used measures of maternal traits to control for nest quality. However, any measurement of differential environmental quality among cross-fostered nests could also be used. Hopefully, the method described here will contribute to further exploration of the influence of each of these components, not only on nestling immunocompetence but also on other offspring traits.

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