



## Is the relation between colour and immune response mediated by nutritional condition in spotless starling nestlings?

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The hypothesis that nestling coloration is important for parent–offspring communication, because it influences parental feeding decisions, has received strong experimental support. In European starlings, *Sturnus vulgaris*, and Alpine swifts, *Apus melba*, manipulation of ultraviolet reflectance of nestlings' mouth and skin affected the amount of food parents provided, and skin brightness of starling nestlings predicted their T-cell-mediated immune response. Therefore, a link between nestling coloration and immunity, mediated by parental effort, was suggested. We explored this hypothesis by experimentally feeding some spotless starling, *Sturnus unicolor*, nestlings while leaving others in the same nest as a control. First, we found a significant effect of food supplementation on nestlings' immune response, which is a requirement for the hypothesis. Second, we confirmed in spotless starlings the association between skin brightness and ability to raise an immune response. However, this correlation disappeared when we controlled for between-nest variation. These results suggest that parental feeding preference is not the only factor explaining nestling immunity, and that covariation between mean brood nestling coloration and parental quality, and/or intrinsic (i.e. genetic) quality of nestlings, may explain the association between immunity and coloration of nestlings. Finally, within-nest variation in nestling coloration partially explained immune responses because food supplementation had more effect on nestlings with brighter skin. We discuss these results as possible evidence of nestling coloration partially reflecting intrinsic characteristics that affect both ability to produce efficient immune responses and parental feeding preferences.

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Nestling coloration has received special attention during recent years mainly because it may be important for parent–offspring communication. The most conspicuous traits that unfeathered nestlings display to their parents are flanges and mouth cavity, and parents may prefer to feed nestlings with the most conspicuous traits (e.g. Kilner & Davies 1998). This preferential feeding by parents of the most conspicuous nestlings has recently received experimental support (Heeb et al. 2003; Jourdie et al. 2004; but see Tschirren et al. 2005), and can be predicted from parents preferentially feeding nestlings that are more easily detectable and/or of better phenotypic quality. For instance, red colour may be attractive for parents because

it could reflect the nestling's level of hunger, or because it could be carotenoid based and thus indicate the health of their offspring (Kilner 1997; Saino et al. 2000a; Saino & Møller 2002). However, the parents' feeding decision in relation to the phenotypic quality of their offspring may be context dependent and, for instance, may vary through the breeding season (Bize et al. 2006).

Nestling coloration may also serve as a cue for locating chicks at the nest. Depending on light and other environmental conditions (i.e. background colour) at the nest, some colours are more easily detected than others. For instance, in dark conditions such as those in hole nests, yellow is more easily detected than red (Heeb et al. 2003). In addition, the spectral irradiance of the nest background is usually minimal at ultraviolet (UV) wavelengths (300–400 nm; Hunt et al. 2003; Jourdie et al. 2004) and, thus, nestlings may reach maximum contrast (i.e. conspicuousness) by showing a peak of reflectance at those

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wavelengths (Hunt et al. 2003). In support of this hypothesis, in most species the reflectance spectra of the mouth and flanges of nestlings peak in the UV (Hunt et al. 2003). More importantly, the manipulation of UV reflectance in the body skin and flanges of nestling starlings, *Sturnus vulgaris*, resulted in a differential increase in body mass of UV-reflecting nestlings just 2 h later (Jourdie et al. 2004).

Whatever the reasons for parents preferring to feed nestlings with a particular coloration (Kilner 1997), this preferential food allocation predicts a relation between nestling colour and the phenotypic quality of fledglings. In accordance with this prediction, Jourdie et al. (2004) found a positive relation between the T-cell-mediated immune response and the brightness of skin reflectance, including both UV and visible light, in starling nestlings. However, evidence for a causal link between UV skin reflectance and condition in offspring is still lacking. Since the nestling's immune response is a trait that depends on nutritional condition (e.g. Saino et al. 1997; Alonso-Alvarez & Tella 2001; De Neve et al. 2004), the relation between brightness and immunity might be mediated by parents preferentially feeding nestlings with more UV reflectance (Jourdie et al. 2004). The relation between nestling phenotypic quality and coloration could also be caused by the skin reflectance of nestlings signalling their immunity and parents feeding nestlings in relation to the expression of the signal, which should in turn brighten the skin further. A significant genetic component of immune response has been detected for nestlings of several species (Saino et al. 1997; Soler et al. 2003b, and references therein). Consequently, a genetic correlation between immune response and nestling coloration might explain not only the relation between these two traits, but also feeding preferences by parents. In any case, if parents differentially feed nestlings with a particular coloration, and these nestlings experience an improvement in their immune response, a direct link between nestling coloration and fitness can be established, since immunocompetence is a major predictor of nestling survival and recruitment (Christe et al. 2001; Møller & Saino 2004; Cichon & Dubiec 2005; Moreno et al. 2005).

We examined the hypothesis that, because of parental feeding preferences, the colour of nestlings influences their nutritional condition, and that this preferential feeding is responsible for the relation between coloration and T-cell-mediated immune response (hereafter phytohaemagglutinin assay, PHA, response) of nestlings. To explore this hypothesis, we tested the effect of food supplementation on the immune response of nestlings. The hypothesis predicts a positive relation between immune response and nestling coloration (Jourdie et al. 2004). This relation could be mediated exclusively by the nutritional condition of nestlings, but there may also be some intrinsic genetically determined potential for immune system development that reflects the reproductive value of offspring (Kilner 1997; Saino et al. 2000b). We explored these possibilities by investigating the effects of nestling coloration and experimental treatment on variation in immune response within and between nests.

## METHODS

### Study Species

The study was carried out in Guadix (37°18'N, 3°11'W), southeastern Spain, during the breeding season of 2005 (April–June), in nestboxes recently (February 2005) installed close to or within colonies of spotless starlings, *Sturnus unicolor*, already established in old buildings in the area. The species is polygynous (Veiga et al. 2001), with a clutch size typically of four or five eggs, and with nestlings usually hatching asynchronously (last egg hatching up to 24 h after the others; Cramp 1998). Nestlings are fed mainly with insects (Motis et al. 1997) by females and, sometimes, also by males (Veiga et al. 2002).

### Experimental Procedure

Three days after the first nestling hatched (i.e. when nestlings were 2–3 days old), each hatchling was weighed and marked with a permanent-colour marker on the tarsus. Hatchlings were ranked according to body mass within each nest. The heaviest nestling was randomly assigned to the food (experimental) or water (control) treatments, and we alternated the treatment of the other chicks in the nest according to body mass rank. The food treatment consisted of 0.2 ml of energy-rich pasta, containing essential micronutrients (minerals, vitamins and amino acids; 20.9 J/g; Nutri-Calorías, Schering-Plough Animal Health, Buena, NJ, U.S.A.), used as an energy and nutritional supplement by veterinarians. The water treatment was 0.2 ml of mineral water. We revisited nests every second day (five visits in total), to re-mark the tarsi and provide more food or water. One possible problem of this experimental approach was that fed nestlings might demand less food from their parents, which might then allocate more food to the rest of the brood. All nestlings in the brood would then effectively be receiving additional food. However, this effect would be conservative in the sense that it would reduce any difference between the treatment and control. Therefore, although nonsignificant effects of this experiment on nestling traits should be considered cautiously, a significant effect on a target trait will indicate that its expression depends on the nutritional conditions experienced by nestlings during development.

About 4 days before fledging, i.e. when they were 13–14 days old, nestlings were ringed, weighed (with a Pesola spring balance  $\pm 0.5$  g), and measured (tarsus length with a digital calliper,  $\pm 0.01$  mm, wing and tail length with a ruler  $\pm 1$  mm). All nestlings were injected subcutaneously with phytohaemagglutinin-P (PHA-P, Sigma Chemical Co., St Louis, MO, U.S.A.) in the wing web to evaluate the in vivo T-cell-mediated immune response following standardized protocols (e.g. Cheng & Lamont 1988; Lochmiller et al. 1993; Soler et al. 2003a). Briefly, after we measured wing web thickness (with a Mitutoyo digital pressure-sensitive micrometer, model ID-C1012 BS,  $\pm 0.01$  mm), we injected nestlings subcutaneously in the right wing web with 0.2 mg of PHA dissolved in 0.04 ml of physiological saline solution (Bausch & Lomb Co.,

Rochester, NY, U.S.A.). The left wing web was injected with 0.04 ml of physiological saline solution. We measured the thickness of each wing web at the injection site before and 24 h after the injection and estimated the T-cell-mediated immune response as the change in thickness of the right wing web (PHA injection) minus the change in thickness of the left wing web. We measured the thickness of each wing web three times, which was highly repeatable (repeatability = 97.2%,  $F_{97,196} = 106.7$ ,  $P < 0.0001$ ) and so we used the mean value in subsequent analyses.

Experimental procedures were licenced by the Consejería de Medio Ambiente, Dirección General de Gestión del Medio Natural de la Junta de Andalucía. None of the nests in the study were deserted. The PHA injection is routinely used in studies of ecological immunology and is assumed not to affect nestling survival (Merino et al. 1999). One of the nestlings that we injected with PHA died within 24 h, but this was due to its poor condition (body mass of dead nestling: 41 g; mean body mass of nestlings of the same age in our studied population  $\pm$  SD =  $75.8 \pm 9.38$  g). Furthermore, our food provisioning improved the T-cell-mediated immune response of experimental nestlings, which is a good predictor of recruitment (Moreno et al. 2005). The treatment did not affect probability of survival (26 nestlings died, 17.8%; experimental: 14; control: 12; chi-square test:  $\chi^2_1 = 0.26$ ,  $P = 0.61$ ); mortality was less than that reported by Cramp (1998) for natural cavities (29.0%) and nestboxes (minimum = 21.7%). Therefore, our study did not affect starling welfare.

### Estimating Nestling Colour

Following the protocol of Jourdie et al. (2004), when nestlings were 4–5 days old (just before the second experimental feeding), we measured nestling coloration on the mouth, the surrounding flanges and the head skin of all nestlings of a nest. Reflectance spectra (300–700 nm) were recorded with an Ocean Optics S2000 spectrometer, connected to a deuterium–halogen light (D2-W, mini) by a coaxial reflectance probe (QR-400-7-UV-vis), and the OOIBase32 operating software (Ocean Optics, Inc., Dunedin, FL, U.S.A.). Reflectance was always measured with the probe placed at a constant distance and reaching the object at 45°. Measurements were relative and referred to a standard white reference (WS-2) and to the dark, which we calibrated before measurement of each nestling.

We measured mouth colour by gently keeping the gape open and introducing the probe to the centre of the upper mouthpart. To measure flange colour, however, we closed the nestling's gape and placed the probe on the angle of the mouth flanges, thus avoiding confusion with the mouth coloration. We decided to differentiate between mouth and flange coloration because these areas may have different functions in parent–offspring communication (Kilner & Davies 1998). Finally, we measured skin coloration on the head, close to the ear, trying to avoid growing feathers. All colour measurements were made three times and variation between nestlings was larger

than variation within nestlings (repeatability > 55%,  $F_{162,325} > 4.7$ ,  $P < 0.0001$ ), justifying the use of mean values per nestling.

For each nestling we calculated the average values of their spectra for mouth, flanges and skin. From these spectra, and following methodology described in Jourdie et al. (2004), we calculated the median maximal value of UV reflectance (M1: median (320 and 360 nm)), the median baseline reflectance value (M2: median (440 and 480 nm)) and the median reflectance in the visible spectrum (M3: median (540 and 700 nm)). Spectral brightness of mouths and flanges was then obtained as  $(M1 - M2) + (M3 - M2)$ , while spectral brightness of head skin was calculated as  $(M1 - M2) + (M3 - M2) + (M1 - M3)$ . We also estimated the percentage of UV (300–400 nm) reflectance in relation to that of the complete spectrum (300–700 nm), which correlated significantly with estimates from Jourdie et al.'s (2004) methodology (Pearson correlation:  $r_{106} > 0.35$ ,  $P < 0.0003$ ). Alternative approaches to measuring nestling coloration have been used in other studies (Hunt et al. 2003; Bize et al. 2006), but we have followed Jourdie et al.'s (2004) methods to replicate their results and resolve the underlying mechanism.

### Statistical Tests

The frequency distributions of morphological (body mass and tarsus and wing lengths) and coloration variables did not differ significantly from normal distributions, and, thus, we used parametric statistical tests. Specifically, to analyse variation in nestling immune response we used general linear models (GLM) with experimental treatment as a fixed factor and nest identity as a random factor. Nestling coloration was introduced as a covariate in the model when we explored its relation with immune response after controlling for the effect of the experiment and nest identity. We also introduced in the model the interaction between nest identity and treatment, which is a random factor that tests for differences in treatment effects between nests. Finally, the interaction between nestling coloration and treatment effect was also introduced in the model to test for a possible differential effect of food supplementation depending on nestling coloration.

To evaluate whether the relation between nestling coloration and immune response was mainly due to between- or within-nest covariation of these two variables, we ran GLMs with type I and III decomposition of sums of squares. In type III decomposition of sums of squares (orthogonal estimated effects) the order in which the factors are introduced in the model does not affect the estimation of their effects on the dependent variable, whereas the use of type I implies that the effect of a target factor is estimated after controlling for the effect of previous factors on the dependent variable (e.g. Statsoft 2001). Therefore, if the effect of nestling coloration on immune response varied depending on either the use of type I or III decomposition errors or position of factors in the models (i.e. before or after nest identity), this would suggest that the relation between nestling coloration and

immune response was mainly due to covariation between these variables at the nest level.

Information on all studied variables was collected for 106 nestlings from 39 nests. For all statistical tests we used Statistica 6.0 (Statsoft 2001) and  $P$  values are two tailed.

## RESULTS

Figure 1 shows the reflectance spectra of skin, mouth and flanges of spotless starlings. We found a positive relation between brightness of skin and level of T-cell-mediated immune response in nestlings (using mean brood values as independent data points; regression analysis:  $\beta \pm \text{SE} = 0.36 \pm 0.15$ ;  $t_{38} = 2.37$ ,  $P = 0.023$ ). However, no other colour variable (spectral brightness of flanges and mouth, see Methods) explained significant amounts of variation in nestling immune response (mean brood values as independent data points; Pearson correlation:  $0.05 < r_{38} < 0.14$ ,  $P > 0.37$ ). Morphological variables of nestlings (body mass and wing and tarsus length) were not significantly correlated with any of the nestling colour variables used (Pearson correlations: body mass:  $0.03 < r_{38} < 0.26$ ,  $P > 0.10$ ; wing length:  $-0.14 < r_{38} < -0.03$ ,  $P > 0.4$ ; tarsus length:  $-0.20 < r_{38} < 0.16$ ,  $P > 0.28$ ). Therefore, in subsequent analyses we used only skin brightness as a measure of nestling coloration, and level of T-cell-mediated immune response as a measure of nestling phenotypic quality.

Food supplementation significantly affected nestling immune response (GLM, type III decomposition of sums of squares; nest identity as a random factor and food

supply treatment as a fixed factor; the interaction between nest identity and treatment was also included in the model:  $F_{1,37.9} = 4.92$ ,  $P = 0.033$ ), but not other traits such as body mass ( $F_{1,34.6} = 0.12$ ,  $P = 0.73$ ) and tarsus ( $F_{1,37.6} = 0.93$ ,  $P = 0.34$ ) and wing length ( $F_{1,29.3} = 1.17$ ,  $P = 0.29$ ). Food-supplemented nestlings showed a larger immune response ( $\bar{X} \pm \text{SE} = 0.66 \pm 0.03$  mm) than control nestlings ( $0.59 \pm 0.03$  mm). Between-nest variation in the level of immune response was larger than within-nest variation ( $F_{38,34} = 3.13$ ,  $P = 0.0005$ ). The effect of the experiment was similar in most starling nests (interaction between nest identity and experimental treatment:  $F_{32,34} = 0.84$ ,  $P = 0.68$ ), which validates our experimental approach. Finally, these results were independent of brood size since the effect of our experiment did not vary in relation to brood size (GLM, similar to that explained before but including the interaction between food treatment and brood size in the model:  $F_{2,40.5} = 1.96$ ,  $P = 0.15$ ).

When brightness of nestling skin was introduced as a covariate in the previous model, it did not explain a significant proportion of variance in nestling immune response (Table 1). This seemed to contradict the significant association between these two variables reported above. However, in this last analysis, between-nest variation in nestling coloration was statistically controlled by including nest identity in the model, and brightness of nestling skin varied significantly between nests ( $F_{38,67} = 3.35$ ,  $P < 0.0001$ ). This means that a particular nestling was more similar in coloration to its nestmates than to nestlings from other nests. Therefore, it is possible that between-nest covariation in nestling coloration and immunity explained the association we detected between these two variables when using mean brood values. In

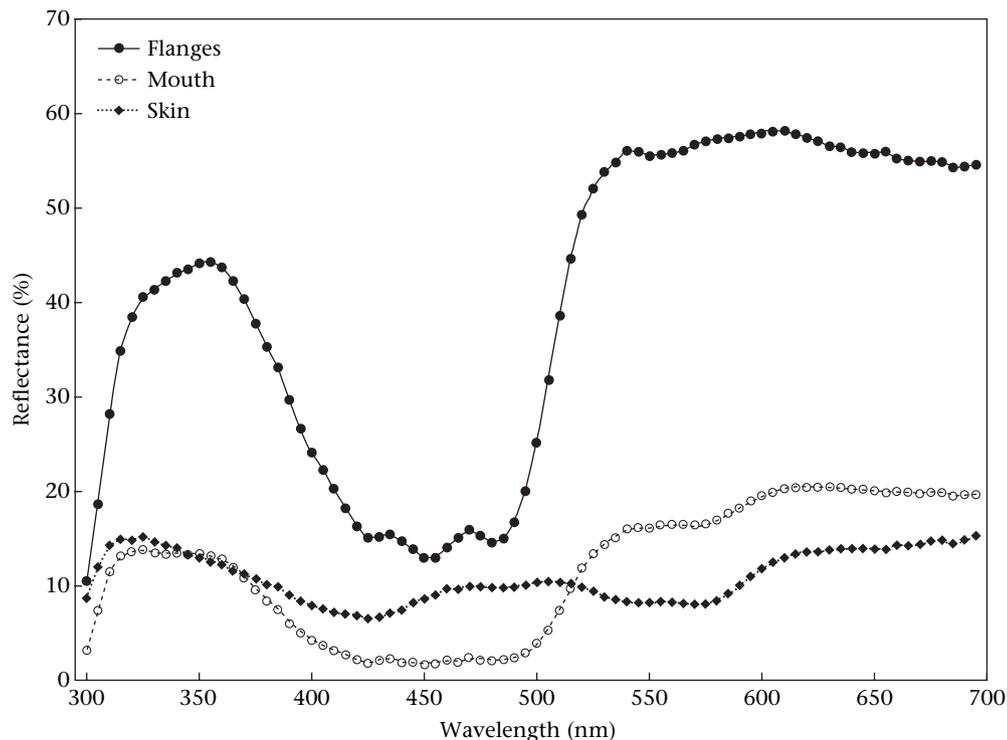


Figure 1. Reflectance spectra (median values) from mouth, flanges and body skin of spotless starling nestlings.

**Table 1.** General linear models explaining T-cell-mediated immune response of nestlings (dependent variable) with skin brightness of nestlings as a covariate, food treatment as a fixed factor, and nest identity as a random factor

	Mean square/error	df	F	P
Type III decomposition				
Skin brightness (fixed) (1)	0.049/0.030	1,32	1.65	0.21
Food treatment (fixed) (2)	0.096/0.030	1,34.0	3.21	0.08
Nest identity (random) (3)	0.082/0.028	38,33.5	2.93	0.001
(1)*(2) (fixed)	0.148/0.030	1,32	4.95	0.033
(2)*(3) (fixed)	0.028/0.030	32,32	0.93	0.58
Error	0.030			
Type I decomposition				
Skin brightness (fixed) (1)	0.378/0.074	1,43.9	5.14	0.028
Food treatment (fixed) (2)	0.152/0.033	1,30.2	4.59	0.04
Nest identity (random) (3)	0.084/0.028	38,24.6	3.029	0.003
(1)*(2) (fixed)	0.125/0.028	1,35.6	4.46	0.042
(2)*(3) (fixed)	0.028/0.030	32,32	0.93	0.58
Error	0.030			
Type I decomposition				
Nest identity (random) (3)	0.092/0.028	38,23.8	3.33	0.001
Skin brightness (fixed) (1)	0.034/0.029	1,63.9	1.17	0.28
Food treatment (fixed) (2)	0.186/0.028	1,23.4	6.74	0.016
(1)*(2) (fixed)	0.125/0.028	1,35.6	4.46	0.042
(2)*(3) (fixed)	0.028/0.030	32,32	0.93	0.58
Error	0.030			

The interaction between experimental treatment and nest identity was maintained in the model to adjust degrees of freedom conservatively to approximately the number of nests with nestlings of both treatments (food supplemented and control). Sums of squares were decomposed by using type III (orthogonal) and type I (hierarchical) methodologies. Results from introducing nest identity as the first or the third variable in the model are shown.

accordance with this interpretation, when we ran the above model but used a type I decomposition of sums of squares (this approach estimates the contribution of all factors in the model taking into account the order of the factors) and introduced skin brightness before nest identity in the model (i.e. the covariate was not controlled for between-nest variation; see *Methods*), all variables explained a significant proportion of residual variance in nestling immune response (Table 1). However, when nest identity was the first variable introduced in the model (and thus all other factors were controlled for nest identity), the effect of skin brightness was no longer significant (Table 1). These results suggest that the relation between skin coloration and nestling immune response was mainly due to between-nest covariation of the two variables, whereas within-nest variation in immune response is better explained by experimental treatment.

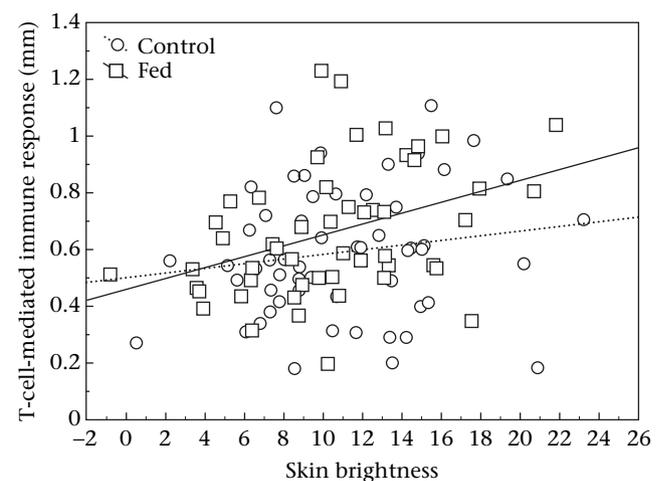
Finally, in accordance with the importance of within-nest variation of skin brightness in explaining the immune response of nestlings, we found that the interaction between experimental treatment and nestling skin brightness

explained a significant proportion of the variance in nestling T-cell-mediated immune response (Table 1). The experiment had more effect on nestlings with brighter skin (Fig. 2).

## DISCUSSION

We found support for the hypothesis that the level of T-cell-mediated immune response in spotless starling nestlings is a nutrition-dependent trait, because food-supplemented nestlings developed a stronger immune response than control ones. This result suggests that a biased parental investment in some nestlings showing traits that are attractive to parents would result in a relation between the expression of those traits (that affect parental investment) and nestling immunity. Such a relation may have important implications, because the level of T-cell-mediated immune response is a good predictor of nestling survival and recruitment in at least some species (Christe et al. 2001; Møller & Saino 2004; Cichon & Dubiec 2005; Moreno et al. 2005). The elaboration of traits attractive to parents could be directly linked to nestling reproductive value not only by parents feeding the most detectable nestlings, but also by parents adaptively and preferentially feeding nestlings with the highest reproductive value.

Nestling mouth coloration affects parental investment (e.g. Gotmark & Ahlstrom 1997; Saino et al. 2000a; Heeb et al. 2003; Jourdie et al. 2004) and, consequently, a relation between this trait and nestling immune response can be predicted. Furthermore, it has been experimentally demonstrated that UV reflectance of both skin and mouth in starling and Alpine swift, *Apus melba*, nestlings affects parental food provisioning (Jourdie et al. 2004; Bize et al. 2006), a result that also predicts a relation between skin coloration and immunity of nestlings. In accordance with this scenario, Jourdie et al. (2004) found a positive relation between skin brightness and the level of T-cell-mediated immune response in starling nestlings. We also



**Figure 2.** Relation between T-cell-mediated immune response (mm; dependent variable) and skin brightness in food-supplemented and control spotless starling nestlings. Regression equations are:  $Y(\text{control}) = 0.499 + 0.008X$ , and  $Y(\text{experimental}) = 0.457 + 0.019X$ .

found such a relation in spotless starling nestlings (Fig. 2). In addition, the within-nest variation in both nestling colour and immune response was lower than between-nest variation. If the relation between nestling coloration and immunity was due to differential parental investment in the most brightly coloured nestlings within a brood as hypothesized by Jourdie et al. (2004), the relation should still hold after we controlled for variation caused by nest identity. However, when this variation was controlled for, nestling coloration no longer significantly explained the level of T-cell-mediated immune response of nestlings. We can conclude that the relation between nestling coloration and immunity was mainly due to between-nest differences in nestling colour that covaried with differences in parental quality and/or genetic quality of nestlings.

Within nests, variation in nestling coloration explained the nestling immune response, because the effect of food supplementation on the immune response was stronger in nestlings with bright skin colour (Fig. 2). If nestling colour reflected not immunocompetence but preferential feeding of the most detectable nestlings by parents (Gotmark & Ahlstrom 1997; Heeb et al. 2003; Jourdie et al. 2004) we would, however, expect the extra food to have a differential positive effect on nestlings of low nutritional condition (i.e. low value of skin brightness). Although we measured nestling coloration only during the second experimental feeding (i.e. visit), we can be sure that the experiment did not affect nestling coloration significantly, because food-supplemented and control nestlings did not differ in skin brightness (results not shown). Therefore, the significant interaction between nestling coloration and experimental treatment cannot be explained by the experiment affecting both nestling colour and immunity. Instead, this interaction suggests that nestlings with bright skin used extra food to improve their ability to produce a strong immune response in a more efficient way than pale nestlings. These results are consistent with nestling coloration being a signal, not only of condition (e.g. Kilner 1997), but also of intrinsic nestling characteristics that predict the PHA immune response at fledging, a signal that parents adaptively use to make feeding investment decisions (Saino et al. 2000a, b; Hunt et al. 2003).

To conclude, we suggest that the relation between nestling immunity and coloration could be explained not only by parents preferentially feeding the most detectable nestlings (Jourdie et al. 2004), but also by the existence of intrinsic characteristics of nestlings that are signalled by their coloration and that predict their ability to produce a strong T-cell-mediated immune response.

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