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No association between measures of immunity in nestling pied flycatchers (*Ficedula hypoleuca*)

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Costs of immunity are widely believed to play an important role in life history evolution, but many studies of ecological immunology have considered only single aspects of immunity. Young of altricial birds, while dependent primarily on innate immunity and maternally derived antibodies for immune defence, have to develop all components of the immune system which could generate resource-based trade-offs among different arms and between growth and immunity. We conducted a study on nestling pied flycatchers *Ficedula hypoleuca* in which we measured levels of natural antibodies (NAbs) and hemolysis, estimated serum immunoglobulin levels and obtained a specific measure of inflammation after inoculation of an antigen, phytohaemagglutinin (PHA). Thus we obtained independent estimates to explore the relationships among different arms of the immune system. We found that no immune variable measured was associated with any other variable at the individual and brood levels. This indicates that different aspects of immunity are independent and difficult to integrate in a general measure of immune response capacity in altricial nestling birds. We found that only NAbs was negatively associated with tarsus length at the age of 7 days, but not later. Thus, the evidence for resource-based trade-offs between nestling growth and immunity is weak in our study population.

Introduction

The immune system of an organism comprises its main defence mechanism against pathogens (Zuk & Stoehr 2002, Davison *et al*. 2008). Besides the obvious benefits in terms of animal health and survival (Hõrak *et al*. 1999, Christe *et al*. 2001, Ardia *et al*. 2003, Hanssen *et al*. 2004), immune responses also convey costs, including a higher risk of autoimmune diseases (Råberg *et al*. 1998) and the depletion of energy or other resources that could be used for other organismic functions (Sheldon & Verhulst 1996, Martin II *et al*. 2003). Individual immune responses have also been shown to vary with environmental conditions during development or reproduction (Morales *et al*. 2004).

The immune system is highly complex and is generally classified into two main components: innate and acquired immunity (Janeway

& Travers 1996). Innate immunity is particularly important during the initial stages of development and is the primary means of controlling bacterial infections (Roitt *et al.* 1998), which include factors present in the blood before antigenic exposure as well as natural antibodies produced by B cells of the adaptive branch (Forsman *et al.* 2010). Acquired immunity is usually classified into humoral and cell-mediated responses (Roitt *et al.* 1998). The humoral response, which includes B cells, acts against extracellular pathogens while the cell-mediated response mainly attacks intracellular pathogens (Roitt *et al.* 1998).

Immunocompetence is sometimes defined as "the magnitude and effectiveness of an animal's immune response" (Adamo 2004), assuming that a greater magnitude of an immune response is better for anti-parasite defence, although a maximum immune response is not necessarily optimal (Viney *et al.* 2005). Vinkler and Albrecht (2011) propose to use a more rigorous terminology, defining it as the ability to produce anti-parasite or anti-antigen immune responses. There is thus some controversy concerning the term "immunocompetence", although it is meant to summarize the effectiveness of one or more components of the immune system against potential parasite infections. However, studies in natural populations frequently evaluate it with a single immunoassay, while the reliability of one component of immunity to measure the overall strength and efficacy of the immune system has been questioned (Norris & Evans 2000, Adamo 2004, Matson *et al.* 2006). In the literature, the correlations between the diverse indices of immunity measured have shown different results at the individual, family or species level (Matson *et al.* 2006, Mendes *et al.* 2006, Forsman *et al.* 2008). Thus some authors have found negative correlations among components of immunity (González *et al.* 1999, Møller & Petrie 2002, Buchanan *et al.* 2003), which have been interpreted as based on energetic trade-offs within the immune system. However, other studies have found positive associations between components of immunity, which have been interpreted as indicating a general ability to fight disease and pathogens (Møller *et al.* 2001, Morales *et al.* 2004, Ardia 2007, Arriero 2009). Furthermore, studies reporting no significant association between components of immunity (Matson *et al.* 2006, Mendes *et al.* 2006, Roulin *et al.* 2007) claim that different immunological responses are triggered by different types of challenges, and may therefore be regulated independently or be under divergent selection and show no association. Only a few of these studies concern altricial nestlings (Forsman *et al.* 2008, Arriero 2009, Palacios *et al.* 2009), for which differences in the rate of development of different arms may preclude any association between measures of immunity and thereby prevent the establishment of a single measure of immunocompetence.

Newly hatched avian nestlings are dependent on innate immunity and maternal immunoglobulins (Apanius 1998, Klasing & Leshchinsky 1999, Pihlaja *et al.* 2006). Adaptive defences, on the other hand, are poorly developed in nestlings and take more time to become fully functional. Differential rates of development of these main arms of the immune system are likely explained by the different costs and processes involved in the ontogeny of each component (Klasing & Leshchinsky 1999, Palacios *et al.* 2009). A trade-off between different components of the immune system during nestling growth would be predicted if the development and maintenance of different aspects of immunity implies different costs and compete for resources with other physiological activities (Deerenberg *et al.* 1997, Norris & Evans 2000). Innate immune function is especially important to altricial nestlings as their relatively short incubation periods may result in poorly developed immune systems at hatching, and as their stay in the nest may result in greater exposure to parasites (Ricklefs 1992, Ardia & Schat 2008). Altricial nestlings also experience strong selective pressures to grow rapidly to fledge (O'Connor 1984). This suggests that the rate of maturation of immune defenses reflects an evolutionary trade-off with growth rate and tissue maturity required to fledge (Soler *et al.* 2003, Tschirren & Richner 2006).

In this study, we explored associations between different arms of the immune system at the nestling stage in altricial birds as exemplified by the pied flycatcher (*Ficedula hypoleuca*), a model organism for eco-immunological studies (Ilmonen *et al.* 2003, Kilpimaa *et al.* 2004, Morales *et al*. 2004, Grindstaff *et al*. 2006, Morales *et al*. 2006, Moreno *et al*. 2008). We also aimed at detecting associations between nestling growth and immune activity, although in a non-experimental setting. To these ends, we measured the activity of natural antibodies (NAbs) and of the complement cascade as components of the innate immune system. NAbs serve as recognition molecules capable of opsonizing invading microorganisms and initiating the complement enzyme cascade, which ends in cell lysis (Ochsenbein & Zinkernagel 2000). The acquired immune system was measured through the injection of the mitogen phytohemagglutinin (PHA), and by quantifying total immunoglobulin (Ig) levels. These maternally derived antibodies may bind to antigenic targets, and thereby preventing the stimulation of the neonatal immune mechanisms (Apanius 1998, Starck & Ricklefs 1998, Klasing & Leshchinsky 1999). PHA has been used in several studies in wild birds, and has been used to measure the T-cell mediated inflammation, thereby providing a good measure of immune response (Moreno *et al*. 1998, Martin *et al*. 2001, Moreno *et al*. 2001, Tella *et al*. 2002). Martin *et al*. (2006) confirmed that the PHA swelling response involves both innate and adaptive components of the immune system. Moreover, although some mild systemic stress could be produced, it has been suggested that PHA does not provoke potential confounding effects associated with physiological stress (Merino *et al*. 1999).

Methods

The study was conducted during the 2009 breeding season in a deciduous forest of Pyrenean oak (*Quercus pyrenaica)* at an elevation of 1200 m a.s.l. in Valsaín, Segovia Province (40°54´N, 4°01´W), Spain. A study of a population of pied flycatchers breeding in nest-boxes in that area has been conducted since 1991 (Sanz *et al*. 2003). Nest-boxes are cleaned every year after the breeding season. They were checked daily for nest-building activity by pied flycatchers, and the dates of clutch initiation, clutch sizes, and numbers of fledged young were recorded.

The pied flycatcher is a small passerine bird, which breeds in many forested areas of the Palaearctic region (Lundberg & Alatalo 1992). It breeds naturally in tree cavities, but if nest-boxes are provided, they are preferred over natural cavities. Egg laying in the population under study typically begins in late May, and clutch sizes range from 4 to 7 eggs with a mode of 6 eggs (mean ± SD = 5.5 ± 0.6). The female incubates alone and receives part of her food from her mate (Moreno *et al*. 2011). Young are brooded by the female only up to day 8 (day 1 = hatching day) (Sanz & Moreno 1995). Both sexes feed the young. Young fledge within 14–16 days of hatching (Lundberg & Alatalo 1992). In our study area this occurs in the second half of June.

A sample of 78 nests with four to six chicks was used for this study. We randomly sampled two chicks per nest from 58 nests, one per nest from 14 nests, and finally three per nest from 6 nests, i.e. 148 chicks in total. Only nestlings that produced a faecal sample to be used in another study (González-Braojos *et al*. 2012a) were blood-sampled, which explains the different numbers of nestlings per nest. Nestlings were measured and weighed at the ages of 7 and 13 days. Tarsus length was measured with a digital calliper to the nearest 0.01 mm, mass was obtained with a Pesola® spring balance (precision of 0.25 g), and wing length was measured with a stopped ruler to the nearest mm. Chicks were banded on day 7 with numbered aluminium rings. Blood was collected from nestlings of pied flycatchers on day 13 by puncturing the brachial vein and collecting two heparinised capillaries, blood being subsequently transferred into Eppendorf tubes and stored in a cooling box. In the lab, we centrifuged the Eppendorf tubes at 12 000 rpm for 2 min on the day of collection. Plasma and cells were separated and stored at –20 °C until analyses in the lab.

Hemagglutination-hemolysis assay

To estimate the levels of circulating NAbs and complement, we used the procedure developed by Matson *et al*. (2005). The agglutination part of the assay estimates the interaction between NAbs and antigens in rabbit blood, producing blood clumping. The lysis part of the assay estimates the action of complement from the amount of hemoglobin released from the lysis of rabbit erythrocytes. Quantification of agglutination and lysis is achieved by serial dilution in polysterene 96-well assay plates, using the dilution step at which the agglutination or lysis reaction is stopped, i.e. column 3 is a score of 3 for hemolysis and column 8 is a score of 8 for agglutination (*see* Matson *et al.* 2005 for more details). Although lysis scores ranged from 1 to 3, hemolysis only occurred in 33 out of 108 responses (hemolysis is not a general phenomenon, *see* De Coster *et al.* 2010, Matson *et al.* 2005). Therefore, scores of lysis were treated as a binary variable, i.e. 0 (score = 0, no lysis), or 1 (score > 0, lysis). We used fresh rabbit blood with Alsever's anticoagulant (HemoStat Laboratories, Dixon, USA), 96 round well assay plates and an HP Photosmart Essential 3.0 scanner that was set to professional mode, with document-type colour film (48 bit colour and 300 dpi). Whole rabbit blood was stored at 4 °C. After determination of the level of hematocrit, we diluted the blood to obtain a solution of 1% of erythrocytes.

The protocol for hemolysis and hemagglutination is as follows. The plasma samples were thawed and homogenized using a vortex. Subsequently, 25 μl of plasma was pipetted into columns 1 and 2 followed by the addition of 25 μl of 0.01 M phosphate buffered saline (PBS) into all wells, except for column 1. The contents of the column 2 wells were serially diluted (1:2) through column 11. Well number 12 contained only the dilution of erythrocytes and PBS, thus serving as a negative control. Subsequently, 25 μl of the 1% solution of rabbit blood was added into all wells. The assay plate was then covered and shaken for 10 s followed by incubation for 90 min in a bath at 37 °C. The assay plate was then removed from the bath and left at ambient temperature at an angle of 45° for 20 min. Plates were then scanned. Afterwards, plates were kept at room temperature for an additional 70 min and scanned for the second time to record maximum lytic activity. All tests were made blindly by S.G.-B. As 40 samples were not usable due to insufficient volume of blood plasma, we were able to measure samples from only 108 individuals.

Immunoglobulin assay

To estimate IgY levels in plasma, we used the procedure developed by Martínez *et al.* (2003). In brief: ELISA plates (Maxi-sorp, Nunc, Rochester, NY, USA) were coated with serial dilutions of serum (100 μl) in carbonate–bicarbonate buffer (0.1 M, pH 9.6, overnight at 4 °C) in order to determine the linear range of the sigmoid curve. Later, the plates were blocked with defatted milk diluted in PBS-Tw buffer for 1 h at 37 °C (200 μl). Antichicken conjugates (Sigma A-9046, MO, USA) were added at 1/250 dilution in PBS-Tw followed by incubation for 2 h at 37 °C (100 μl). The dilution of antichicken antibody was selected after a previous study to achieve the maximum slope in the linear range. In addition, antichicken antibodies were diluted without any protein (i.e. BSA, gelatine, defatted milk, etc.) which eliminates unspecific binding. After incubation with a substrate comprising ABTS (2,2´-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)) and concentrated hydrogen peroxide diluted to 1/1000 for 1 h at 37 °C, absorbances were measured at $\lambda = 405$ nm using a plate spectrophotometer. Under these conditions, we achieved the maximum values of absorbance. Once the linear range of the sigmoidal curve was achieved for pied flycatcher nestlings, we chose the data obtained using the serum dilution nearest to the centre of the linear range. We could use 139 samples for this assay.

Phytohaemagglutinin (PHA) injection

PHA is a plant-derived mitogen that stimulates the recruitment of leucocytes involved in both adaptive and innate immune responses at the site of injection, producing a measurable tissue swelling (Martin *et al.* 2006, Forsman *et al.* 2010). This is commonly used in evolutionary ecology to estimate T-cell-mediated immunity, although it also reflects other components of the immune system such as major histocompatibility complex molecules (Moreno *et al.* 1999, Morales *et al.* 2006). We used the protocol without control wing proposed by Smits *et al.* (1999). Twelve-day old nestlings were injected with 0.02 mg of PHA in 0.02 ml of PBS into the left wing web, after measuring the thickness at the point of injection. Three measures of the thickness were taken with a digital spessimeter with constant pressure (Mitutoyo 7/547, Tokyo, Japan) to the nearest 0.01 mm. After 24 h, three new measurements of the thickness of wing webs at the point of injection were taken (repeatabilities were 0.99). The immune response was estimated as the difference between the average initial and average final measurements. All the injections and measurements were made by the same person (S.G.-B.). Only 138 nestlings could be correctly measured (4 missing due to failure to inject the correct amount, another 4 were not injected by mistake, and for 2 we could not obtain the pre-injection measure).

Data analysis

IgY levels were normalized by square-root transformation. PHA response and hemagglutination were normally distributed. Scores of lysis were treated as a binary variable, i.e. 0 (score = 0, no lysis), or 1 (score > 0, lysis) (De Coster *et al.* 2010).

To explore the relationships among immunological variables at the individual level, we performed mixed model ANOVA for each variable with brood ID as random factor and the other variables as covariates. For hemolysis, we used GLIMMIX with a binomial distribution. To examine correlations between immunological variables at brood level, we used means per brood in linear correlations.

To estimate the associations between biometrical variables on days 7 and 13, as well as between changes in the biometrical variables between these days and each immunological variable, we used linear mixed models using Satterthwaite's correction for estimating degrees of freedom (Satterthwaite 1946) with SAS 9.1 (SAS Institute Inc., Cary, NC, USA). Three linear mixed models were run, with one normally distributed immune variable (IgY level, PHA response, hemagglutination) as a dependent variable, and hatching date, brood size, tarsus length, mass and wing length of nestlings at 7 or 13 days of age or the difference in measures between both ages as independent variables. In total, three analyses for each immunity and biometric variables were conducted (for day 7, day 13 and the difference between the measures on days 13 and 7). Nest was included as a random factor.

To test lysis, we used GLIMMIX with a binomial distribution, with nest as a random factor and using Satterthwaite's correction. In this analysis, we included the same variables as in the earlier ones.

Final models were produced by a backward deletion procedure until models with only significant effects were obtained ($\alpha = 0.05$).

Degrees of freedom in the different analyses are not the same given the different numbers of samples available for the various immunity measures (*see* above).

Table 1. Means and standard errors (SE) of immune variables.

	n	Mean	SE
IgY levels (absorbance)	139	0.219	0.003
PHA (mm)	138	0.255	0.007
Hemagglutination (titre)	108	7.399	0.119
Hemolysis (titre)	108	0.550	0.093

Results

The variables of acquired and innate immunity, i.e. PHA response, IgY level and hemagglutination were not correlated at the individual level (Tables 1 and 2) and at the brood level (Table 3).

Table 2. Mixed model ANOVA of different measures of immunity with brood ID as a random factor.

	df	*F*	*p*
IgY level			
PHA response	1,80.8	0.09	0.766
Hemolysis	1,79.4	0.01	0.926
Hemagglutination	1,63.8	2.12	0.150
PHA response			
IgY level	1,80.5	0.19	0.666
Hemolysis	1,80.6	0.01	0.939
Hemagglutination	1,64.7	0.02	0.886
Hemagglutination			
IgY level	1,81	1.50	0.224
PHA response	1,81	0.03	0.868
Hemolysis	1,81	0.12	0.731

Table 3. Linear correlations between brood means of different measures of immunity; as hemolysis is not linear, we used a nonparametric Spearman rank correlation. We only included nests in which we had the three measures of immunity ($n = 58$).

	IgY level	PHA response	Hemagglutination	Hemolysis
PHA response	$r = 0.038$, $p = 0.776$	–		
Hemagglutination	$r = 0.099$, $p = 0.455$	$r = 0.018$, $p = 0.890$	–	
Hemolysis	$r_s = –0.108$, $p = 0.418$	$r_s = 0.072$, $p = 0.590$	$r_s = 0.063$, $p = 0.638$	–

Hemolysis at the individual level was not correlated with any variable (PHA response: $F_{1,93.7} = 0.44$, $p = 0.509$, IgY: $F_{1,72.7} = 2.18$, $p = 0.144$, Hemagglutination: $F_{1,88.3} = 0.02$, $p = 0.885$). In the analyses of nestling measures on day 7, only hemagglutination was negatively correlated with tarsus length (Table 4), while other immune variables were not correlated with any biometrical measure. Brood size showed a negative association with IgY in the full model (Table 4). None of the immune variables were correlated with biometrical variables of nestlings at the age of 13 days or with the changes in the biometrical variables between 7 and 13 days of age (all $p > 0.10$). Rank in the mass hierarchy on days 7 and 13 showed no associations with any immunity measure (all $p > 0.30$).

Table 4. Linear mixed models for IgY level, PHA response and hemagglutination as dependent variables, and generalized mixed model for hemolysis. We included nest as a random factor and hatching date, brood size, wing length, body mass and tarsus length on day 7 as covariables using the Satterthwaite correction for estimating degrees of freedom. Final models were obtained from full models by successive backward deletion of variables until the variance explained did not significantly improve the model ($\alpha = 0.05$). p value indicating significance is set in boldface.

	Estimate	df	F	p
IgY level				
Full model				
Hatching date	0.000	1,75.4	0.23	0.621
Brood size	–0.009	1,80.6	5.97	0.052
Wing length on day 7	0.002	1,124	1.93	0.313
Body mass on day 7	–0.007	1,119	1.40	0.157
Tarsus length on day 7	0.006	1,121	0.02	0.364
PHA response				
Full model				
Hatching date	–0.001	1,66.8	1.00	0.320
Brood size	0.000	1,68.1	0.01	0.939
Wing length on day 7	–0.006	1,119	1.50	0.222
Body mass on day 7	0.015	1,112	2.13	0.146
Tarsus length on day 7	0.013	1,120	0.84	0.362
Hemagglutination				
Full model				
Hatching date	0.007	1,94	0.07	0.790
Brood size	0.153	1,94	1.40	0.238
Wing length on day 7	0.046	1,94	0.34	0.563
Body mass on day 7	–0.100	1,94	0.43	0.512
Tarsus length on day 7	–0.408	1,94	3.14	0.079
Minimal model				
Tarsus length on day 7	–0.367	1,98	5.40	**0.022**
Hemolysis				
Full model				
Hatching date	–0.045	1,66	0.47	0.497
Brood size	–0.282	1,58.9	0.90	0.348
Wing length on day 7	0.143	1,95.2	0.69	0.409
Body mass on day 7	–0.316	1,93.1	0.87	0.353
Tarsus length on day 7	0.068	1,97.9	0.02	0.884

Discussion

We did not find any correlation between different measures of the innate and acquired immune systems at the individual and brood means levels. We only found a negative correlation between tarsus length on day 7 and hemagglutination, while no other association between measures of the immune system employed and biometrical variables of nestlings at ages of 7 and 13 days and their growth between these ages have been detected.

The first result underlines the problems in obtaining a general measure of immunocompetence, and emphasizes the importance of measuring different aspects of the immune system due to their statistical independence and their complexity, including numerous well-defined, but interacting components (Blount *et al*. 2003, Adamo 2004, Matson *et al*. 2005, Matson *et al*. 2006, Salvante 2006). Since different types of infections (viruses, bacteria, etc.) are controlled by different types of immune responses, a single measure of immunity is not sufficient to evaluate every kind of immune capacity (Adamo 2004). Moreover, correlations between various immunological variables and resistance to specific diseases appear to be generally pathogen-dependent (Adamo 2004).

There is conflicting evidence concerning the relationships between different measures of immunity in adult birds. In relation to humoral and cell-mediated immunity, some authors have reported a positive correlation between these two arms (Møller *et al*. 2001, Morales *et al*. 2004), while others have shown the opposite relation (González *et al*. 1999, Johnsen & Zuk 1999, Møller & Petrie 2002, Buchanan *et al*. 2003, Arriero 2009). In fact, this discrepancy was found in females of the same species at three sites and was attributed to differences in condition between these sites (Ardia 2007). Other studies included measures of innate immunity (hemagglutination-hemolysis, plasma bactericidal activity, etc.); some did not find correlations between these different arms of the immune system (Matson *et al*. 2006, Mendes *et al*. 2006). However, Forsman *et al*. (2008) found that in the house wren (*Troglodytes aedon*) and the tree swallow (*Tachycineta bicolor*) nestlings nestlings the humoral immune response was negatively related to the PHA response and positively to plasma bactericidal activity (*Escherichia coli* killing capacity). However, the associations between different measures of immunity were not significant among individual nestlings within broods. Palacios *et al*. (2009) showed that innate immune components would develop earlier than adaptive components in the tree swallow (*Tachycineta bicolor*) nestlings, and indicated a lack of correlation between a measure of innate immunity (hemagglutination/hemolysis) and two measures of adaptive immunity (PHA response and IgY level), which implies a lack of constraints, synergism or trade-offs in these particular measures in these nestlings. This may be due to the differences in the onset and rate of development of different components of the immune system which may preclude any association at the individual level (Palacios *et al*. 2009). However, an absence of trade-offs between different arms of the immune system may be expected under good conditions for nestlings with respect to climate, nutrition or infection.

Innate immunity is particularly expensive to growing young because the inflammatory response induces anorexia and diverts nutrients needed for growth to the acute phase response (Klasing 1994, Klasing & Leshchinsky 1999). In nestling birds, some studies have shown trade-offs between the nutrients required for growth and those needed to mount an immune response (Klasing & Leshchinsky 1999, Szép & Møller 1999, Soler *et al*. 2003, Brommer 2004, Pihlaja *et al*. 2006). Mauck *et al*. (2005) showed that in Leach's storm-petrel chicks, agglutination titres increased with chick age and that there was an inverse relationship between NAbs and growth rate. It is not clear that the simple maintenance of immune function is costly in the absence of infection (Klasing 1998), though maintenance is apparently costlier for the innate immune system than for the adaptive immune system (Råberg *et al*. 2002). The higher cost of the innate immune system may explain our result that the only association between nestling biometry and immunity was found for NAbs. This trade-off was only detected at 7 days of age. Nestlings are experiencing the fastest skeletal growth before 10 days of age (Lundberg & Alatalo 1992), which possibly explains why the association with NAbs was not found at 13 days.

Several studies of nestlings have reported a positive association between body mass and PHA response (Saino *et al*. 1997, Brinkhof *et al*. 1999, Hõrak *et al*. 2000, Westneat *et al*. 2004, Moreno *et al*. 2005, Morales *et al*. 2006, Roulin *et al*. 2007, Forsman *et al*. 2010). However other studies have found no association of body mass with cell-mediated immune response in nestlings (Hõrak *et al*. 1999, Martin *et al*. 2001, Tella *et al*. 2002, Moreno *et al*. 2005, Roulin *et al*. 2007). Brzek and Konarzewski (2007) found that the association between PHA response and body mass was negative when food was scarce, positive when resources were abundant while there was no significant correlation under intermediate conditions. In agreement with this, we found in the same study population significant positive associations between nestling mass and PHA response in years when nestlings attained high fledging masses [1999, mean mass on day 13 (SE): 13.68 g (0.18), and 2000, mean mass (SE): 13.73 g (0.11)[but not in a year with slightly lower fledging masses [2002, mean mass (SE): 13.20 g (0.14)] (Moreno *et al*. 2005). Thus, positive growth–immunity associations may only be found when nestlings attain higher masses before fledging, which suggests that growth–immunity trade-offs may only be detectable under very poor conditions for breeding, with no significant association in intermediate conditions. In 2009, the mean mass (SE) was 13.35 g (0.10) which is similar to that in 2002 and intermediate with respect to nestling growth in 2010 [nestlings that fledged only attained 12.53 g (0.30)]. Our results thus agree with those experimentally obtained by Brzek and Konarzewski (2007). In general, we did not find strong evidence for associations between nestling growth and development of immune responses in the specific conditions experienced in the study year, although our study is not experimental.

To conclude, we could not confirm the existence of a general axis of immunocompetence among individual nestlings or among broods, nor evidence of resource-based trade-offs among different components of the immune system. This absence of associations could be due to differences in the onset and rate of development of the different immunity components. We only detected associations between early skeletal growth and innate immunity, which may reflect the higher costs of developing this arm of the immune system in altricial nestlings.

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