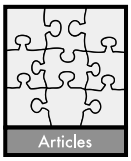

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Within-brood size differences affect innate and acquired immunity in roller *Coracias garrulus* nestlings

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The immune system has a crucial importance determining animal health and survival. Its maintenance and activation are costly and usually trade with other physiologically costly functions. In birds, number and size of siblings in a nest are likely to determine the development of immunity. Indeed lower immunocompetence is expected in large than in small broods. Moreover, in asynchronous breeders, siblings are expected to differ in immunocompetence because asynchrony produces marked size hierarchy within-broods. Here we studied the effect of environmental conditions at the nest, chick sex and size, and natural mass differences among siblings due to hatching asynchrony on the development of the innate and acquired immune systems in the threatened non-size dimorphic asynchronous breeder European roller *Coracias garrulus*. Constitutive innate immune function was estimated by using a new technique proposed by Matson et al. (2005). Natural Antibody (NAb) and Complement levels, innate immunity, varied with the mass difference between each chick and its heaviest sibling. NAb levels were higher in late-hatched siblings compared to early-hatched ones, indicating that the smallest offspring in each brood has the most developed innate immune system. This relationship was independent of the nest environment. The heterophil/lymphocyte (H/L) ratio, which may indicate the level of stress, was higher in the heaviest siblings of each brood. In addition, the H/L ratio and white blood cell count (WBC) of nestlings, measures belonging to both the innate and acquired arms of the immune system, were related to conditions suffered in the nest. Our results may be explained by differential allocation of resources by female rollers as a way to improve the survival of the whole brood.

Disease is one of the most important evolutionary agents (Haldane 1949). Animals face pathogenic or parasitic infections by the activation of their immune system (Zuk and Stoehr 2002). Immune response is, thus, a physiological mechanism enhancing animal health and survival (Johnsen et al. 2000, Råberg and Stjernman 2003, Møller and Saino 2004). However, the maintenance of the immune system in absence of infection (e.g. Lochmiller and Deerenberg 2000, but see Klasing 1998), and mounting an immune response (Bonneaud et al. 2003), are nutritional and energetically demanding processes that may monopolize many of the nutrients and energy that could be used for other costly functions (Sheldon and Verhulst 1996, Lochmiller and Deerenberg 2000). For instance, immunity may trade with nestling growth (Saino et al. 1997, Fair et al. 1999, Soler et al. 2003, Mauck et al. 2005), investment in reproduction (Festa-Bianchet 1989, Moreno et al. 1999, Bonneaud et al. 2003, Ardia 2005), and body condition (Sanz et al. 2004). Therefore, immunity can be disadvantaged during stressful periods as the result of reallocation of resources towards other energetically costly functions.

Chick immunocompetence in birds is mediated by parents and by environmental conditions. Parents influence genetically their offspring's immune system and may influence the environment in which the offspring develops by parental effects. Furthermore, number and size of siblings in a nest can determine the degree of competition within broods (Mock and Parker

1997) and hence the energetic stress suffered by each chick during its growth. This stress is likely to affect the development of immunity because chicks in poor condition usually have low immunocompetence (Saino et al. 1997, Alonso-Álvarez and Tella 2001). It seems obvious that chicks in larger broods should suffer more energetic stress than those in smaller broods (e.g. Naguib et al. 2004). In addition, we can predict that older chicks suffer less energetic stress since they are more competitive than the smaller ones. However, the way in which parents may allocate resources within their broods to reduce sibling competition, might change this to a different pattern from that predicted (Martínez-Padilla et al. 2004).

The immune system of vertebrates consists of two branches, a non-specific, innate branch and a more specific, acquired branch (Male and Roitt 2000). The innate immune system provides non-specific initial protection to parasites and infections. Meanwhile, the acquired immune system provides later and more specific protection against foreign agents. The two branches are constituted by defensive cellules (cellular component), and soluble molecules or cell associated receptors which respond to specific antigens (humoral component). Although the innate and acquired immune systems seem to be combinatorial systems (Fearon 1997), each compartment of them indicates variations in different aspects of individual condition (Ots et al. 1998, Matson et al. 2005). Thus, it seems appropriate to simultaneously investigate different parts of the immune system when studying its ecological and/or evolutionary sources of variation (Matson et al. 2005, 2006, Mendes et al. 2006).

In this study we measured innate and acquired immune systems in chicks of a wild population of a non-dimorphic bird, the European roller *Coracias garrulus*. We measured natural antibodies and the complement cascade as innate humoral immunity. Natural antibodies (NAb) recognise and attach to invading organisms and also initiate the complement cascade (Ochsenbein and Zinkernagel 2000). Their levels have been shown to parallel general disease resistance (Parmentier et al. 2004). Regarding the complement cascade, it recognises and kills invading organisms by lysing cells. Its deficiency is related to a range of diseases (Matson et al. 2005). We measured the relative amount of leucocytes (Total White Blood Cell Count, WBC hereafter) and the heterophil/lymphocyte ratio (H/L hereafter) as components of both the innate and acquired immune systems. Leucocytes include cells belonging to both the innate (heterophils) and acquired (lymphocytes) arms and protect against various pathogenic antigens. High levels of leucocytes circulating are symptomatic of stress syndrome and inflammatory processes (Ots et al. 1998). Heterophils, as part of the white blood cells, protect in a non-specific way against antigens. Meanwhile, lymphocytes are highly specific defensive cells. The ratio H/L is used as an estimator of stress in response to infectious diseases, starvation and psychological disturbance (Ots et al. 1998). Therefore, these two measures could provide information on individuals' health.

Rollers are particularly suitable for investigating the link between hatching asynchrony and immune function development within broods. Incubation begins before clutch completion, thus nestling rollers usually hatch at one day intervals (Cramp and Simmons 1988), which results in patent size hierarchies within broods (Sosnowski and Chmielewski, 1996, this study Figs. 1 and 2). The main aim of this study was to assess the effect of environmental conditions at the nest, chick sex, size and mass, and natural mass differences among siblings due to hatching asynchrony on the development of the immune system in the roller. We predicted that: (i) different pairs should have chicks with different immunity as a function of the interaction between parental quality and environmental conditions during the rearing period (e. g. Saino et al. 1997, Chin et al. 2005). In addition, we expected that (ii) chicks within-broods should differ in their immunity because they

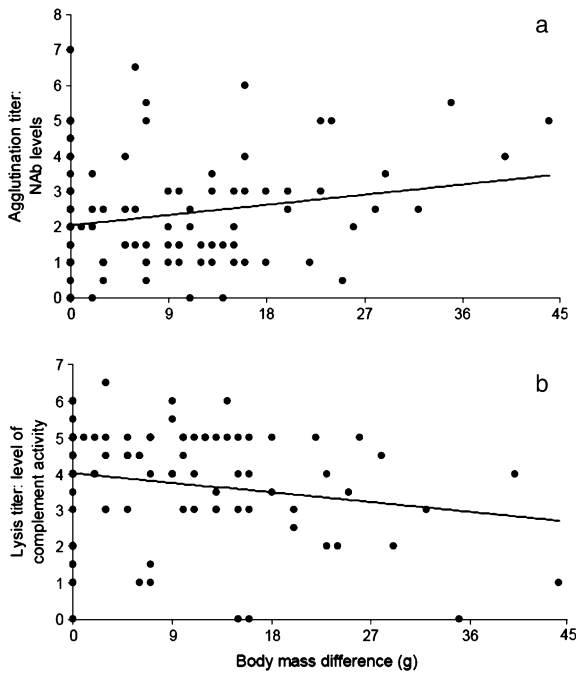

Fig. 1. Relationship between the size position of roller chicks in their broods with: (a) natural antibody levels, and (b) level of complement activity of these chicks. NAb level was calculated at the step where agglutination stopped and level of complement activity at the step where lysis stopped. Mass position of each chick was calculated as the difference between its weight and the weight of its largest sibling.

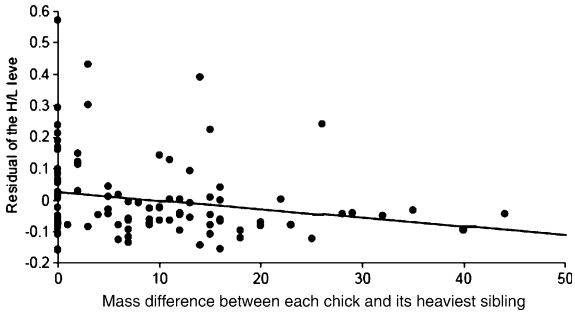

Fig. 2. Effect of body mass difference between each chick and its heaviest sibling on the Heterophil/Lymphocyte ratio of roller nestlings. The y-axis represents the residuals from the general linear mixed model performed controlling for the effect of brood size as a covariable and of nest as a random factor.

presumably are under different nutritional stress as a consequence of hatching asynchrony (e.g. Martínez-Padilla et al. 2004). Also, because the sex of nestlings may influence the development of the immune function (Grossman 1985, Moreno et al. 2001, Dubiec et al. 2006), we considered the sex of nestlings in our analyses. We have not, however, a clear prediction concerning the sex of the nestlings due to the absence of sexual-size dimorphism in this species.

Materials and methods

Study system

The study was conducted during the 2005 breeding season in an unwooded area of the Cáceres province, in western Spain (39°27′N, 6°20′E) where rollers breed in nestboxes placed on electric poles crossing the area. The area is characterized by the predominance of dry pastures.

The roller is a threatened secondary hole-nesting bird (Cramp and Simmons 1988) that uses nest-boxes where natural cavities are scarce (Avilés and Sánchez 1997). It is a sexually monomorphic and monochromatic species (Avilés 2006). The roller is a socially monogamous species, both sexes incubate the eggs, brood and feed the young but the female takes a larger share. Incubation takes 20 d and rearing takes 24 d (Cramp and Simmons 1988).

Nest boxes were monitored every 10 days from early May to fledging to determine laying dates, clutch sizes, and fledging success. Hatching order in broods was not directly established because a higher frequency of visits to nests would have produced disturbance to such a threatened species. Thus, we considered that mass hierarchy reflected hatching order. This assumption is supported by one-day visits during the entire hatching period to five nests in the same area (J. M. Avilés unpubl. data). In these nests, chicks hatched during the first three days of the hatching period grew at a similar rate while chicks hatched after that time grew slower. Moreover, in these nests mass hierarchy always reflected hatching order. When the older chick in each brood was 19 days old, chicks were weighted to the nearest gram with a Pesola spring balance and their wing lengths measured with a rule to the nearest 1 mm. Additionally 225 µl of blood were extracted by brachial venipuncture. Blood collected was stored refrigerated until centrifugation and plasma removal. Then, plasma was frozen for storage until assessing innate immune response. Blood cells were stored in ethanol for later sexing. Also, a drop of blood was smeared on a slide, air-dried and fixed in ethanol until measuring acquired immune response.

Nestling size was measured by wing length and body mass. We used these two measurements because nestling body mass may be more related to condition than wing length. We also calculated the wing length and mass difference between each chick and its larger or heavier sibling at 19 days of age to measure the rank of a chick in the within-brood size/mass hierarchy. These two ranks were highly correlated in each chick ($F_{1,89} = 74.75$, $P < 0.001$, regression coefficient = 0.53), after accounting for the random effect of the nest ($Z = 0.20$, $P = 0.42$) and consequently we only used the mass difference in our analyses to avoid collinearity.

Sex identification

Individuals were sexed by using a polymerase chain reaction (PCR) amplification based on the technique used by Fridolfsson and Ellegren (1999). DNA was isolated from the red blood cells by boiling them in 100 µl of 50 mM NaOH for 20 min in a thermocycler. PCR amplification was performed in 20 µl volumes on an Applied Biosystems GeneAmp PCR System 9700. Final concentrations were: 5 mM MgCl2, 0.2 mM dNTPs (each; Bioron GmbH), 0.25 µM (each) 2550F/2718R primers (Fridolfsson and Ellegren 1999), 0.098 µg/µl BSA (Amersham Biosciences), 0.5 U Taq DNA polymerase (Bioron GmbH) and 1 µl raw extract. Thermal profile used was: 94°C for 2 min, 55°C for 30 s, 72°C for 1 min, followed by 36 cycles (92°C for 30 s, 52°C for 30 s, 72°C for 30 s), and a final 72°C for 5 min step. PCR products were separated in 3% agarose gels run in standard TE buffer and visualized by SyBRSafe (Invitrogen) staining.

Measuring immunity

Assessment of innate humoral immunity was made by using the new assay described in detail by Matson

et al. (2005). Briefly, this assay is based on NAb-mediated complement activation and red blood cell agglutination. The agglutination reaction measures the interaction between NAb and antigens and the lytic reaction measures the amount of hemoglobulin released from the lysis of exogenous erythrocytes, which is a function of the amount of lytic complement proteins present in the sampled blood. The assay is specially designed for small or threatened wild birds because it requires only a small (100 μl) blood sample and because information can be collected in a single visit to the nest. Quantification is done by serial dilution of plasma samples and assessment of the dilution step at which either the agglutination or lysis reaction against the same amount of rabbit blood cell suspension stopped. Plates are vortexed for 10 s at a low speed, and set to incubate at 37°C for 90 min. After incubation plates are tilted at a 45° angle along their long axis for 20 min at room temperature, and then scanned (Microtek Scanmaker 5900, Carson, USA) using the positive transparency (top-lit) option and a full-size image (300 dpi). Subsequently, plates are kept at room temperature for an additional 70 min and scanned for a second time to record maximum lytic activity. We then quantified agglutination (measures NAb) and complement-mediated lysis by assessing the dilution stage (on a scale from 1 to 12) at which these two reactions stopped (further details in Matson et al. 2005).

The measure of acquired immunity was made by examination of blood smears that were stained with azure-eosin. Total number and proportion of different types of leucocytes were estimated in slides. This was made on the basis of an examination of a total of 100 leucocytes under oil immersion. Estimates of the WBC were obtained by counting the number of leucocytes per approximately 10,000 erythrocytes. Differential leucocyte counts were obtained by multiplying their proportions with WBC. In the analyses, only data for lymphocytes and heterophils as the most numerous immune cells are used.

Statistical analyses

Chick was used as the statistical unit in analyses. However, because nestlings in a given nest share parents and environmental conditions, the nest was introduced as a random factor in all statistical models. Immunological variables were NAb level, complement-mediated lysis, WBC and H/L ratio. NAb data were $\log_2$-transformed to achieve normality. We performed linear mixed models (LMM) with the MIXED procedure of SAS (SAS 1999) to analyse the relationship among immunological variables accounting for the random effect of the nest. LMM were also used to analyse if the immunological variables, as dependent variables, were affected by the nestling sex, as a fixed factor, by nestling wing length, nestling mass, position of the chick in the within-brood mass hierarchy and brood size of the nest, as covariables, and by the nest as a random effect. Laying date might affect nestlings' immunology in this species because its reproductive parameters has been shown to decline seasonally (Avilés et al. 1999). However, we did not introduce this variable in analyses because laying date and brood size correlated in the study system ($F_{1,34} = 11.82$, $P < 0.002$) and thus we considered that introducing brood size in models we accounted for laying date.

Model selection was carried out by backward stepwise procedures, testing the significance of each variable one by one, and removing only the variable that resulted in the largest increase in model fit. The nest as a random effect was an exception to that rule because it was always retained in statistical models. The result is the most adequate model for explaining the variability in the response variable.

In this study, 37 nests with 114 nestlings were considered. Variations in sample size among statistical analyses were due to the fact that some morphological or physiological data were unavailable for some broods or nestlings.

Results

Relationships between components of the immune system

The two innate components measured here, i.e. NAb level and complement-mediated lysis, were negatively related ($F_{1,68} = 219.73$, $P < 0.001$, regression coefficient $= -0.399$) after controlling for the random effect of the nest ($Z = 1.30$, $P = 0.10$). On the other hand, WBC and H/L ratio were positively correlated ($F_{1,70} = 9.74$, $P < 0.003$, regression coefficient $= 45.90$), after controlling for the significant random effect of the nest ($Z = 2.28$, $P = 0.01$). Regarding the relationship among innate and mixed components of immunity, WBC was correlated neither with the NAb levels ($F_{1,62} = 0.00$, $P = 0.96$; nest effect: $Z = 2.09$, $P = 0.02$), nor with the complement-mediated activity ($F_{1,62} = 0.10$, $P = 0.76$; nest effect: $Z = 2.11$, $P = 0.02$). The H/L ratio was correlated neither with the NAb levels ($F_{1,62} = 1.65$, $P = 0.20$; nest effect: $Z = 2.03$, $P = 0.02$), nor with the complement-mediated activity ($F_{1,62} = 0.54$, $P = 0.46$; nest effect: $Z = 1.96$, $P = 0.02$).

Innate immunity: natural antibodies and complement activity

NAb levels were positively related to mass difference between the target chick and its largest sibling (Table 1), i.e. lighter chicks of each brood had higher level of NAb compared to heavier ones (regression coefficient = 0.015, Fig. 1a). NAb levels, however, were not affected either by the nest of origin, the number of siblings sharing the nest (brood size), the sex of the chick, and the wing length and mass of the chick during blood sampling (Table 1).

The level of complement activity was negatively related to mass differences (regression coefficient = −0.03, Fig. 1b) between the target chick and its heaviest sibling in a brood (Table 1). This result involves that the lighter chicks of each brood had less complement activity. This result was independent of the nest in which the chicks were being raised, brood size, sex, wing length and mass of the chick (Table 1).

Mixed immunity: leucocyte counts

When we controlled for the brood size of the nest, the H/L ratio was negatively related to mass difference between the target chick and its heaviest sibling (Table 1), i.e. heavier nestlings inside broods had higher H/L levels (Fig. 2). Moreover, H/L was dependent of the nest in which the chick was being raised (Table 1). Neither sex, nor nestling wing length or mass influenced H/L (Table 1).

No relationships were found between WBC and chick wing length, chick mass, chick sex, brood size and mass difference (Table 1). WBC was only dependent of the nest in which the chick was being raised (Table 1).

Discussion

Our results show the importance of the position of chicks in the mass hierarchy of a brood to determine their innate and acquired immunity. The relationship found between the NAb level and difference between body mass of the chick and its heaviest sibling, which is likely to be the oldest, indicates that these antibodies in roller nestlings increase with differences to the heaviest sibling, i.e. their levels are higher in late-hatched siblings compared to early-hatched ones. This means that NAb levels depend on within-brood mass hierarchy and that the lightest offspring in each brood has a more developed innate immune system than their heavier

Table 1. Determinants of innate and acquired immune arms in nestling rollers. Linear mixed models for each immune variable were used with: 1) nestling size measured by wing length and nestling mass, mass difference between the chick and its heaviest sibling and brood size as covariables, 2) nestling sex as a fixed factor, and 3) the nest as a random factor. Non-significant terms were backward eliminated when their removal improved the model fit, otherwise they remained in the model. Retained terms are in bold type.

Response term	Sources of variation	Statistic	P-value
NAb level	Sex	$F_{1,66} = 0.57$	$P = 0.45$
	Wing length	$F_{1,67} = 0.98$	$P = 0.32$
	Body mass	$F_{1,67} = 2.61$	$P = 0.11$
	Mass difference	$\mathbf{F_{1,68} = 4.70}$	$\mathbf{P = 0.034}$
	Brood size	$F_{1,67} = 2.11$	$P = 0.15$
	Nest	$Z = 1.40$	$P = 0.09$
Level of complement activity	Sex	$F_{1,66} = 0.08$	$P = 0.78$
	Wing length	$F_{1,67} = 1.76$	$P = 0.19$
	Body mass	$F_{1,67} = 1.42$	$P = 0.24$
	Mass difference	$F_{1,68} = 4.00$	$P = 0.049$
	Brood size	$F_{1,66} = 0.0$	$P = 0.96$
	Nest	$Z = 0.34$	$P = 0.37$
H/L index	Sex	$F_{1,69} = 1.67$	$P = 0.20$
	Wing length	$F_{1,68} = 0.0$	$P = 0.98$
	Body mass	$F_{1,69} = 0.37$	$P = 0.55$
	Mass difference	$\mathbf{F_{1,70} = 4.89}$	$\mathbf{P = 0.03}$
	Brood size	$\mathbf{F_{1,70} = 1.35}$	$\mathbf{P = 0.25}$
	Nest	$\mathbf{Z = 1.90}$	$\mathbf{P = 0.03}$
WBC	Sex	$F_{1,68} = 0.22$	$P = 0.64$
	Wing length	$F_{1,70} = 2.30$	$P = 0.13$
	Body mass	$F_{1,69} = 0.54$	$P = 0.47$
	Mass difference	$F_{1,68} = 0.15$	$P = 0.70$
	Brood size	$F_{1,70} = 1.00$	$P = 0.32$
	Nest	$\mathbf{Z = 2.24}$	$\mathbf{P = 0.01}$

siblings because high levels of NAb seem to parallel general disease resistance (Parmentier et al. 2004).

Different interpretations could explain the relationship between Nab levels and within-brood mass hierarchy. First, higher levels of NAb in late compared to early-hatched chicks could reflect a higher level of infection in the former individuals. However, contrary to all the other immunoglobulin molecules (Pereira et al. 1986, Boes 2000), the level of NAb does not depend on previous exposure to specific antigens. Moreover, if junior chicks were more infected than senior chicks, their level of WBC and/or the H/L index should be more elevated in junior than in senior chicks because these indexes express stress in response to inflammatory processes and infectious diseases. However, we found exactly the opposite pattern and late-hatched chicks showed lower H/L ratio than their older siblings. A second explanation for our results is that females might allocate more immunoglobulins (Ig) in the last eggs in order to benefit late-hatched young more than early-hatched ones. Nestling mortality not attributable to nest or adult predation and thereby due to brood reduction is very low (9.09% of the $n = 143$ eggs hatched did not produce a fledging in our study population). Thus, differential allocation of inmunoglobulins within broods could be the mechanism by which asynchronous rollers may avoid detrimental effects of brood reduction. Saino et al. (2001) found the same immunocompetence pattern within broods of barn swallows *Hirundo rustica*. Barn swallows have also hatching asynchrony and brood reduction seems not to be a common reproductive strategy in their population (Saino et al. 2001). In all the other species in which within-brood variations in immunocompetence were measured, immunity was either similar among siblings or higher in senior than in junior siblings (house martin *Delichon urbica*: Christe et al. 1998, barn owl *Tyto alba* and blue tit *Parus caeruleus*: Roulin et al. 2003, black-headed gull *Larus ridibundus*: Müller et al. 2003), irrespectively of the species specific level of hatching asynchrony or brood reductionism. Nevertheless, maternal Ig seem to contribute little to the NAb level as indicated by the low level of agglutination by plasma from young chicks (Matson et al. 2005). However, perhaps a little contribution is enough to mediate within-brood variation in NAb or this variation is mediated by other maternally controlled substances. Indeed, and as a third possibility to our results, mothers could deposit decreased yolk androgen (immunosuppresors) levels over the laying sequence to improve the advantages of bigger chicks, as a way of hormonal favouritism (Schwabl et al. 1997), thus prejudicing their immune system. This androgens deposition pattern has been found in two non-reductionist species but with hatching asynchrony: the american coot *Fulica americana* (Reed and Vleck 2001) and the zebra finch *Taeniopygia guttata* (Gil et al. 1999), but also in a siblicidal species such as the cattle egret *Bubulcus ibis* (Schwabl et al. 1997). This mechanism of hormonal favouritism would favour increased growth and worse immunity of senior compared to junior chicks as the consequence of the existing trade-off between growth and immunity (Soler et al. 2003). This explanation is supported by the high H/L values found in early compared to late-hatched nestlings. This could indicate a high growth rate in old nestlings that would produce nutritional stress and the high levels of the ratio H/L. Also, unpublished data in which nests were visited daily suggests that older siblings grow faster than younger ones (J. M. Avilés unpubl. data). Nevertheless, only an experiment would allow distinguish among the different possible mechanisms at work.

However, we found that the level of complement activity in nestlings of roller broods had the opposite pattern than the NAb level, i.e. the complement level decreased with the increase in body mass difference between each chick and its senior sibling irrespective of the nest. This result is probably due to the fact that the lysis titres reflect the interaction of NAb and complement, not only the complement (Matson et al. 2005).

The advantage shown by late-hatched nestlings within each brood seems to be extended to the H/L index because in roller nestlings this index increased when mass differences decreased. This pattern shown for H/L ratio is not the consequence of older chicks showing a more developed acquired immune system because our analyses revealed that the values of H/L ratio were independent of wing length, which is a variable that is likely to reflect nestling age. Since the H/L index indicates stress due to inflammatory processes, infectious diseases or nutritional restrictions, it is reasonable to conclude that junior nestlings are the less stressed nestlings within broods of rollers. This is in agreement with the results obtained in the kite *Milvus migrans* in which senior males had the maximum stress in broods, probably as a consequence of their greater activity and aggressiveness to impose their control in the brood hierarchy (Blanco et al. 2006). In the roller, senior chicks seem also to be dominant because they grow faster than junior siblings (J. M. Avilés unpubl. data). Furthermore, there is some evidence of sibling aggression from senior to junior siblings within a brood (D. Parejo pers. obs.).

Interestingly, we found a nest effect when analysing factors influencing WBC and H/L ratio but not innate immunity (Table 1). The explanation to this could be found in the fact that the acquired immunity measures the response of the individual to foreign agents while the innate immunity measures the level of existing defence even in the absence of infection. That is, the acquired immune system may be highly dependent of the environment and hence of parental condition.

Therefore, the dependence of the H/L index and the WBC of the nests may indicate that different broods are under different parasite load and/or level of infections. In contrast, the pattern found for the NAb levels within-broods seems to be consistent through the different nests and then NAb levels seem to be more the consequence of genetic or maternal effects than of environmental conditions.

No effect of the nestling sex was found in any of our analyses. Contrarily to size dimorphic birds, in the roller both sexes are similar in size, thus it is not surprising the lack of a sex effect on the development of the within-brood immune system because the main effect was mediated by the size hierarchy and this is independent of the sex in size non-dimorphic birds.

Conclusions

Our study shows the existence of within-brood variations in roller chick's immunity suggesting that junior siblings within each brood show the best immunocompetence. Since the link between immunocompetence and future survival and then fitness is well established (e.g. Christe et al. 1998, 2001, Moreno et al. 2005), our results could indicate that roller junior chicks are provided with better arms to survive compared to their older siblings. However, we cannot discard that better immunity in junior chicks compared to seniors was a by-product of the immunosuppressive effect of a higher allocation of testosterone by the female to the chicks. Therefore, the link between chick immunity and maternal allocation of Ig and testosterone in eggs within-broods remains to be elucidated.

Furthermore, this is the first time, to our knowledge, that a suite of immune system measures has been applied to study within-brood variations of immunocompetence in a bird species. Despite the importance of these variations in animal life history strategies and fitness, only a few studies have been focused on within-brood variations in one measure of immunity (see however Christe et al. 1998, Saino et al. 2001, Müller et al. 2003, Roulin et al. 2003). Moreover, the study of several immune components, although of pivotal importance, has not been faced many times (but see Ots et al. 1998, Matson et al. 2006, Mendes et al. 2006). Finally, we have successfully used a very new and low invasive technique to assess constitutive innate immune function in a wild population of a threatened bird. Our success in doing that in the roller leads us to recommend this technique with other threatened species for conservational purposes.

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