

Post-fledging recruitment in relation to nestling plasma testosterone and immunocompetence in the spotless starling

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Summary

1. Social and ecological conditions experienced by individuals during early life can strongly influence their development and survival. Nestlings of many species present important variations in plasma androgens that can be associated with begging and sibling competition and may translate into fitness effects, since broods with higher testosterone (T) production may have better body condition and higher fledging success. However, the positive effects of androgens may be counterbalanced by a reduction of immune defences and a greater susceptibility to diseases.

2. In this study we examined the potential relationships between natural variation in plasma T, immunity and post-fledging survival rate in nestlings of the spotless starling (*Sturnus unicolor*).

3. We found that nestlings with higher cellular-mediated immune responses (CMI; measured as a swelling response to phytohaemagglutinin injection) were more likely to be recruited in the population than nestlings with lower CMI responses. Males presented higher CMI response than females, possibly due to differences in competitive advantage over food. We also found that CMI response was negatively related to T levels, as predicted by the immunocompetence handicap hypothesis. However, despite this reduction in CMI response, we failed to find an association between nestling T levels and survival prospects. Our results add to the evidence of the role played by immune defences in determining survival prospects in natural populations.

4. In conclusion, our study reveals that CMI response can be considered as a good predictor of post-fledging recruitment. As far as we know, this is the first study attempting to evaluate the relationship between nestling T and post-fledging survival. Our results suggest that the potential benefits accrued by high levels of T in sibling competition during the nestling stage do not translate into increased survival.

Key-words: cellular-mediated immune response, immunocompetence handicap hypothesis, plasma testosterone, survival

Introduction

Survival is a key life-history trait and thus it is important to understand what factors might affect it and how. In birds, the variables most frequently associated with post-fledging survival are hatching date and body mass. Previ-

ous studies have found that individuals who fledge early in the season present a higher probability of survival (Perrins 1965; Cooke, Findlay & Rockwell 1984; Harris, Halley & Wanless 1992; but see Monrós, Belda & Barba 2002; Greño, Belda & Barba 2008). Similarly, individuals with a higher body mass at fledging may have higher survival rates than lighter ones (e.g. Perrins 1965; Tinbergen & Boerlijst 1990; Magrath 1991; Both, Visser & Verboven 1999; Naef-Daenzer, Widmer & Nuber 2001; Monrós, Belda & Barba 2002; but see Stienen & Brenninkmeijer 2002; Greño, Belda & Barba 2008). However, body mass and

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fledging date could in fact be mere correlates of some specific condition or health factor driving survival probability after fledging. For example, it is known that pathogens and parasites affect survival of their avian hosts (Janeway *et al.* 1999) and that nestling immune function parameters are associated with survival in the nest (Christe, Møller & de Lope 1998; Horak *et al.* 1999; Merino, Møller & de Lope 2000) post-fledging recruitment (Christe, Møller & de Lope 1998; Christe *et al.* 2001; Lobato *et al.* 2005; Moreno *et al.* 2005) and adult survival (Saino, Calza & Møller 1997; Soler *et al.* 1999; Møller & Saino 2004). As the maturation of immune function may take up to several weeks after hatching (Klasing & Leshchinsky 1999), rearing conditions experienced by nestlings may substantially affect the development of immunity. Thus, social and ecological conditions that individuals experience during early life can influence their development and survival (Lindström 1999). Particularly, nutritional stress during early development has been shown to modify growth, immunity and behaviour (reviewed in Monaghan 2008) thereby affecting fitness via an array of traits. Hence siblings' competitive abilities to acquire food within a brood may be an important determinant of survival.

Since rivalry for parental care between siblings is the first situation of social competition an organism encounters after birth (Mock & Parker 1997), physiological adaptations allowing nestlings to maximize their competitive potential should be strongly selected. Nestlings of many species present important variations in plasma androgens that can be associated with begging and sibling competition (Goodship & Buchanan 2006; Naguib *et al.* 2004; Quillfeldt *et al.* 2006; but see Quillfeldt *et al.* 2007) and may translate into real fitness effects, as broods with higher testosterone (T) production have better body condition (Quillfeldt *et al.* 2006) and higher fledging success (Goodship & Buchanan 2006). However, the positive effects of androgens may be counterbalanced by a reduction in immune defences and a greater susceptibility to diseases. To date, several studies have found that the maintenance of elevated circulating levels of androgens may come at the cost of reduced immunocompetence (reviewed in Owen-Ashley, Hasselquist & Wingfield 2004), increased basal metabolic rate (Buchanan *et al.* 2001), increased levels of stress hormones (Ketterson & Nolan 1992), decreased resistance to oxidative stress (Alonso-Alvarez *et al.* 2007) and ultimately reduced survival (Redpath *et al.* 2006). Since high quality individuals are expected to suffer less from costs associated with T production (Folstad & Karter 1992), individual variation in plasma T levels may reflect differences in the underlying individual quality (i.e. intrinsic factors such as genetic or maternal effects) or environmental variation (i.e. extrinsic factors such as the social and ecological environment). Thus, plastic secretion of androgens may function as a powerful tool that animals can use to adapt themselves to varying environmental conditions (Dufty, Clobert & Møller 2002). For instance, hormonal induction of aggressive behaviour by T occurs when the risk of social competition is high and the opposite when the risk

of social competition is low (Wingfield *et al.* 1990; Tarlow, Wikelski & Anderson 2001; Ferree, Wikelski & Anderson 2004). Accordingly, experimental studies have found that nestling T levels varied in response to both induced nutritional stress (Goodship & Buchanan 2006) and brood size manipulations (Naguib *et al.* 2004; Gil *et al.* 2008; but see Núñez-de la Mora, Drummond & Wingfield 1996; Tarlow, Wikelski & Anderson 2001). Furthermore, an increase in brood size negatively affects cell mediated immune response (Saino, Calza & Møller 1997; Horak *et al.* 1999; Naguib *et al.* 2004; Gil *et al.* 2008), probably because of energetic costs (Saino, Calza & Møller 1997; Horak *et al.* 1999; Lochmiller & Deerenberg 2000), or alternatively due to the immunosuppressive effects of endogenous T in nestlings (Fargallo *et al.* 2007).

Environmental conditions during early development may differentially affect male and female offspring (McClure 1981; Clutton-Brock 1991). For example, in sexually size-dimorphic species the larger sex may be more vulnerable to poor rearing conditions given its greater resource requirements (Weatherhead & Teather 1991; Torres & Drummond 1997; Hipkiss *et al.* 2002; Fargallo *et al.* 2006), or alternatively, it may dominate limited food resources due to its competitive superiority in sibling rivalry (Bortolotti 1986; Anderson *et al.* 1993; Oddie 2000; Fargallo *et al.* 2003, 2006). This sex-specific sensitivity to resource availability during growth may have far-reaching consequences upon fitness (Metcalf & Monaghan 2001). Since immunity may compete with growth, any resource allocation trade-off between growth and immune function should also be dependent on nestling sex (Fargallo *et al.* 2002, 2007; Müller, Dijkstra & Groothuis 2003; Chin *et al.* 2005; Dubiec, Cichon & Deptuch 2006; Lobato *et al.* 2008). In addition, inherent characteristics in sex phenotype, such as the high levels of testosterone in males, oestrogen in females (Owens & Short 1995; Olsen & Kovacs 1996) or different sensitivity to similar levels of hormones (Müller *et al.* 2005) can determine differential vulnerability to environmental conditions between sexes.

In this study we examined the potential relationships between natural variation in plasma T, immunity and the post-fledging survival rate in nestlings of the spotless starling. Since rearing environment influences nestling T levels, we predicted inter-nest variation in plasma T levels. Additionally, we expected individual T levels to be higher in larger broods with more sibling competition than in smaller broods with less sibling competition. Since androgens may compromise immune function in nestlings, we examined the variation of cell-mediated immune response (CMI) in relation to differences in circulating T levels. We expected a negative correlation between nestling T and CMI response in both sexes. Finally, we explored the probability of survival and local recruitment in relation to both plasma T and CMI. We expected that both nestling competitive capacity (nestlings with higher T levels) and nestling immune capacity (nestlings with higher CMI response) would be good predictors of recruitment probability.



Fig. 1. Nestlings of spotless starling at 20 days of age inside a nest-box.

Materials and methods

STUDY AREA AND GENERAL PROCEDURES

This study was conducted during two consecutive breeding seasons (2004 and 2005) in Soto del Real, Madrid (central Spain), where a colony of spotless starlings (*Sturnus unicolor*; Fig. 1) have been breeding in nest boxes since 2003. The facultative polygynous spotless starling is a dimorphic species in plumage and size (with males being the larger sex), which is closely related to the European starling (*Sturnus vulgaris*). Females lay around five eggs per clutch and can breed once or twice in a season (first and second clutches), sometimes laying a replacement clutch when another is lost. Incubation is usually performed by females and males vary considerably in the amount of parental care they provide (Moreno *et al.* 1999; Veiga & Polo 2003).

Nests were visited every day to determine laying date, clutch size and the exact hatching date of the first chick in each brood. The nestling period in our population is 20–22 days long, and during this time all nests were monitored periodically and nestlings were ringed, weighed and measured at 6 days of age. We measured beak, tarsus and wing lengths using a digital sliding calliper to the nearest 0.01 mm. To measure the cell-mediated immune response (CMI) we used the phytohemagglutinin (PHA) test which is a commonly used technique for measuring T-cell mediated immunocompetence not only in birds but also in fishes, amphibians, reptiles and mammals. The technique involves a subcutaneous injection of a mitogen (PHA) and measurement of subsequent swelling as a surrogate of T-cell mediated immunocompetence. Thus, on day 14 all nestlings were injected subcutaneously in the centre of the left wing web with a 0.05 mL of a 5 mg mL⁻¹ solution of PHA (L8754; Sigma Aldrich, Madrid, Spain). A measurement of the thickness of the wing web was taken prior to the injection and 24 h later with a micrometer (Mitutoyo Co., Tokyo, Japan) to the nearest 0.01 mm. The difference in the thickness of the wing web before and after PHA injection was used as response estimate for the cell-mediated immune response. The swelling induced by the PHA injection provides a useful measurement of immune activity showed by several types of immune cell (Martin *et al.* 2006; Tella *et al.* 2008), and correlates with measures of fitness in a variety of bird species (Saino, Calza & Møller 1997; Tella *et al.* 2000). At 15 days of age, measurements of body size and body mass were taken and a blood sample (800 µL) was extracted for hormone assays

and molecular sexing. Nestlings were removed from the nest-box one at a time. Blood samples were taken immediately after removing them out of the nest-box and once nestlings were bled measurements of CMI and body size were performed. Time of handling never exceed 5 min per nestling. Similar handling conditions in our study population have shown not to influence nestling T levels (Gil *et al.* 2008).

Local recruitment of ringed chicks as breeders to the population was monitored during the four subsequent years (from 2005 to 2008), as most females are expected to recruit 1 year after hatching and most males 2 years after hatching (Cordero *et al.* 2001). Recruits were captured inside the nest boxes during the first year they attempted to breed.

SEX DETERMINATION AND HORMONE MEASUREMENT

DNA was extracted from blood using the PUREGENE protocol (Gentra Systems, Minneapolis, USA). Around 20 ng of each chick DNA solution was used in a polymerase chain reaction to amplify a part of the CHD-W gene in females and the CHD-Z gene in both sexes (Griffiths *et al.* 1998). The results were validated using adult birds of known sex and if the case, when recruitments were captured as adults.

Blood samples for hormone analysis were taken from the jugular vein with a syringe containing traces of heparin to avoid clotting. Samples were kept cool for the rest of the day (maximum 6 h) and subsequently centrifuged at 7345.25 g for 10 min. The separated plasma was stored in microtubes at -80 °C until analyses were performed. T was extracted twice with a volume of 10X diethyl ether from 300 µL plasma samples and resuspended in an equal volume of assay buffer (Cayman Chemicals, Ann Arbor, MI, USA). The extraction recovery of cold spiked samples was high (average = 97.4%, SD = 4.4). Hormone concentrations were determined in duplicate using a commercially available enzyme immunoassay (Cayman Chemicals) following the manufacturer's protocol. The assay is 100% specific for testosterone, 27.4% for 5α-dihydro-testosterone (5α-DHT), and 3.7% for androstenedione. The intra-assay coefficient of variation was 9.30%, and the inter-assay coefficient of variation after correction by means of linear regressions of these standards, was 7.37%. Linearity was checked by serial dilutions of a pool, which provided a linear slope that did not differ from expected values.

STATISTICS

Body size of chicks at 6 and 15 days old was estimated by combining wing length, tarsus length and beak length in a principal component analysis. PC1 explained 87.2% (eigenvalue 1.74) of the variation in these measurements at day 6 and 69.9% (eigenvalue 2.09) of the variation in day 15.

In order to evaluate the potential relationships between natural variation in plasma T, immunity and post-fledging survival rate in nestlings of the spotless starling we included in the analyses nestlings that reached 15 days old and from which a blood sample and a measurement of CMI response were taken. We examined differences in T levels of nestlings using General Linear Mixed Models (GLMM) in which we included nest as random factor, year, sex and clutch number (first and second clutches) as fixed factors and body size (PC1 scores at day 15) and number of nestlings per brood in day 15 as covariates ($N = 256$ nestlings from 125 broods). Interactions within these variables were included in the initial model.

To analyse variation in CMI of nestlings we used a GLMM in which we included the same explanatory variables as used in the

previous model and plasma T levels was included as a covariate ($N = 247$ nestlings from 115 broods). The following interactions were included in the initial model: year \times sex \times clutch, year \times sex, year \times clutch and clutch \times sex.

Post-fledging recruitment of individual nestlings (recruited vs. non-recruited) was examined using Generalized Linear Mixed Models analyses with binomial error and logit link (GLIMMIX Macro of SAS) where nest was included as random factor and year, clutch number, number of nestlings per brood in day 15, sex, body size (PC1 scores at day 15), body mass, plasma T level, and CMI as exploratory variables.

We used the bias-corrected version of Akaike's Information Criterion (AICc) to identify the best approximating statistical models explaining the response variables from a set of alternative candidates (Burnham & Anderson 2002). This information criterion identifies the models accounting for the most variation with the fewest variables (lower AICc values indicate a better approximation; Boyce *et al.* (2002)). Models were ranked on the basis of the differences between the AICc of a given model and the AICc of the highest ranked model (Δ AICc) and those models that were separated by less than 2 AICc points were considered as equally probable (Burnham & Anderson 2002). To test for the importance of variables when the effect of other variables was accounted for, a forward variable-selection process using the AICc value was used. Models proposed are those in with lower AICc (Table 1). Analyses were performed using SAS statistical software (SAS Institute Inc., Cary, NC, USA). The statistical significance of differences between categories of the same variable was computed using the LSMEANS statement of SAS. Degrees of freedom were calculated following the Satterthwaite method. Residuals from the models were normally distributed.

Results

T LEVELS (TABLE 2)

There was significant variation between nests in T plasma levels (GLMM, nest: $Z = 3.48$, $P < 0.0001$). Overall T

levels were not different between first and second clutches (first clutches mean \pm SD: 67.63 ± 35.92 pg mL⁻¹, second clutches mean \pm SD: 63.37 ± 26.63 pg mL⁻¹). The interaction between clutch number and year was significant (GLMM, clutch number: $F_{1,135} = 7.66$, $P = 0.006$; clutch number \times year: $F_{1,135} = 24.50$, $P < 0.0001$). This is because in year 2004 first clutches presented higher T levels than second clutches, while in 2005 no differences were found. T levels were not different between sexes and neither body mass, body size nor number of siblings within a brood affected T plasma concentrations (GLMM all $P > 0.05$).

IMMUNITY (TABLE 2)

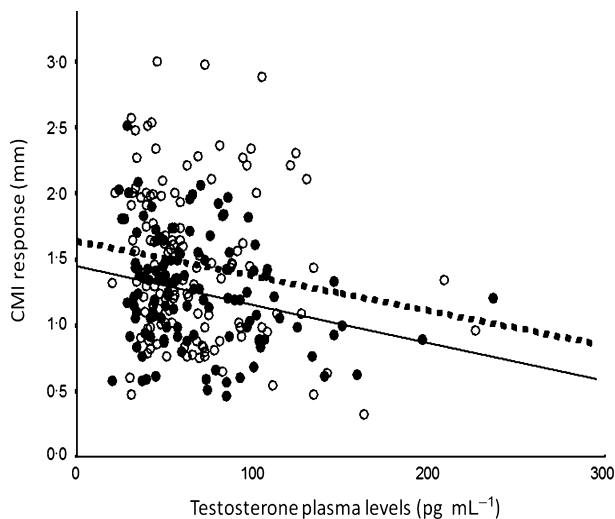
Cell-mediated immune response differed between nests and between years (GLMM, nest: $Z = 4.26$, $P < 0.0001$; year $F_{1,124} = 27.27$, $P < 0.0001$). On average, first clutches presented a higher CMI response than second clutches (first clutches: mean \pm SD, CMI = 1.32 ± 0.54 mm, range = 0.32 – 3 mm, $n = 230$; second clutches: mean \pm SD, CMI = 1.11 ± 0.45 mm, range = 0.32 – 2.56 mm, $n = 66$; GLMM, clutch number: $F_{1,127} = 4.93$, $P = 0.03$). Males presented higher CMI than females (males: mean \pm SD, CMI = 1.33 ± 0.58 mm, range = 0.32 – 3 mm, $n = 134$; females: mean \pm SD, CMI = 1.11 ± 0.46 mm, range = 0.46 – 2.52 mm, $n = 122$; GLMM sex: $F_{1,197} = 14.24$, $P = 0.0002$). In both sexes, CMI negatively correlated with plasma T concentration (GLMM plasma T: $F_{1,242} = 7.70$, $P = 0.006$; Fig. 2). This relationship was not driven by extreme values in T levels, if values higher than 196 pg mL⁻¹ are removed, the same relationship is maintained (data not shown). CMI response was unrelated to body mass, body size and number of siblings within a brood (GLMM, all $P > 0.05$) and therefore these variables were excluded from the final model.

Table 1. Ranking of the most probable models [lower Akaike's Information Criterion (AIC) values] explain nestling T levels, CMI response and local recruitment. The term 'clutch' refers to clutch number (first or second breeding attempt). Initial models with all variables included and their AIC are presented in the first row of each dependent variable

Dependent variable	Terms in the model	AIC	Δ AIC
T initial	Nest, year, sex, clutch, body mass, size, siblings	-65.0	
	year \times sex \times clutch, year \times sex, year \times clutch, clutch \times sex		
	Nest, year, clutch, sex, year \times clutch	-124.3	-90.1
	Nest, year, clutch, size, year \times clutch	-146.5	-30.8
T final	Nest, year, clutch, siblings, year \times clutch	-149.0	-8.6
	Nest, year, clutch, year \times clutch	-155.1	-6.1
CMI initial	Nest, year, sex, clutch, body mass, size, siblings, plasma T	-461.2	
	year \times sex \times clutch, year \times sex, year \times clutch, clutch \times sex		
	Nest, year, sex, clutch, plasma T, clutch \times year	-526.1	-69.8
	Nest, year, sex, clutch, plasma T, sex \times year	-527.2	-4.9
CMI final	Nest, year, sex, clutch, plasma T, clutch \times sex	-527.4	-3.8
	Nest, year, sex, clutch, plasma T	-531.0	-3.6
Recruitment initial	Nest, year, sex, clutch, body mass, size, siblings, plasma T, CMI	1330.8	
	Nest, year, sex, clutch, size, siblings, CMI	1325.6	34.2
	Nest, year, sex, clutch, siblings, CMI	1325.0	29.0
	Nest, year, sex, clutch, siblings, plasma T, CMI	1297.0	28.4
Recruitment final	Nest, year, sex, clutch, size, siblings, plasma T, CMI	1296.6	0.4

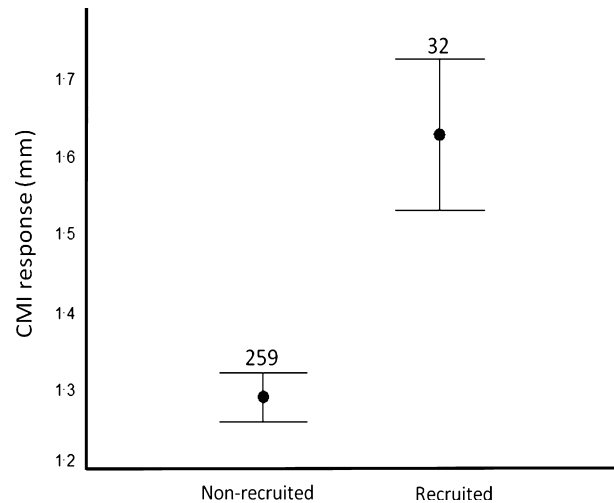
Table 2. Final models [lower Akaike's Information Criterion (AIC) values] explain nestling T levels, CMI response and local recruitment

Dependent variable	Effect	d.f.	F value	P
Testosterone	Clutch number	1, 135	7.66	0.0064
	Year	1, 135	0.29	0.5934
	Clutch × year	1, 135	24.5	0.0001
CMI response	Plasma T	1, 242	7.7	0.0059
	Clutch number	1, 127	4.93	0.0281
	Year	1, 124	27.27	0.0001
	Sex	1, 197	14.24	0.0002
Local recruitment	Nestling size	1, 238	0.13	0.7181
	Number of siblings	1, 238	0.3	0.5852
	Nestling plasma T	1, 238	0.02	0.8868
	Nestling CMI	1, 238	10.6	0.0013
	Sex	1, 238	0.46	0.4964
	Clutch	1, 238	2.18	0.1412
	Year	1, 238	9.05	0.0029

**Fig. 2.** Cell-mediated immune response (CMI) measured as swelling response to PHA injection (mm) in relation to T plasma levels in male (open circles, dotted line) and female (full circles, solid line) nestlings. Values presented correspond to measures of T levels. (GLMM test: $F_{1,242} = 7.70$, $P = 0.006$; GLMM sex: $F_{1,197} = 14.24$, $P = 0.0002$).

POST-FLEDGING RECRUITMENT (TABLE 2)

After controlling for the effect of the year (GLIMMIX year: $F_{1,238} = 9.05$, $P = 0.002$), the CMI response of nestlings was the only variable that significantly predicted local recruitment: recruitment probability increased with nestling CMI response (GLIMMIX CMI response: $F_{1,238} = 10.60$, $P = 0.001$; Fig. 3). Recruits and non-recruits did not differ in their plasma T levels as nestlings (GLIMMIX plasma T: $F_{1,238} = 0.02$, $P = 0.88$), or body size (GLIMMIX size: $F_{1,238} = 0.13$, $P = 0.72$). The probability of recruitment was not affected by the number of siblings within a brood (GLIMMIX siblings: $F_{1,238} = 0.30$, $P = 0.58$) and was not different between males and females (GLIMMIX sex: $F_{1,238} = 0.46$, $P = 0.50$) nor between first and second clutches (GLIMMIX clutch number: $F_{1,238} = 2.18$, $P = 0.14$). Although these

**Fig. 3.** Nestling cell-mediated immune (CMI) response (Mean \pm SE) in relation to the probability of local recruitment. (GLIMMIX, $F_{1,238} = 10.60$, $P = 0.001$).

variables were not significant in relationship to local recruitment, they were not excluded from the final model because the model including them presented the smaller Akaike information criterion (AIC).

Discussion

Our study aimed to examine whether differences in plasma T and immunity affected the probability of post-fledging recruitment in nestlings of spotless starling. Evidence that immune function may constitute an important determinant of survival in nestling birds is increasing (Gonzalez, Sorci & de Lope 1999; Horak *et al.* 1999; Merino, Møller & de Lope 2000; Christe *et al.* 2001; Møller & Saino 2004; Lobato *et al.* 2005; Moreno *et al.* 2005; Hylton *et al.* 2006; Nadolski *et al.* 2006). Our results add evidence of the role played by immune defences in determining survival prospects in natural populations since we found that nestlings with higher CMI response were more likely to be recruited in the population than nestlings with lower CMI response. We found that males presented higher CMI responses than females, and this might be related to differences in competitive advantage over food. Available studies on nestling birds dealing with inter-sexual differences in nestling immune response have not provided uniform results (probably due to differences in sexual dimorphism among studied species): since some studies have found inter-sexual differences in CMI response (Fargallo *et al.* 2002; Müller, Dijkstra & Groothuis 2003; Tschirren, Fitze & Richner 2003; Dubiec, Cichon & Deptuch 2006), whereas others have not (Tella *et al.* 2000; Saino *et al.* 2002; Jovani *et al.* 2004; Müller *et al.* 2005; Lobato *et al.* 2008). Despite an overall lower female immune response, the probability of post-fledging recruitment was not different between sexes, thus suggesting that the effects of CMI response on survival override sexual differences.

Several studies have found an association between body mass and food intake and CMI response in adult and juvenile birds (reviewed in Alonso-Álvarez & Tella 2001; Fargallo *et al.* 2002, 2003; Laaksonen *et al.* 2004; Martínez-Padilla 2006). After controlling for sex differences, we found no association between body mass and size and CMI response in spotless starlings. First clutches, however, had higher CMI responses than second clutches. This result can be explained by at least two non mutually exclusive possibilities. Firstly, environmental conditions may affect the expression of the immune response in birds (e.g. food supply; Saino, Calza & Møller 1997; Sorci, Soler & Müller 1997). The other possibility is that high-quality birds breed earliest in the season thus conferring their nestlings with better immune defences because of their higher condition (environmental or genetic). Indeed, we found inter-nest variation in CMI response suggesting that there is potential for parents' quality to play a role in nestling immune defence development (Brinkhof *et al.* 1999).

Surprisingly, although nestlings reared in first clutches presented a higher CMI response than nestlings reared in second clutches, first and second clutches presented similar survival probabilities. Recruiting probabilities typically decline with date in many avian species (Perrins 1965; Cooke, Findlay & Rockwell 1984; Harris, Halley & Wanless 1992; but see Monrós, Belda & Barba 2002; Greño, Belda & Barba 2008). Although our results showed that the effects of CMI response on post-fledging recruitment are important *per se* and not due to their correlation with timing of reproduction, it is important to note that nestlings that did not reach the fledging age are not included in the analyses. In our study population first clutches produce more fledgings than second clutches (I. López-Rull & D. Gill, unpublished data) however once nestlings have fledged the survival disadvantage of being born late in the breeding season seems to disappear.

Previous work has shown that variation in brood size can affect sex-specific immunity differently in passerines (Saino, Calza & Møller 1997; Horak *et al.* 1999; Naguib *et al.* 2004; for starlings see Chin *et al.* 2005) being the larger sex the one that exhibits a negative relationship between immune response and brood size (Saino, Calza & Møller 1997; Horak *et al.* 1999; Chin *et al.* 2005). However, in our study we found no relationship between number of siblings and CMI response in males or females suggesting that possible sexual differences in CMI response in relation to number of siblings within a brood are too slight to be detected under natural conditions and that they may be more tightly linked with parents' quality. Also, as sex differences in the CMI response may be associated with different sex-sensitivity to environmental conditions (Fargallo *et al.* 2002; Chin *et al.* 2005; Dubiec, Cichon & Deptuch 2006), it is possible that under certain rearing conditions (e.g. strong parasite pressure, low food availability) variation in CMI in relation to number of siblings might appear in nestlings. In accordance with the absence of a relationship between number of siblings and CMI response, the probability of post-fledging recruitment was not related to brood size.

Since immune response appears highly resource-dependent (Alonso-Álvarez & Tella 2001) and nutritional stress experienced in early life has been shown to affect the development of immunity, we hypothesized that nestling competitive capacity for food acquisition within a brood would be a good predictor of local recruitment. Nestlings of many species present important variations in plasma androgens that can be associated with sibling competition (Goodship & Buchanan 2006; Naguib *et al.* 2004; Quillfeldt *et al.* 2006; but see Quillfeldt *et al.* 2007). Experimental studies have found that nestling T levels varied in response to both induced nutritional stress and brood size manipulations (Goodship & Buchanan 2006; Naguib *et al.* 2004; Gil *et al.* 2008; but see Núñez-de la Mora, Drummond & Wingfield 1996). Our study showed marked differences between nests in T levels. However, contrary to our predictions we found that number of siblings within a brood did not affect T plasma concentrations. Neither did we find a significant relationship with nestling body mass or size. Moreover, despite our expectations of high levels of circulating sex steroids benefitting individuals by optimizing social competitiveness (Wingfield *et al.* 1990), we failed to find an association between nestling T levels and survival prospects. This result may be due to the costs associated to production and maintenance of circulating androgens. We found that CMI response was negatively related to T levels, as predicted by the immunocompetence handicap hypothesis (Folstad & Karter 1992), and shown before in other species at the nestling stage (Naguib *et al.* 2004; Gil *et al.* 2008). Thus our study gives support to the idea that positive effects of androgens may be counterbalanced by a reduction of immune defences. However, given that CMI was positively related to recruiting probability the lack of association between nestling T levels and survival prospects is particularly surprising. Our study thus suggests that negative immune effects brought about by producing and maintaining circulating T levels, are not enough to decrease survival prospects. Given that this is a correlational study and that T levels were not modified, we can speculate that natural differences in T levels in this species are limited in nature by selection. This would imply that whatever benefits are accrued by high levels of circulating sex steroids, either at the nestling or the adult stage (Wingfield *et al.* 1990), these levels are never raised over a certain limit which would harm survival.

We did not have a clear prediction about sex differences in nestling T since previous studies in this species have found contradicting results (Müller *et al.* 2007; Gil *et al.* 2008). Because in our study no sex differences were observed in circulating levels of testosterone and the negative relationship between CMI response and T levels was consistent in males and females our data do not support the idea that sex differential vulnerability observed in CMI response is associated with sexual differences in levels of or sensitivity to circulating testosterone. In agreement with this result sex differences in post-natal cell-mediated immunity in kestrels were not explained by sex differences in circulating testosterone levels (Fargallo *et al.* 2007). Another possibility to explain nonsexual differences in circulating T levels may be that juvenile

starlings are photorefractory until they are almost 12 months and then need to experience short days before they can respond to long days and become sexually mature (McNaughton, Dawson & Goldsmith 1992). Thus, if testicular genesis of T is not occurring in nestling starlings, the endogenous production of T in nestlings and its natural variation may be due a non-gonadal source which may not necessarily vary between sexes. So far the mechanisms underlying testosterone production in nestling passerine birds remain poorly understood, however, our findings provide data on an important ecological pattern irrespective of whether the mechanism has been completely elucidated or not.

We found that variation in plasma T levels was greater between than within nests supporting the idea that rearing environment influences nestling T levels. We did not have a directional prediction about how nestling T could differ between first and second clutches because although there should be a decrease in food availability during the breeding season, clutch size is greatly reduced for later laid clutches in starlings (Feare 1984; Smith, Ottosson & Sandell 1994; Christians, Evanson & Aiken 2001; I. López-Rull, unpublished data), thus reducing the number of potential competitors within a nest. The interaction between clutch number and year was significant, probably signalling that intra-annual environmental conditions vary between years, and have some effect on plasma T levels.

In conclusion, CMI response of nestlings was the only variable that significantly predicted local recruitment: nestlings having higher CMI response were more likely to be recruited than nestlings with lower CMI response. Although competitive abilities for food acquisition may be an important determinant of survival, neither number of siblings within a brood nor nestling T levels were associated with the probability of recruitment. Possibly, given the immune-suppressive effects of androgens, T may only be secreted during short periods of high aggression. Thus, our data do not allow confirmation of the absence of discrete differences in T levels that could latter affect post-fledging survival. Future studies dealing with competitive abilities of food acquisition in relation to survival would require sampling right at the time of sibling competition (e.g. when parents feed offspring). Also, experimental studies are needed to understand the potential relationships between androgens, immune response and survival.

Acknowledgements

We are grateful to E. Bulmer, M. Gil, M. Muñoz, K. Deuptch, J. Kamps and I. Espinosa for their help during fieldwork and to L. Pérez Rodríguez and J.A. Fargallo for their valuable comments on the manuscript. ILR and PC were supported by PhD grants from CONACYT (México) and CPS was supported by an I3P contract from the Comunidad de Madrid. Research was funded by grants from the Spanish Ministerio de Educación y Ciencia (BOS2002-00105 and CGL2005-05611-C02-01). The experimental protocol used in this research was approved by the Environment Department of the Autonomous Community of Madrid. Permission to work in the study area was granted by the Ayuntamiento de Soto del Real and the Consejería de Medio Ambiente of the Comunidad Autónoma de Madrid. This manuscript is a contribution of the field station 'El Ventorrillo'.

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Received 3 May 2010; accepted 24 August 2010

Handling Editor: Jennifer Grindstaff