

## Relative importance of factors affecting nestling immune response differs between junior and senior nestlings within broods of hoopoes *Upupa epops*

Manuel Martín-Vivaldi, Magdalena Ruiz-Rodríguez, María Méndez and Juan J. Soler

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Animals should invest in the immune system to protect themselves from parasites, but the cost of immune responses may limit investment depending on resource availability. In birds' broods, senior and junior chicks in size hierarchies face different rearing conditions, and thus we predicted that factors affecting immune response should differ between them. In asynchronously hatched hoopoe *Upupa epops* broods, we found that the immune response of senior nestlings was not related to their body condition, but positively related to risk of parasitism (which was indirectly estimated by laying date). This suggests that their immunocompetence is not limited by access to resources, and they can differentially invest in immune response with increasing risk of parasitism. On the other hand, immune response of junior nestlings was related to their body condition, but secondarily also to risk of parasitism. Our results agree with previous studies that have found significant influence of nutritional status and risk of parasitism on nestlings immune defence, but show that the effects of these environmental factors on nestling immunocompetence differ between nestlings occupying high and low rank positions in size hierarchies. The possible influence of maternal effects on the results found is also discussed.

*M. Martín-Vivaldi (correspondence), M. Ruiz-Rodríguez and J. J. Soler, Estación Experimental de Zonas Áridas, Consejo Superior de Investigaciones Científicas, 04001 Almería, Spain. – M. Méndez, Departamento de Biología Animal, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain. Present address of M. Martín-Vivaldi: Departamento de Biología Animal, Facultad de Ciencias, Universidad de Granada, 18071 Granada, Spain. E-mail: mmv@ugr.es*

Parasite attacks may cause severe damages to hosts, and thus the ability to mount an effective immune defence is a primary factor determining individual fitness. For instance, in some birds, the level of immune response explains nestling and fledging success (Christe et al. 1998, González et al. 1999), but also the probability of survival to the next breeding season (Soler et al. 1999b). Therefore, immunocompetence is of central importance in evolutionary ecology (Sheldon and Verhulst 1996, Schmid-Hempel 2003).

Despite that nestling immunocompetence has a genetic component, most within species variation in immune response is caused by environmental factors

(Brinkhof et al. 1999, Christe et al. 2000, 2001, Tella et al. 2000a, Soler et al. 2003b). Food availability during development is one of the main factors affecting nestling immunocompetence (Chandra and Newberne 1977, Gershwin et al. 1985, Saino et al. 1997, Møller et al. 1998, Soler et al. unpubl. data). Moreover, body mass of nestlings, which is related to availability of resources during development, usually predicts the level of nestling immune response (Christe et al. 1998, 2001).

The second primary environmental factor affecting nestling immunocompetence is risk of parasitism, which is positively related to laying date (De Lope 1993a). Parasite infestations may cause very strong detrimental

effects on hosts (De Lope 1993b) and, thus, risk of parasitism should favour an increase in nestling immune response. In accordance with that, a positive relationship has been found between immunocompetence and different variables related to risk of parasitism both at the interspecific (Møller and Erritzoe 1996, 1998, 2002, Møller 1997, Møller et al. 2003, Martin et al. 2001) and at the intraspecific level (Tella et al. 2001). Thus, the need for an effective immune system increases with the advance of the breeding season, because parasite pressure increases as well (Merino et al. 2000, Christe et al. 2002). However, availability of resources may also decrease as the season progress, which should affect to body condition of nestlings, but also to the immune system development (Sorci et al. 1997). Therefore, variation of immune response of nestlings should be explained by their body mass, but also by risk of parasitism experienced during development.

In asynchronously hatched broods, senior nestlings usually enjoy advantages in the access to food (Mock and Parker 1997). Therefore, within the same nest, junior and senior nestlings differ in availability of resources that could be invested in immune function and, thus, immune system of junior nestlings would be more constrained by food availability than that of senior nestlings. If that is the case, resource availability (i.e. body condition) should be a main determinant of variation in immunocompetence for junior, but not for seniors nestlings.

The effect of risk of parasitism on immune response may also differ between senior and junior nestlings within the same nest. Differences in food availability for junior and senior nestlings may increase as the season progress, mainly because food availability usually decreases with the advance of the breeding season (Przybylo et al. 2001). Therefore, given that risk of parasitism also increases as the season progress (see above), but junior would be in relatively poorer physical condition than senior nestlings, they may have relatively less resources to allocate in immune response in situations of high risk of parasitism (i.e. late laying dates), and so the effects of risk of parasitism would differ between junior and senior nestlings within the same nest. Nevertheless, a differential investment by females in last-laid eggs (as suggested for barn swallows by Saino et al. 2001), or dependent on risk of parasitism (Saino et al. 2002a, on laying date De Neve et al. 2004a), may compensate disadvantages of junior nestlings, and may favour adaptive levels of immunocompetence in relation to risk of parasitism also in junior nestlings. Thus, depending on the existence of differential maternal effects on late-laid eggs and/or in relation to risk of parasitism it can be expected both that laying date is a predictor of immune response only in senior nestlings, or that both senior and junior nestlings increase immune defence with laying date. In any case, in species with marked brood size hierarchies, maternal effects in last

eggs do not completely compensate junior disadvantages caused by their late hatching, giving rise to nestlings greatly differing in body condition within the same brood. Therefore, it is possible that the variables that better explain immune response of nestlings differ within a brood depending on differences in nestling condition caused by hierarchies.

Here, we test this hypothesis by studying T-cell mediated immune response and parasitism of nestling hoopoes *Upupa epops* by ectoparasitic flies *Carnus hemapterus*. Since probability of parasitism increases as the season progress (see results), we predict that level of immune response in whole broods should be positively related to laying date while controlling for the possible indirect effect of variation in body mass with date (prediction 1). Moreover, hoopoes hatch asynchronously, establishing a marked brood hierarchy with siblings greatly differing in body mass (Bussman 1950, Kubik 1960, Gupta and Ahmad 1993, Baldi and Sorace 1996, Martín-Vivaldi et al. 1999). In this species females feed preferentially to first hatched nestlings (Martín-Vivaldi et al. 1999) suggesting that food availability for senior nestlings is less restricted than for junior nestlings. Therefore, first hatched nestlings should increase immunocompetence as a response to an increase in risk of parasitism without any effect of body mass (prediction 2), while immune response of junior nestling should mainly be explained by their body mass, although additional effects of laying date while controlling for body condition may be expected.

## Material and methods

### Study area and species

The study was carried out in the springs of 2002–2004 in Hoya de Guadix (37°18'N, 3°11'W), southern Spain, a high altitude plateau at approximately 1000 m above sea level, where we have studied the population of hoopoes breeding in natural holes since 1992. The vegetation is sparse in the area, including cultivated cereals, some remains of holm oaks forests, grows of almond trees, and olive trees and other tree crops in irrigated areas surrounding villages. Hoopoes are more abundant in the olive crops and holm oak remains.

Hoopoes are medium sized coraciiformes that feed on ground and subterranean invertebrates and nest in a variety of holes that are re-used successfully by different individuals for many years (Cramp 1998). Females lay one or, at most, two clutches along the breeding season, between February and June, and clutch size ranges from 4 to 12 eggs in western Europe, although the most common sizes are 6–8 eggs (Martín-Vivaldi et al. 1999). Incubation is performed only by the female, and starts usually with the first or second egg, producing a complete hatching asynchrony in which eggs usually

hatch at 24 h or even greater intervals (Bussman 1950, Gupta and Ahmad 1993, Baldi and Sorace 1996, Cramp 1998). This generates a marked size hierarchy within the brood that frequently causes the death of the last chicks (Bussman 1950, Kubik 1960, Baldi and Sorace 1996, Martín-Vivaldi et al. 1999). In our population, during the 1991–1995 breeding seasons, brood reduction by the death of some nestling occurred in more than 90% of successful nests, and the proportion of eggs per clutch that failed to produce fledglings was very high (49%, Martín-Vivaldi et al. 1999). This causes that brood sizes at the second half of the nestling period only rarely exceed five nestlings. Females incubate alone and stay within the nest during incubation and the first week of the nestling period, being fed by males. Afterwards both pair members carry food to the nest until fledging of chicks when they are 24–30 days old (Baldi and Sorace 1996, Cramp 1998, Martín-Vivaldi et al. 1999).

The ecto-parasitic fly *Carnus hemapterus*, (Diptera: Carnidae) is a 2-mm long blood-sucking fly that parasitises nestling birds (Walter and Hudde 1987, Kirkpatrick and Colvin 1989, Dawson and Bortolotti 1997, Roulin 1998). It uses a wide diversity of bird species as hosts (Capelle and Whitworth 1973, Dawson and Bortolotti 1997, Grimaldi 1997), although seems to parasitise preferentially hole-nesting species (Grimaldi 1997). *Carnus hemapterus* flies usually overwinter as pupae in birds nests. After their emergence, adults are initially winged, but typically lose their wings once they locate a suitable host (Roulin 1998, 1999). Flies are assumed to colonise nest hosts actively during the winged phase of their life cycle (Grimaldi 1997, Roulin 1998, 1999).

All adult breeding hoopoes were colour ringed. During the study period we surveyed 84 clutches including first, second and replacement clutches (20, 36 and 28 in 2002, 2003, and 2004, respectively). Many clutches failed either to predation (33 clutches) or desertion (2 clutches), leaving 49 successful broods that survived up to age of ringing. Among these, five were first or second clutches laid by repeated pairs in different breeding attempts and, to avoid pseudo-replication, we have excluded their data on their second attempt. However, when a female (one case) or a male (one case) laid two clutches with different partners, both have been included in the analyses. Thus our sample size is of 44 successful broods. However, there was a nest in which we took body measurements but did not inject PHA, and therefore analyses involving immune response include a case less than those only including biometric data. Also, since juniors are defined as those nestlings situated after the second position in the size hierarchy (see below), four broods with less than three nestlings are not included in the analyses for junior nestlings, and therefore sample sizes differ between categories.

During the winter 2002–2003, 150 nest boxes were installed scattered in the study area to make easier the work with the species. In 2002 all the 11 clutches used where laid in natural holes, in 2003 six nests were in natural holes and 10 in nest boxes, and in 2004 all pairs ( $n=17$ ) bred in nest boxes. There were not differences between natural nests and nest boxes in the 2003 breeding season in prevalence of parasitism (chi-square = 1.95,  $P=0.16$ ), intensity of parasitism (one-way anova  $F_{1,15}=0.79$ ,  $P=0.39$ ), mean immune response of nestlings (one-way anova  $F_{1,14}=1.53$ ,  $P=0.24$ ), or number of chicks fledged (one-way anova,  $F_{1,15}=0.04$ ,  $P=0.84$ ), therefore both types of nests have been considered together for subsequent analyses.

Brood sizes of the nests included in the analyses at age of taking measurements ranged between two (four cases) and six nestlings (two cases; mean  $\pm$  SE =  $3.89 \pm 0.15$ ,  $n=44$ ).

### **T-cell-mediated immune response, ectoparasite load, and nestling hierarchy**

Nests were visited regularly to record laying date, clutch size and hatching date. When the oldest chick was 18–25 days old, we opened the nest, ringed, weighed, and measured nestlings, as well as estimated their parasite load by counting the number of haematomas and dry blood spots traces of the activity of flies *Carnus hemapterus* (Soler et al. 1999a) on the belly and underwings. We used a four-values scale for number of blood spots: 1: 0 to 10 spots, 2: 11 to 20 spots, 3: 21 to 40 spots, and 4: more than 40 spots. This scale was applied to each nestling, but as a variable reflecting ecto-parasitism of the entire brood, we used mean values of pecks found in all nestlings.

A phytohemagglutinin-P (PHA-P, Sigma Chemical Co.) injection was used to evaluate the in vivo T-cell mediated immune response of nestlings (Cheng and Lamont 1988). We injected nestlings subcutaneously in the left wing web with 0.2 mg of PHA dissolved in 0.04 ml of physiological saline solution after removing feathers on the area and marking the point of injection with an indelible felt-tip pen. The right wing web was injected with 0.04 ml of saline solution (Bausch and Lomb Co.). We measured the thickness of each wing web at the pre-marked injection site with a digital pressure-sensitive micrometer (Mitutoyo, model ID-CI012 BS; to the nearest 0.01 mm) before and 24 h after the injection. The T-cell mediated immune response or wing web index was then estimated as the change in thickness of the left minus that of the right wing web (Lochmiller et al. 1993). Measurements of each wing web on each occasion were repeated three times, and the mean value was used in subsequent analyses. Inflammatory response to PHA injection have been shown to reflect ecto-parasite

induced mortality across species of birds, estimated as differences in breeding success between heavily and non-parasitized broods (Martin et al. 2001). These estimations included parasitism by *Carnus* flies and, thus, T-cell immune response of hoopoes should depend on the level of parasitism selection pressure exerted by *Carnus* flies.

We estimated hatching order of chicks within a brood by comparing bill and wing length at age of ringing. This is a good indicator of differences in age since both structures grow along the whole nestling period and do not reach its final size before fledging (Bussman 1950, Krištín 1994, own unpubl. data). Despite there is sexual size dimorphism in adult hoopoes' bill and wing length (Cramp 1998, own unpublished data), at the age of our measurements neither of these two body parts have finished growing in nestlings, and therefore differences among them should reflect age and not the final size to be acquired. Moreover, the fact that ranking is maintained between the early and late nestling period (see below) clearly show that the measurements indicate the hatching order and not sex differences. When there was disagreement between the relative sizes of these two structures, we used the one with the greatest percentage of difference. Other studies have also used differences in wing length to establish hatching order within the brood (Roulin et al. 2003 for kestrels *Falco tinnunculus*).

For the comparison between nestlings occupying high and low positions in the hierarchy we distinguished two groups within each brood. The first group called "seniors" included the first and second nestlings in the hierarchy. The second group, called "juniors" included all the remaining nestlings. For the analyses we used mean values of the variables of interest for each group in each brood. This estimate seems appropriate because: (a) the most common brood size in our data is four nestlings, and therefore most broods are divided in two halves, and (b) when studying whether nestlings can change their positions in the hierarchy along the nestling period, we found that for 10 nests with available information, only nestlings occupying ranks 1 and 2 in the hierarchy interchanged their position from early (oldest chick with 8–13 days) to late nestling period (oldest chick with 19–24 days). Such changes occurred in five out of 10 broods (50%, against the expected frequencies of no change, Fisher exact test  $P=0.033$ ). In no case did a nestling of one of the two groups ("juniors" and "seniors") change of category between early and late nestling period.

The number of nestlings per brood within the group "juniors" varies depending on brood size. To check that results are not affected by this fact, we performed analyses both including all broods, or only broods with less than five nestlings, and we always obtained similar

Table 1. Results of All Effects General Regression Models exploring the influence of several factors on immunocompetence (response to an injection with PHA) for the whole brood (mean values of all nestlings), senior (mean values of first and second nestlings) and junior (mean value of the remaining nestlings) hoopoe nestlings. Analyses are performed: (a) including all brood sizes, and (b) including only brood sizes up to 4 nestlings. Variables marked with asterisk on P-value entered as significant predictors using a Forward Stepwise Model (with year and tarsus length forced to enter) instead of the All Effects Model presented.

	F a/b	df a/b	P a/b
<b>Brood</b>			
Body mass	5.45/4.27	1	0.026*/0.049*
Tarsus length	0.94/0.47	1	0.340/0.501
Laying date	7.55/6.88	1	0.010*/0.015*
Brood size	1.06/1.41	1	0.323/0.246
Brood age	0.75/0.06	1	0.393/0.804
Year	3.08/1.20	2	0.059/0.318
Intensity of infection	0.00/0.13	1	0.967/0.724
Error		34/24	
<b>Senior nestlings</b>			
Body mass	0.53/1.24	1	0.470/0.276
Tarsus length	0.00/0.11	1	0.981/0.738
Laying date	5.48/4.82	1	0.025*/0.038*
Brood size	0.25/0.73	1	0.624/0.400
Brood age	0.42/0.00	1	0.521/0.979
Year	1.87/0.48	2	0.170/0.624
Intensity of infection	0.11/0.54	1	0.743/0.468
Error		34/24	
<b>Junior nestlings</b>			
Body mass	10.27/5.98	1	0.003*/0.024*
Tarsus length	2.80/1.82	1	0.105/0.192
Laying date	5.60/3.95	1	0.025*/0.061*
Brood size	0.02/0.07	1	0.885/0.799
Brood age	0.84/0.08	1	0.367/0.779
Year	4.45/1.76	2	0.020*/0.198
Intensity of infection	0.20/0.31	1	0.661/0.587
Error		30/20	

results (Table 1). Results were also the same comparing predictors of immune response only of the first and last hatched nestlings per brood, or even performing one separated analysis for each of the rank positions (data not shown). Moreover, in all analyses brood size was included as a covariable, to control for the possible effect of differences in the number of nestlings. Therefore, we are confident that the results found are not caused by a particular way of defining groups but are a real fact.

## Statistics

The variables studied did not differ significantly from normal distributions (Kolmogorov-smirnov tests, all  $P > 0.1$ ) and, thereby, we used parametric statistics. We used All Effects and Forward Stepwise General Regression Models to explore the effects of several potential predictors on the immune response of nestlings by using the Statistica 6.0 (StatSoft 2001) software. In such models we included as predictors laying date, body mass, tarsus length (to control body mass for body size thus obtaining an estimate of condition independent of size), brood size, brood age (age of the first hatched nestling), intensity of the infection by flies in the brood, and year. When comparing values for seniors and juniors within the same nest we used paired t-tests. To analyse the relationship between laying date and the intensity of the infection, as good as to check for dependence between body mass and laying date, we used quadratic regressions with laying date corrected for year differences (One-way ANOVA, inter-annual differences in laying date:  $F_{2,36} = 24.43$ ,  $P < 0.001$ ; there were not inter-annual differences in intensity of the infection or body mass,  $P > 0.05$ ). Finally, to analyse the relationship between laying date and the prevalence of parasites while controlling for year differences we used Generalized Linear Models with binomial distribution, because prevalence also differed among years (Pearson  $\chi^2 = 9.38$ ,  $P = 0.009$ ).

## Results

The intensity of parasitism by flies in nests of hoopoes tended to increase as the season progressed and, although it decreased at the end of the season, the linear term is statistically significant (quadratic regression  $R = 0.370$ ,  $F_{2,41} = 3.24$ ,  $P = 0.049$ ;  $b_0 = -2.63 \pm 1.90$ ,  $t(41) = -1.39$ ,  $P = 0.174$ ;  $b_1$  (linear term)  $= 0.102 \pm 0.040$ ,  $t(41) = 2.55$ ,  $P = 0.015$ ;  $b_2$  (quadratic term)  $= -0.0005 \pm 0.0002$ ,  $t(41) = -2.52$ ,  $P = 0.016$ , Fig. 1b). On the other hand, although the prevalence of the infection tended to increase with laying date (logistic regression,  $\chi^2 = 3.66$ ,  $df = 1$ ,  $n = 44$ ,  $P = 0.056$ , Fig. 1a) the effect did not persist after correcting for year

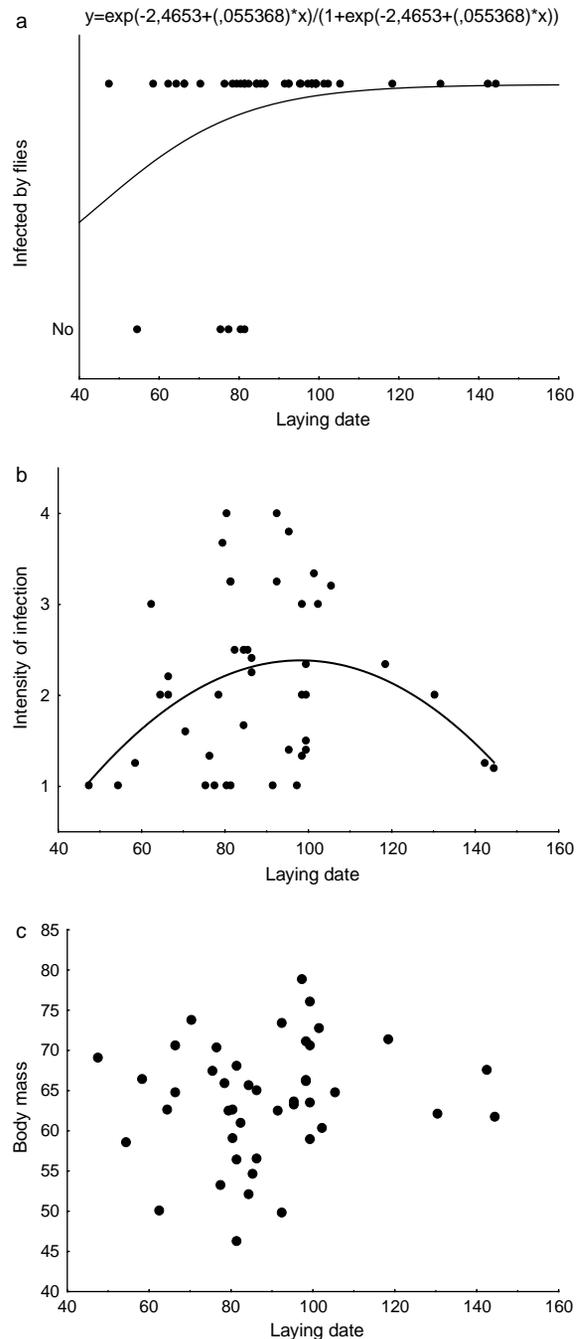


Fig. 1. Relationship between laying date and (a) prevalence of parasitism, (b) intensity of the infection, by *Carnus hemapterus* flies on hoopoe broods, and (c) brood mean body mass (g), after correcting for year differences in laying date. Laying date = 1 corresponds to first day of February.

differences in a Generalized Linear Model (Wald Stat. = 2.46,  $P = 0.117$ ). The results on intensity of the infection allow the use of laying date as a variable related to risk of parasitism. Moreover, mean body mass did not result significantly related to laying date either in first or second order coefficients (quadratic regression

$R = 0.144$ ,  $F_{2,41} = 0.44$ ,  $P = 0.650$ ;  $b_0 = 59.86 \pm 15.62$ ,  $t(41) = 3.43$ ,  $P = 0.0004$ ;  $b_1$  (linear term)  $= 0.037 \pm 0.330$ ,  $t(41) = 0.11$ ,  $P = 0.911$ ;  $b_2$  (quadratic term)  $= 0.0001 \pm 0.0020$ ,  $t(41) = 0.04$ ,  $P = 0.970$ , Fig. 1c).

In accordance with prediction 1, average level of T-cells mediated immune response of all siblings in broods resulted positively related to laying date (Table 1).

Due to differences in rank in the hierarchy, senior were of larger body mass than junior nestlings in the same nest (paired-t test,  $t = 5.06$ ,  $n = 40$ ;  $P < 0.001$ , seniors mean (SD)  $= 66.27$  (7.02), juniors mean (SD)  $= 60.83$  (8.98)). Furthermore, when analysing factors affecting nestling immune responses in a General Regression Model, body mass, laying date, and year explained significant variation of junior-nestlings' immune response. Body mass of junior nestlings was the main predictor (Table 1, Fig. 2), but laying date also entered in both the All Effects Model and the Forward Stepwise analysis (Table 1). In the case of senior nestlings, only laying date was a significant predictor of immune response independently of using All Effects or Forward Stepwise Models (Table 1, Fig. 2).

Differences between senior and junior nestlings in the relative contribution of body condition and laying date in explaining variation in nestling immune response were significant for body condition (comparison of partial correlation coefficients from Forward Stepwise Models,  $P = 0.046$ , seniors  $= 0.141$ , juniors  $= 0.542$ ), but not for laying date (comparison of partial correlation coefficients from Forward Stepwise Models,  $P = 0.944$ , seniors  $= 0.361$ , juniors  $= 0.375$ ). The effect of body condition on immune response tended to be stronger in junior nestlings although the difference did not reach significance (comparison of standardised partial regression coefficients from Forward Stepwise Models,  $t = 1.80$ ,  $df = 80$ ,  $P = 0.076$ , seniors ( $\pm SE$ )  $= 0.136$  (0.157), juniors ( $\pm SE$ )  $= 0.517$  (0.139)), while the effect of laying date on immune response was very similar in both age classes (comparison of standardised partial regression coefficients,  $t = 0.02$ ,  $df = 80$ ,  $P = 0.976$ , seniors ( $\pm SE$ )  $= 0.497$  (0.211), juniors ( $\pm SE$ )  $= 0.506$  (0.218)).

In order to evaluate the consistency of our results among years, we repeated the analyses including the interaction between year and each of the two main predictors of immune response. In all the models (both in All Effects and Forward Stepwise Models), body condition persisted as a significant predictor of juniors immune response, and its interaction with year was not significant neither for junior (All Effects General Regression Model  $F_{2,26} = 1.69$ ,  $P = 0.205$ ) nor for senior nestlings (All Effects General Regression Model  $F_{2,30} = 2.62$ ,  $P = 0.089$ ). Similarly, laying date persisted as a significant predictor of seniors immune response, and its interaction with year was not significant (All Effects General Regression Model  $F_{2,30} = 1.77$ ,  $P = 0.187$ ). However, the effect of laying date for junior nestlings

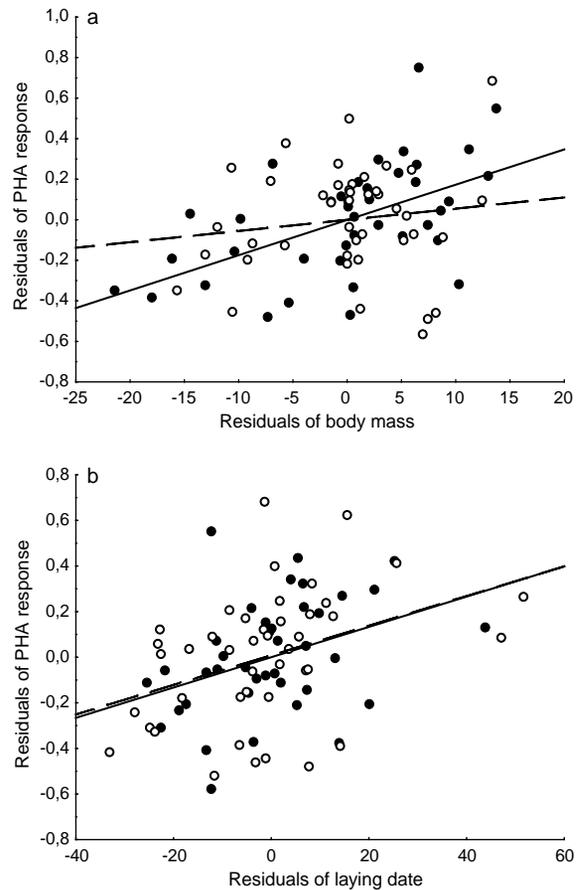


Fig. 2. Relationship between residuals of immunocompetence (response to an injection with PHA) of nestlings and (a) body mass residuals, and (b) laying date residuals, after the final Forward Stepwise Models in Table 1. For Fig. 2a, the variables: PHA response (of both categories) and body mass of juniors were corrected for year, tarsus length, and laying date; while body mass of seniors was corrected only for year and tarsus length. For Fig. 2b, the variables: PHA response and laying date for seniors were corrected for year and tarsus length; PHA response and laying date for juniors were corrected for year, tarsus length and body mass. Filled points and continuous lines are data for junior chicks, dotted lines and empty points are data for senior chicks.

disappeared in both All Effects and Forward Stepwise Models after including the interaction between year and laying date and the effect of such interaction was significant in the final Forward Stepwise Model ( $F_{2,32} = 4.58$ ,  $P = 0.018$ ).

## Discussion

We found that the intensity of parasitism by *Carnus* flies in hoopoe broods tended to increase with date. This result is in accordance with previous findings of increasing infestations by ectoparasites along the breeding season (De Lope 1993a, Merino and Potti 1995,

Soler et al. unpubl. data). *Carnus hemapterus* is the most common ectoparasite in hoopoe nestlings and it should act as a vector of other parasites (see Soler et al. 1999a). Moreover, body mass of nestlings did not vary along the breeding season and, thus, the effect of laying date on nestling immune response can be considered as reflecting risk of parasitism, and not food abundance in our studied population of hoopoes. Since we also included the actual intensity of infection in nests as a potential predictor in the analyses, but this variable never entered significantly in the models, it is the risk of parasitism associated to laying date, and not the real infection experienced by the brood the factor influencing immune response.

Nestling immunocompetence is affected by risk of parasitism and quality and quantity of food received during development, which has received, either comparative, observational, and experimental support (see reviews in Zuk and Stoehr 2002, Schmid-Hempel 2003). Our results also confirm that both variables are important predictors of nestling immunocompetence in hoopoes since we found that mean T-cells immune response of nestlings increased as the season progressed (and laying date is related to risk of parasitism but not to body mass of nestlings) and was positively related to mean body condition in the same statistical model.

The effects of both variables have usually been studied independently in different species or study years. However risk of parasitism and food availability for nestling may be related (see introduction) and, thus, both variables should be included in the same models to detect relative contribution of each one explaining nestling immune response. In addition, most studies trying to detect factors affecting nestling immunocompetence use whole broods as measurement units (Merino et al. 2000, Saino et al. 1997, Christe et al. 2001), or introduce body mass of each nestling just for controlling for this variable in mixed models with nests as a random factor (see for instance Tella et al. 2000b, Soler et al. 2003a, De Neve et al. 2004b) but do not discuss within brood differences in the relative contribution of different factors. However, within brood variation in variables related to immune response may also help to understand factors affecting this important life history trait. This is because variation in environmental conditions experienced by nestling in high and low positions in the nest hierarchy can be predicted. Therefore, we hypothesised that the relative importance of the factors affecting immune response may also differ between nestlings occupying such positions within the same nest. This approach is much more robust than that of using mean-values of immune response per nest and exploring its relationship with environmental factors, because siblings are genetically related and, thus, by focussing on differences between them, the genetic component of

the studied trait and its possible relationship with environmental variables is, at least partially controlled.

The first environmental condition for which junior and senior nestlings may differ is availability of resources, which is a prime factor explaining level of nestling immune response. Hoopoes, by hatching asynchronously, establish a very clear body size hierarchy within the brood and thus, as in other species, senior nestlings should always get their needs satisfied before junior nestlings are fed (Massemin et al. 2002). This fact is confirmed by the observation that hoopoe females preferentially feed first hatched nestlings (Martín-Vivaldi et al. 1999).

Risk of parasitism could also be higher for the smaller nestlings because they would be the tasty chicks for parasites, thereby diminishing parasite selection pressure for nestlings of higher rank (Christe et al. 1998). However, we have found that parasite intensity suffered by senior and junior nestling of hoopoes was similar (Martín-Vivaldi and Soler unpubl. data) and, therefore, such possibility can be excluded in this species. Another possible influence of ranking in the effect of risk of parasitism, could derive from the existence of a trade-off between investing in immune response and investing in growing or sustaining basic physiological needs for surviving (Soler et al. 2003a and references therein). Since junior nestlings have more limited access to food than senior nestlings, they may be constrained to adapt their investment in immune response to the current risk of parasitism. However, they could still be selected for investing the few resources available differentially in immune system at the expenses of growing if this improves survival. Also, it is possible that females differentially invest in eggs components depending on risk of parasitism (see below). Therefore, despite differences in availability of resources between junior and senior nestlings may suggest different abilities to increase immune response with date, other outcomes are possible and predictions do not point clearly in one direction.

Our results are in accordance with the hypothesis of differences related to ranking in the hierarchy, since laying date was the only predictor of T-cell mediated immune response of senior, but body condition was the main predictor for junior nestlings, although laying date also entered as a second predictor in importance in the case of junior nestlings (Table 1). The fact that body condition of seniors was not related to immune ability suggests that the size hierarchy generated by hatching asynchrony in the hoopoe ensures that nestlings in the higher positions always get enough resources for adequate development of the immune system. This possibility is in accordance with previous results showing that feeding rate of adult hoopoes correlates with the number of nestlings surviving to fledging, but does not affect body mass of the senior nestlings (Martín-Vivaldi et al. 1999). On the other hand, junior nestlings seem to be

constrained in this regard by the availability of food when competing with their older siblings. This suggests that, even in the case that hoopoe females invested differentially in the last eggs laid with substances that enhance immune ability (as suggested by Saino et al. 2001 in barn swallow), to compensate the disadvantages of late hatched nestlings (as shown in other species with hormones that improve nestling growing and competitiveness Schwabl 1993, 1996, Schwabl et al. 1997, Lipar et al. 2003, Eising et al. 2001, Eising and Grootuis 2003, Müller et al. 2004, but see Royle et al. 2001 for a possible-context dependence role of such differential investment), such investment would not be enough to ensure full development of immune ability which would still depend on the amount of food received.

Laying date explained significant variation in immune response of both senior and junior nestlings, and the effect of this factor on immune response was very similar for both age classes. However, in the case of juniors, the effect of laying date was not consistent among years as shown by the significant effect of its interaction with year. This inter annual variation in the effect of laying date for junior nestlings could be related to differences in food availability among years and thus on the possibility of differentially to invest in immune system depending on the resources available. In a study with *Ciconia ciconia*, Jovani et al. (2004) showed that body condition was a consistent predictor of immune response in nestlings among years, however they also found that the effect of laying date varied among years, highlighting that there could be temporal variability on the relationships between cellular immune response and individual traits. Our results agree with them in the type of traits with and without inter-annual variability but, in accordance with our hypothesis of differences between nestling categories, also suggest that such temporal variation in environmental conditions affects in different ways to junior and senior nestlings. The fact that such inter annual variation was not present in senior nestlings again suggests that thanks to their competing advantages seniors always have plenty of resources to invest in the immune system.

Since our study is descriptive, we cannot infer strong conclusions about causality from our results, nor can we rule out the possibility that other variables not considered may explain them. Nevertheless, together with risk of parasitism and body condition, the other main factor known to affect immune ability of nestlings are maternal effects (depositing antibodies, carotenoids or lysozymes in the eggs, Blount et al. 2002, Gasparini et al. 2002, Koutsos et al. 2002, 2003, Saino et al. 2002a, b, c, 2003). Also, several studies have shown that cellular immune response may differ between sexes both in adults (Moreno et al. 2001, Barbosa and Moreno 2002) and nestlings (Fargallo et al. 2002, Tschirren et al. 2003, Jovani et al. 2004). However, we believe that such effects

are not good candidates to explain the differences between senior and junior nestlings found in this study.

Regarding sex, the only possibility that the differences found between senior and junior nestlings are due to sex is that the sex ratio is consistently skewed to one sex in the first part of the hatching sequence and to the other sex in the second part of that sequence. Although this is possible if hoopoe females control adaptively the sex of embryos in the laying sequence, the fact that our results persist considering different brood sizes, and even using only the first and last hatched nestlings per brood, or performing one separated analysis for each of the rank positions (see Materials and methods), clearly suggests that differences are due to rank.

Regarding maternal effects, first, the absence of an effect of body condition on immunocompetence of seniors could be explained by maternal effects, if females preferentially introduce important resources for nestling immunocompetence in first laid eggs (Saino et al. 2002a,b), to increase advantages of first hatched nestlings. However, in such scenario we should consistently find higher immune responses in senior nestlings, which is not the case (Martín-Vivaldi and Soler, unpubl. data).

Second, females could benefit junior nestlings by introducing important resources for immunocompetence differentially in the last eggs laid. Such differential maternal effects would compensate in some degree their disadvantages and ameliorate differences with senior nestlings (as suggested by Saino et al. 2001 in barn swallows *Hirundo rustica*), however, cannot be responsible of the differences found in this study.

Finally, maternal effects could directly produce a pattern of increasing immune response of nestlings with date (as found for both seniors and juniors in this study), if females increase the amount of resources deposited in all eggs along the breeding season. Maternal effects related with date (De Neve et al. 2004a) or risk of parasitism associated to male quality (Saino et al. 2002a) have been demonstrated in other species, and may be one of the reasons explaining the effect of laying date on immunocompetence of junior nestlings despite their limited access to food. In this case, to distinguish between date-related differential investment of the available resources by chicks, and date-related differential investment by females in the eggs, an experimental approach is needed. Even thus, our results clearly show that the immune responses of senior and junior nestlings in hoopoe brood hierarchies are affected by environmental factors in a different way as showed by comparisons of partial correlation and regression coefficients.

In summary, we propose that, because environmental conditions experienced by different nestlings in a brood can vary in species with asynchronously hatched clutches, factors explaining level of nestling immune response could also vary within a nest. Although experimental manipulation of environmental conditions

is necessary for further support of the hypothesis, our results clearly suggest that this is the case within hoopoe broods.

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