

Competition for resources modulates cell-mediated immunity and stress hormone level in nestling collared doves (*Streptopelia decaocto*)

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Abstract

Competitive stress imposed by hatching asynchrony may affect developmental trajectories of offsprings by regulating resource allocation between growth and other fitness-related traits. For instance, the down-regulation of immunity is a commonly observed phenomenon under stressful conditions. However, physiological mechanisms that regulate resources allocation to growth and immune functions in response to competition for resources, as well as inter-sexual differences in physiological strategies, are still poorly investigated. To partially fill this gap, we first conducted a descriptive study on chicks of the collared dove (*Streptopelia decaocto*), a species producing two chicks per brood. Our results show that first hatchlings (seniors) were bigger, showed lower baseline corticosterone levels (CORT) and showed a higher cell-mediated immunoresponsiveness (CMI) than late hatchlings (juniors). However, when controlling for body size, only CMI remained weaker in junior chicks suggesting differences in strategies of resource allocation between siblings. Interestingly, CORT in juniors increased with increasing within-brood hierarchy. But, while within-brood variation in CMI followed the opposite pattern of variation in CORT, we found no evidence that inter-individual variation in CMI was directly related to CORT. In addition hatching-rank related differences in body size, CMI and CORT was similar between sexes.

To ensure that the lower phenotypic value expressed by juniors was not fully related to a lower quality of the late-laid egg, we experimentally suppressed the competitive stress experienced by juniors by removing the first-laid egg (i.e. the egg normally producing a senior chick). In the absence of their brood mates, juniors were bigger, had lower levels of CORT and showed a higher CMI than juniors raised in two-brood chicks, suggesting that body size, CMI and CORT in juniors were modulated by the competitive stress.

Overall, this study suggests that juniors respond to within-brood competition by elevating CORT and down-regulating CMI. In this context, the role of CORT, as a mechanism regulating physiological strategies related to growth and immunocompetence is discussed. © 2007 Elsevier Inc. All rights reserved.

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1. Introduction

Many phenotypic traits at adulthood are influenced by developmental trajectories in early life (Lindström, 1999), so environmental conditions experienced during ontogeny

may have substantial long-term effects on individual performance (Metcalf and Monaghan, 2001). Among environmental factors acting on developing individuals, availability and accessibility of dietary resources are crucial components that may constrain individuals' developmental trajectories, presumably through physiological trade-offs between fitness-related traits (Stearns, 1992; Mock and Parker, 1997; Naguib et al., 2004).

In altricial and semi-altricial bird species, nestlings' access to dietary resources relies exclusively on parental

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provisioning. Accordingly, brood size and competition among related siblings may have important life history consequences through allocation to fitness-related traits that compete for the same limited resources. Current evidence that a nutritional stress may impair crucial traits originates from experimental enlargements of brood size. For instance, nestlings raised in enlarged broods have been shown to express a reduced growth, a lower survival and a weaker immune responsiveness than nestlings raised in control or reduced broods (Tinbergen and Boerlijst, 1990; Saino et al., 1997; Hōrak et al., 1999; Naguib et al., 2004). A similar conclusion can be drawn from the frequently observed covariance between the hatching order and nestlings' phenotypic traits. Indeed, as a result of their competitive disadvantage, the youngest nestlings have often been shown to express impaired growth and immunocompetence (Christe et al., 1998; Roulin et al., 2003; Müller et al., 2003).

Interestingly, if these findings support the hypothesis that competition for limited resources may limit the expression of a large array of fitness-related traits, they also suggest that allocation trade-offs between growth and immune defences may shape individual development and performances (Norris and Evans, 2000). In support of this view, it has been shown that size at fledging may have profound long lasting effects on fitness (Amundsen and Slagsvold, 1998) and that exaggerated allocation of resources to immune defences may impair nestlings growth (Brommer, 2003; Soler et al., 2003), presumably because immune defences are also resources demanding functions (Lochmiller and Deerenberg, 2000; Ots et al., 2001; Martin et al., 2002; Eraud et al., 2005; but see Hōrak et al., 2003; Verhulst et al., 2004). In this context, early investment into immune defences is thought of as a part of evolutionary strategies in nestlings. More specifically, immunodepression during a nutritional stress would be a trigger for organisms to promote certain resources saving from a physiological function that is not directly related to immediate survival (Sheldon and Verhulst, 1996; Fargallo et al., 2002; Ilmonen et al., 2003). However, the physiological mechanisms that govern allocation trade-offs between growth and immune functions are poorly understood in wild bird populations.

Vertebrates have evolved hypothalamic–pituitary–adrenal functions such as the production of glucocorticoids to trigger physiological and behavioural adjustments that enable individuals to cope with changes in environmental conditions (Wingfield and Ramenofsky, 1999). In birds, the main glucocorticoid is corticosterone. Based on the findings that corticosterone has immunosuppressive properties (Padgett and Glaser, 2003), and that nestlings experiencing poor developmental conditions up-regulate their corticosterone production (Harvey et al., 1980; Nuñez-de la Mora et al., 1996; Kitaysky et al., 1999, 2001a), it is therefore thought that corticosterone might play a pivotal role in mediating trade-offs between growth and immune defences during a nutritional stress. However, few field

studies have simultaneously examined nestlings' growth, corticosterone levels and immune performances in relation to developmental conditions (Ilmonen et al., 2003; Saino et al., 2003). Moreover, the influence of hatching asynchrony on these aspects has been largely neglected although hatching-rank may affect the developmental conditions of nestlings. In addition, when recent studies suggest that physiological strategies related to growth and immunity may differ between sexes of nestlings (Fargallo et al., 2002; Tschirren et al., 2003), it remains to be elucidated whether such differences are governed by a sex-specific up-regulation of corticosterone production.

Therefore this study aimed to investigate (i) how environmental conditions experienced by growing individuals (in particular competition imposed by the hatching-rank) may generate allocation trade-offs between growth and immunity, (ii) whether the secretion of corticosterone may trigger these trade-offs and finally, (iii) whether responses to environmental conditions may differ between sexes. We used the collared dove (*Streptopelia decaocto*) as a suited model since this species presents useful biological traits. Firstly, clutch size rarely differs from two eggs, therefore allowing within-brood variation to be examined independently of clutch size and interestingly, the hatching is asynchronous (Cramps, 1985). Secondly, during early life, chicks face limited resources (Blockstein, 1989) since they are exclusively fed on a secretion (crop milk) which is produced in a limited amount (Murton et al., 1974) at the expense of parents' body fluids (Denbow, 2000).

Practically, we assessed hatching rank-related growth, cell-mediated immunity and corticosterone levels. If nutritional and competitive stress imposed by the hatching pattern constrained the allocation of limited resources to growth and immunity, we predicted that latter-born chicks (juniors) would have depressed growth and immune defences compared with first-borns (seniors). In addition, as a supposing concerning the role of corticosterone, we speculated higher baseline levels of corticosterone in juniors than in seniors. Previous studies have, however, shown that females may manipulate egg quality along with the laying sequence by depositing variable amounts of some immunomodulating compounds (Schwabl, 1993; Lipar et al., 1999; Eising et al., 2001; Royle et al., 2001; Groothuis and Schwabl, 2002; Hayward and Wingfield, 2004). Accordingly, irrespective of growth-stress induced trade-offs, a decline in egg quality along with the laying sequence might result in similar findings because in the collared dove, the hatching order matches the laying order (Eraud et al., unpublished data). Therefore, to test this alternative hypothesis, we manipulate in parallel, the competitive stress experienced by juniors by removing the first-laid egg (i.e. the egg producing a senior chick in unmanipulated broods). If depressed growth and immunocompetence, and increased levels of corticosterone in juniors resulted from the growing conditions experienced in the nest rather than from the egg quality, then juniors would show better growth and cell-mediated immunity and would

also circulate lower baseline levels of corticosterone when raised in the absence of their broodmate.

2. Material and methods

2.1. Study area and species

The study was conducted on a breeding population of collared doves in Deux-Sèvres and Charente-Maritime (western France, 46° N, 0.5° W), from late April to late September 2004. In this species, a complete clutch typically includes 2 eggs which are laid over a period of 2 days. They are incubated for 14 days and hatch over a period of 1–2 days, depending on the onset of incubation. During the 4–5 days post-hatching, chicks are exclusively fed on crop milk dispensed by both parents. Afterward, the proportion of crop milk in the food is gradually replaced with a mix of seeds and green materials. Nestlings fledge at 16–17 days (Cramps, 1985).

2.2. General field procedures for data collection

Only complete clutches of 2 eggs ($n = 60$) were surveyed until hatching date, using a mirror mounted on a telescopic cane. For the nests found during egg laying, eggs were marked with a non-toxic ink according to the laying sequence and nests were subsequently visited every 2 days until the hatching date. For nests found after clutch completion, the calcification of egg shells was examined to determine if clutch was recently completed (the end of eggs is translucent until 3 days after laying, pers. obs.) and intervals between subsequent visits were defined accordingly (every 2 days for freshly completed clutch, daily otherwise). Over the hatching period, nests were visited daily to determine the hatching order. For a given nest, when two chicks were found on the same day, hatching order was accurately determined by presence/absence of remains of the yolk sac on the chick's body and by tarsus colouration (tarsus are pink colored in freshly hatchlings, grey–pink colored soon after hatching and grey on the day after; pers. obs.). Hatchlings were then marked on their right tarsus using a non-toxic permanent marker. For our purposes, first and second-hatched chicks will be denoted as *senior* and *junior* chicks, respectively (Roulin et al., 2003). At 5- and 7-day-old, chicks were weighed using an electronic balance (± 0.25 g) and we measured head length (bill and crane) and left 1st primary feather using a digital calliper (± 0.01 mm). A blood sample (200–250 μ l) was collected from the brachial vein of 5-day-old chicks using sterile needles and heparinized microcapillary tubes. Samples were collected before biometric measurements between 11:00 and 16:00 and they were immediately stored at 2–6 °C until centrifugation. All samples were taken under 2 min following chicks removal from the nest. When senior and junior chicks had to be sampled the same day, they were bled simultaneously by two observers. Otherwise, chicks were bled at the same time of the day, but sequentially, to respect the hatching span. Since seniors were bled first, the associated disturbance might have elevated the baseline level of corticosterone in juniors. However, we found no difference in corticosterone levels between juniors bled simultaneously with their nestmates (2.97 ng/ml ± 0.40) and juniors bled sequentially (2.75 ng/ml ± 0.52 ; $t = -0.51$, $P = 0.61$). We were therefore confident that plasma corticosterone reflected baseline levels since the effects of time and handling stress were minimized by the use of this standardized field procedure (see Romero and Reed, 2005). Blood was centrifuged at 4000 r.p.m. for 15 min. Red cells and the plasma were then stored at -40 °C until DNA extraction and radioimmunoassay analysis. Finally, cellular immunocompetence was assessed at the age of 7 days post-hatching.

2.3. Manipulation of the competitive stress experienced by junior chicks

In parallel to the descriptive part of our study (see previous section), we manipulated competitive stress experienced by juniors while controlling for egg quality. Since the hatching order matched exactly the laying order ($n = 32$ clutches with known laying and hatching sequences), this manip-

ulation was achieved by removing the first-laid eggs, corresponding to senior chicks in 2-egg clutches. Practically, for a set of clutches surveyed during laying, the first-laid egg was removed after clutch completion and replaced by a dummy egg of equal size. Accordingly, manipulated broods contained only one chick from the second-laid egg. This experimental design allowed to investigate the effect of post-natal conditions on the expression of late-hatchling's phenotypic traits—this effect resulting from change in asynchrony and/or brood size *per se*—while controlling for the egg-laying rank. For our purposes, single-hatchlings will be denoted as *single juniors*. On these chicks, biometric measurements, blood samples and analyses were performed as previously described in the above section.

2.4. Corticosterone radioimmunoassay analysis

Total corticosterone levels were assessed by radioimmunoassay at the Center d'Etudes Biologiques de Chizé (CEBC) as detailed in Mauget et al. (1994) and Lormée et al. (2003). Corticosterone was extracted from a 50 μ l plasma sample using diethyl-ether and assayed in duplicates. Corticosterone antibody raised in rabbits against corticosterone-3-(*O*-carboxymethyl) oxime bovine serum albumin conjugate was purchased from Biogenesis, UK. Cross-reaction was 9% with 1-desoxycorticosterone and less than 0.1% with other plasma steroids. Labelled corticosterone (8000 cpm of H^3 -Corticosterone) was purchased from Amersham Pharmacia Biotech, France. We used a Packard liquid scintillation counter β (Model 1600 TR, Canberra) to count the bound fraction after adding scintillant to samples. All samples were run in four assays. Detection limit was 100 pg/ml and the lowest measurement was 0.44 ng/ml. Intra- and interassays coefficients of variation were 9.6 and 11.7%, respectively ($n = 3$ duplicates).

2.5. Cell-mediated immunity

In vivo T cell-mediated immunity (CMI) was assayed in nestlings by measuring the proliferative response of T lymphocytes to an injection of phytohaemagglutinin (PHA). Hypersensitivity test using PHA is a commonly used test in studies investigating immune performance in nestlings (Tella et al., 2000; Fargallo et al., 2002; Müller et al., 2003) because PHA is considered as a method to assess performances of cellular immunity (Cheng and Lamont, 1988). Following Smits et al. (1999), at the age of 7 days, chicks were injected intradermally in a marked site (with a non-toxic ink) of the patagium with 0.5 mg of PHA-P (Sigma Chemicals) dissolved in 0.1 ml of sterile phosphate-buffered saline. Injections were always performed in the left wing to avoid a potential wing effect on immune response. PHA induces a wing web swelling due to the accumulation of T-cells and infiltration of macrophages at the injected site (Smits et al., 1999). To minimise disturbance at nest, the swelling was determined only at one time point post-treatment, namely at 24 h post-injection, in agreement with previous studies (see references above). Accordingly, prior to, and 24 h (± 10 min) after injection, the thickness of the wing web was measured three times to the nearest 0.01 mm with a micrometer (Teclock, Model SM 112, Italia). Repeatability of measurements both prior, and 24 h after injection, was high ($n = 78$ individuals; respectively: $r_{approx} = 0.97$, $P < 0.0001$ and $r_{approx} = 0.99$, $P < 0.0001$; Lessells and Boag, 1987). Initial and final measures were then averaged and difference between means was used as a measure of CMI.

2.6. Molecular sexing

Nestlings were sexed using a polymerase chain reaction (PCR). DNA was extracted from blood samples following the protocol of Wang et al. (1993). The mix PCR was characterized as follows: $1 \times$ Tp, 1.5 mM $MgCl_2$, 0.2 mM deoxyribonucleoside triphosphate, 0.4 μ M of primer 2550F, 0.4 μ M of primer 2718R and 0.5 U AmpliTaq DNA polymerase (*Thermus aquaticus*, Sigma). CHD genes were amplified using a Mastercycler thermocycler (Eppendorf). An electrophoresis was performed at 80 V for 30 min to resolve the amplified products on a 1.8% agarose gel stained with ethidium bromide. Individuals were categorized either males when only the CHD-Z gene was revealed, or females in the presence of both

CHD-Z and CHD-W homologous genes. The technique was used to sex a set of adult birds of known sex from outdoor aviaries ($n = 6$). In all cases the sex was correctly determined.

2.7. Statistical analyses

In growing individuals, a nutritional stress may affect both structural size and energy reserves. Accordingly, to get an integrative measure of 5 and 7-day-old nestlings' condition that encompass both structural size and condition (hereafter referred to as BSC), we used the first principal components (PC1) from Principal Component Analyses (PCA) with body mass, head length and length of the left 1st primary feather as original variables (Green, 2001; Tschirren et al., 2003). The first principal component (PC1) accounted for 82% and 73% of the total variance at 5 and 7 days old, respectively. At both ages, eigenvectors on PC1 for original variables were all >0.54 , indicating that chicks with positive scores on PC1 expressed a better BSC than those with negative scores.

We used General Linear Mixed Models (GLMMs) with the Restricted Maximum Likelihood approach (REML) to explore hatching rank and sex-related differences in baseline corticosterone levels (CORT) and cellular immunity (CMI). Hatching rank (senior vs. junior) and sex were included as fixed factors into the models, hatching date as a covariate, plus all possible two-way interactions. Since chicks from the same nest shared the same parents, nest identity was fitted as a random factor to avoid pseudoreplication. When necessary, data (corticosterone levels) were \log_{10} transformed to normalize the distributions and homogenize the variances. A similar procedure was used to examine variation in BSC. However, not all information was available for all broods at day 5 and 7 post-hatch. Then, separate analyses at 5 and 7 days were performed on BSC to maximize sample size. Models selection followed a stepwise backward procedure where the least significant terms were sequentially removed (McCullagh and Nelder, 1989). Accordingly, final models included only terms explaining a significant amount of variation of the variables of interest, plus nest identity as random factor.

Sex ratio (number of males/brood size) in relation to hatching rank was analyzed using a logistic mixed model with sex of the hatchling as a binary variable response (male vs. female), hatching rank as a fixed factor and nest identity as a random factor to take into consideration the lack of independence of chicks raised in the same nest. For the same reasons, relationships between continuous variables measured at the individual level and including both senior and junior chicks, were tested using mixed linear regressions, fitted with nest identity as a random factor. Simple linear regressions were used otherwise.

The effect of the experimental manipulation on juniors' BSC and CMI was investigated using AN(C)OVA models. When testing for CORT, Welch ANOVA has been used in place of ANOVA as distributions were normal after \log_{10} transformation but the homogeneity of variances assumption was not satisfied. Prior to analyses, treatment groups were controlled for homogeneity in sex-ratio and hatching date. Neither sex-ratio, nor hatching date differed between treatments when testing for differences in BSC, CORT and CMI (sex-ratio: Fisher-exact tests, all P 's > 0.05 ; hatching date: t -tests, all P 's > 0.05). Finally, relationships between variables measured at the individual level were examined using linear regressions. All statistical analyses were performed using JMP 4.1 (SAS Institute).

3. Results

3.1. Sexual dimorphism and hatching rank-related condition

The proportion of males did not differ between hatching positions (seniors: 0.60; juniors: 0.58; mixed-logistic regression testing for the effect of hatching order on nestling sex, Wald $\chi^2 = 0.010$, $P = 0.75$). In addition, overall condition (BSC) did not differ between sexes, neither at day 5 post-hatching nor at day 7 post-hatching (Tables 1 and 2). Irrespective of sex, seniors expressed a higher BSC than juniors (Table 1, Fig. 1a). Overall, these results suggested that a

Table 1
Summary table of GLMMs explaining BSC, CORT and CMI in chicks of Collared dove

Response terms	Explanatory terms	F -value (df)	P	Rejected terms
Overall condition at 5 days old (PCI scores)	Hatching rank	39.61 _{1,36}	<0.001	Sex hatching rank * Sex Hatching date Hatching rank * Hatching date Sex * Hatching date
Overall condition at 7 days old (PCI scores)	Hatching rank	47.72 _{1,32}	<0.001	Sex Hatching rank * Sex Hatching date Hatching rank * Hatching date Sex * Hatching date
Corticosterone (ng ml ⁻¹)	Hatching rank	7.76 _{1,36}	0.008	Sex Hatching rank * Sex Hatching date Hatching rank * Hatching date Sex * Hatching date
Cell-mediated immunity (mm)	Hatching rank	10.17 _{1,31}	0.003	Sex Hatching rank * Sex Hatching date Hatching rank * Hatching date Sex * Hatching date

GLMMs were fitted with nest identity as a random factor using the Restricted Maximum Likelihood approach (REML) and consequently, shrunken effect tests on random effects are not shown. Models selection followed a stepwise backward procedure, where the least significant terms were sequentially removed. Explanatory terms that significantly improved the fit of the models are given with their corresponding F -statistics and P -values. Non-significant terms that were excluded from the models during the selection procedures (at $P > 0.05$) are shown in the *Rejected terms* column.

Table 2

Overall condition, baseline corticosterone levels and T-cell immune responsiveness in males and females chicks of Collared dove raised in broods of two hatchlings

Traits	Males (\pm SE)	<i>n</i>	Females (\pm SE)	<i>n</i>
Overall condition at 5 days old (PCI scores)	-0.061 ± 0.232	47	0.106 ± 0.292	27
Overall condition at 7 days old (PCI scores)	-0.138 ± 0.226	42	0.168 ± 0.321	23
Corticosterone (ng ml^{-1})	2.45 ± 0.27	47	2.31 ± 0.22	27
Cell-mediated immunity (mm)	2.51 ± 0.10	41	2.42 ± 0.10	22

Means are given \pm SE. No significant difference was observed between sexes (see Table 1).

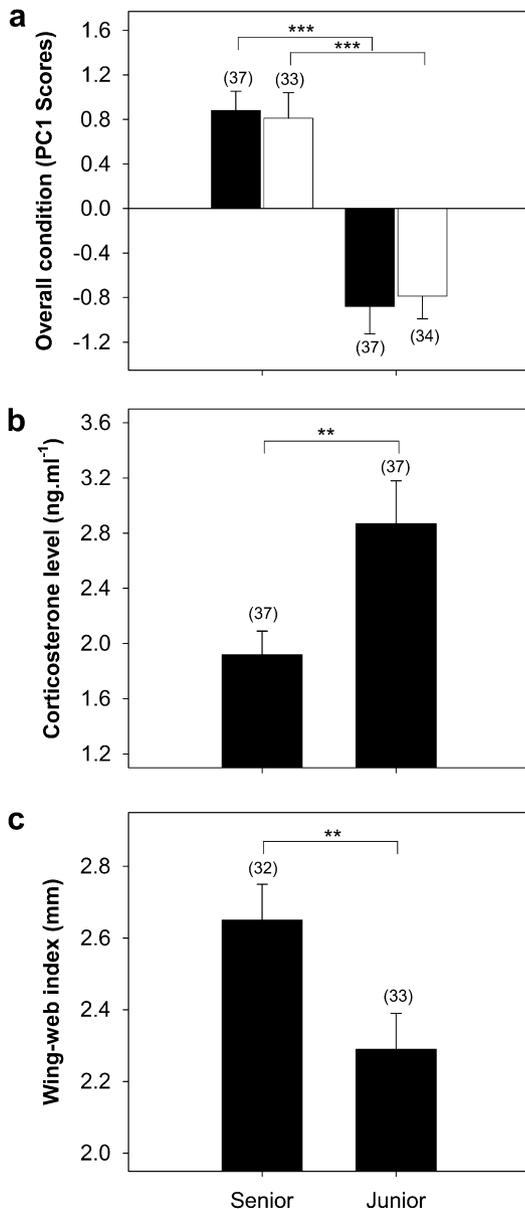


Fig. 1. Overall condition (BSC), baseline level of corticosterone (CORT) and cell-mediated immune responsiveness (CMI) in relation to the hatching order, for chicks of collared dove. First-born chicks are denoted as seniors and late-born ones as juniors. (a) Overall condition at 5 days old (filled bars) and 7 days old (open bars). (b) Baseline level of corticosterone at 5 days old. (c) Cell-mediated immune response at 7 days old (thickness of the wing web 24 h after injection of phytohaemagglutinin minus thickness of the wing web prior to injection). Data are means \pm SE. Sample sizes are given above bars. Symbols denote *P*-values: ** $P \leq 0.01$, *** $P \leq 0.001$.

sexual dimorphism was lacking and that the hatching pattern did not differentially affect chicks' BSC in a sex specific way.

3.2. Corticosterone levels in relation to hatching order and sex

Baseline level of corticosterone (CORT) was significantly affected by hatching rank. CORT was significantly higher in juniors than in seniors (Table 1, Fig. 1b). CORT was similar between sexes (Tables 1 and 2) and we found no interaction between sex, hatching order and hatching date (Table 1). At the individual level, CORT was weakly, but related significantly to BSC, with CORT increasing with decreasing BSC (GLMM; slope: -0.048 ± 0.019 , $F_{1,36} = 5.94$, $P = 0.020$; Fig. 2a). Hatching rank-related CORT was then examined while controlling for variation in BSC, by including BSC as covariate into a model with hatching rank as a fixed factor. Results showed that CORT did no longer remain significantly related to the hatching order (GLMM: $F_{1,35} = 2.84$, $P = 0.101$), indicating that hatching rank-related difference was associated with difference in BSC between senior and junior chicks. Interestingly in juniors, we found a negative relationship between CORT and difference in BSC with their nestmates (PC1 scores for junior minus PC1 scores for senior), indicating that stress hormone level in juniors increased with increasing within-brood size hierarchy (simple linear regression; slope: -0.467 ± 0.17 , $F_{1,35} = 7.40$, $P = 0.01$, Fig. 2b). On another hand, within-brood hierarchy did not affect CORT in seniors (simple linear regression; slope: 0.04 ± 0.10 , $F_{1,35} = 0.16$, $P = 0.693$).

3.3. Cell-mediated immunity in relation to hatching order and sex

Cell-mediated immunity (CMI), as assessed by the wing-web swelling, was significantly related to the hatching order, with juniors expressing a weaker immune response than seniors (Table 1, Fig. 1c). Neither CMI differed between sexes (Tables 1 and 2), nor it was related to the hatching date (Table 1). In addition, we found no interaction between hatching order, sex and hatching date (Table 1). Immune performance was significantly related to BSC measured on the day of injection (GLMM; slope:

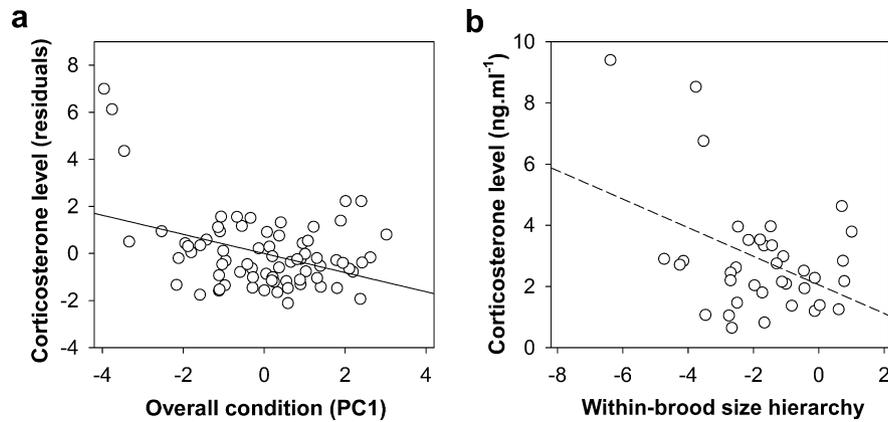


Fig. 2. (a) Relationship between baseline level of corticosterone and overall condition in chicks of collared dove. The y-axis represents the residuals from the GLMM model of the association between random effect of the nest and level of corticosterone. (b) Relationship between baseline level of corticosterone in juniors (open dots and dashed line) and within-brood size hierarchy (PC1 scores for juniors minus PC1 scores for seniors).

0.11 ± 0.05 , $F_{1,31} = 4.82$, $P = 0.036$). BSC was then included into the model to further investigate hatching rank-related immunity while controlling for condition. After inclusion of BSC, results showed that CMI remained significantly weaker in juniors than in seniors (GLMM: $F_{1,30} = 4.65$, $P = 0.039$), indicating that hatching rank-related difference in CMI was not fully related to differences in BSC among siblings.

Although hatching rank-related differences in CMI showed the opposite pattern of hatching rank-related differences in CORT, inter-individual variation in CMI was not significantly related to variation in CORT (GLMM; slope: -0.34 ± 0.31 , $F_{1,25} = 1.21$, $P = 0.282$).

3.4. Effects of the experimental manipulation on Juniors' condition, CMI and CORT

In the absence of their broodmates, single juniors were consistently in better condition (at day 5 and 7 post-hatch; ANOVAs, respectively: $F_{1,44} = 6.00$, $P = 0.018$ and $F_{1,45} = 21.01$, $P < 0.0001$), showed an increased CMI (ANOVA: $F_{1,44} = 18.98$, $P < 0.001$) and had lower CORT than juniors raised in the presence of seniors (Welch ANOVA: $F_{1,25,48} = 4.66$, $P = 0.041$; Fig. 3). CORT was not related to chicks' BSC at 5 days old (simple linear regression: $F_{1,44} = 2.40$, $P = 0.129$), whereas CMI significantly increased when increasing BSC measured at 7 days old (simple linear regression; slope: 0.212 ± 0.07 , $F_{1,44} = 9.79$, $P = 0.003$). BSC was then included into an ANCOVA model to further investigate difference in CMI between treatments while controlling for condition. Results showed that CMI remained significantly higher in single juniors than in juniors raised with their broodmates (ANCOVA: $F_{1,43} = 8.67$, $P = 0.003$). Although the variation of CMI between treatments showed the opposite pattern of CORT (Fig. 3b and c), there was no evidence that inter-individual variation in CMI was significantly related to variation in corticosterone level (simple linear regression: $F_{1,33} = 0.36$, $P = 0.55$).

4. Discussion

4.1. Within-brood variation in overall condition and stress level

Results of the current study show that hatching pattern affected both overall condition and stress levels in collared dove nestlings. More specifically, we found that first-born chicks (seniors) were consistently in better condition than late-borns (juniors) and that variation in levels of corticosterone (CORT) across the hatching sequence followed a reversed pattern, with juniors circulating significantly higher levels of corticosterone than seniors.

Previous studies have reported that maternally derived steroid hormones, including corticosterone, (Hayward and Wingfield, 2004) are transferred to egg yolk and that their concentration is often the highest in late-laid eggs (Lipar et al., 1999; Eising et al., 2001; Groothuis and Schwabl, 2002). Accordingly, this phenomenon might have explained the higher level of CORT found in juniors. However, several of our results suggest that this explanation is unlikely to apply. Firstly, CORT levels in two-brood juniors augmented with increasing within-brood size hierarchy. Secondly, juniors raised in the absence of their nestmates circulated lower levels of CORT. Finally, there is no evidence to date that collared dove females transfer significantly higher levels of CORT in the late-laid egg (mean (se) values over 39 broods: 20.17 pg.mg^{-1} (1.15) for first laid-egg vs. 21.30 pg.mg^{-1} (1.21) for second laid-egg; Eraud et al., *unpublish. data*). However, if our results suggest that maternal stress hormones have not played a direct role at day 5 post-hatch, we cannot completely discard the hypothesis that maternally derived hormones have programmed the HPA axis of birds to respond under stressful conditions. Consequently, how the pre-natal exposure to maternally derived CORT drives a stress response in nestlings needs further work. Nevertheless and contrary to the developmental hypothesis (Schwabl, 1999; Sims and Holberton, 2000), our findings would also suggest that

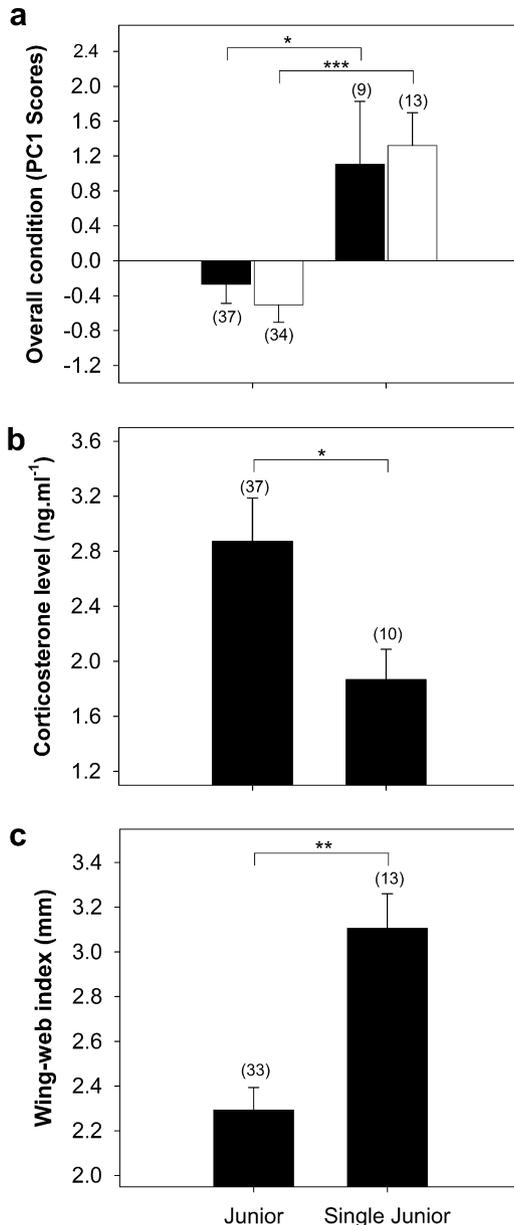


Fig. 3. Effects of experimental manipulation on traits of junior chicks of collared doves. (a) Overall condition at 5 days old (filled bars) and 7 days old (open bars). (b) Baseline level of corticosterone at 5 days old. (c) Cell-mediated immune responsiveness at 7 days old. (thickness of the wing web 24 h after injection of phytohaemagglutinin minus thickness of the wing web prior to injection). Juniors are chicks originating from late-laid egg and raised in the presence of their nestmates and single Juniors are chicks originating from late-laid egg but raised in the absence of their broodmates. Data are means \pm SE. Sample sizes are given above bars. Symbols denote P -values: * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

the adrenocortical responsiveness in collared dove chicks was not suppressed and that the competitive stress might have triggered a stress response in subordinate chicks. Only levels of CORT in juniors but not in seniors, were related to within-brood size hierarchy, clearly suggesting that the stress induced through competition in brood was dependent on the position in the within-brood hierarchy, which was imposed by the hatching pattern. Interestingly, this is

in agreement with previous findings. For instance, it was shown in the kestrel (*Falco tinnunculus*) that the level of stress proteins (Hsp60) of the smallest sibling was positively related to the size difference with the largest one (Martínez-Padilla et al., 2004). In addition, the baseline levels of CORT have been previously reported to covary with brood size and within-brood size hierarchy in several species (Nuñez-de la Mora et al., 1996; Ramos-Fernández et al., 2000; Tarlow et al., 2001; Ilmonen et al., 2003; Love et al., 2003; Saino et al., 2003; but see Sockman and Schwabl, 2001).

In several bird species, chicks have been shown to respond to food shortages by secreting CORT (Freeman et al., 1981; Nuñez-de la Mora et al., 1996; Kitaysky et al., 1999, 2001a; Saino et al., 2003; Walker et al., 2005). In this context, the secretion of CORT is suggested as an adaptive mechanism triggering physiological and behavioral adjustments associated to fitness benefits. Moreover, elevated levels of corticosterone have been shown to regulate aggression between siblings (Kitaysky et al., 1999) and to stimulate begging for food, thus enabling chicks to signal their nutritional status and to restore their condition (Kitaysky et al., 2001b). Accordingly, in the current study, nutritional stress resulting from the competition for resources imposed by the hatching pattern could be the proximate environmental factor that might explain high levels of corticosterone in juniors. In support of this view, we found that the level of corticosterone was inversely related to chicks' condition and that, after controlling for hatching rank-related differences in overall condition, hatching rank-related difference in CORT did not remain significant. The hypothesis that two-brood juniors faced a nutritional deficit in the presence of their nestmates relies on the important assumption that the parents provisioned their chicks with resources in a limited amount. During their first days of life, chicks of collared dove are fed entirely on crop milk which is produced by parents at the expense of their body fluids (Denbow, 2000) and several results are supportive of the hypothesis that its' secretion is limited. For instance, reduced growth rates in crop-milk-dependent chicks have been reported in artificially enlarged broods of several representatives of the columbidae family (Mourning dove: Westmoreland and Best, 1987; Woodpigeon: Murton et al., 1974; Rock dove: Burley, 1980). In addition, it has also been demonstrated that a single parent was unable to raise two crop-milk-dependent chicks (Burley, 1980). Altogether, these results clearly support the hypothesis that crop milk provision by parents is resource-limiting. Hence, competition for resources is likely to be strong between siblings and it is therefore reasonable to consider that the stress experienced by two-brood juniors chicks might result from a competition for limited resources. However, competition between siblings also involves physical/social competition, which could elevate stress levels, as well as food restriction due to finite resources being spread across the brood. Since both these factors were changed in the experimental manipulation,

we acknowledge that it was not possible to discriminate the respective importance of these confounding factors, while the results of this manipulation (i.e. single-juniors expressed a better growth) suggested that food availability and accessibility played an important role.

4.2. Within-brood variation in cell-mediated immunity

In concordance with previous studies (Christe et al., 1998; Müller et al., 2003), our results showed that cell-mediated immunity (CMI) was weaker in late-born chicks than in first-borns. Since seniors and juniors were immune challenged at a similar age (i.e. 7 days old), it is unlikely that this finding was due to the difference in the age-related maturation of immune functions (Apanius, 1998a). Alternatively, differential investment of maternally derived immunomodulating compounds into eggs across the laying sequence (e.g. steroids, carotenoids, immune factors; Royle et al., 2001; Müller et al., 2004; Pihlaja et al., 2006) might have also caused hatching rank-related immunocompetence. However, several of our results clearly suggest that CMI was related to growing conditions (Alonso-Alvarez and Tella, 2001). Firstly, CMI covaried positively with overall condition and secondly, single juniors expressed a higher immunoresponsiveness than juniors raised with their broodmates, although single and two-brood juniors both originated from the late-laid egg.

Furthermore, these results also suggest that in the collared dove, juniors might suffer from higher susceptibility to infections than seniors, which in turn, might promote lower survival prospects (Christe et al., 1998; Hörak et al., 1999; Tella et al., 2000). However, a reduced growth during early life may also have profound effect on their fitness (Amundsen and Slagsvold, 1998; Metcalfe and Monaghan, 2001). In this context, the down-regulation of costly physiological functions which are not directly related to immediate survival is thought to promote fitness benefits by saving resources to sustain growth functions (Brommer, 2003; Soler et al., 2003). Since immunity is a resources demanding function (Lochmiller and Deerenberg, 2000), it is suggested that immunosuppression would allow the reallocation of scarce nutritional reserves away from costly immune responses (Apanius, 1998b). In the line of this hypothesis, our results suggest that chicks expressed contrasted priorities of investment according to their position in the hatching sequence. More specifically, CMI remained weaker in juniors than in seniors, even after controlling for hatching rank-related differences in overall condition. Presumably, juniors might have reduced immune abilities to save resources for growth, and to compensate the disadvantage imposed by hatching asynchrony. However, although we found that hatching rank-related nutritional status and CMI showed an opposite pattern with respect to the variation in CORT levels, it remains unclear whether the secretion of CORT was the proximate physiological mechanism that might have mediated immune functions. Various studies on vertebrates have reached the conclusion

of an immunosuppressive effect of high levels of corticosterone (Saad et al., 1986; Morici et al., 1997; Padgett and Glaser, 2003) and several others have reported that immune performances covary negatively with circulating glucocorticoids (Fowles et al., 1993; Hasselquist et al., 1999; Saino et al., 2003; Berger et al., 2005). However, we found no evidence that the immune responsiveness was significantly correlated to the level of CORT (see also Ilmonen et al., 2003) therefore questioning the assumption that nutritional stress-induced immunosuppression in collared dove chicks, would be mediated through an adrenocortical stress response. However, it should be noticed that if we expected immunocompetence to vary proportionally with CORT levels, we cannot discard the hypothesis that, when above a threshold causing immunosuppression, corticosterone levels would not suppress immunity any further (Berger et al., 2005). Further experimental studies investigating a dose-dependent effect of corticosterone on immune performances are therefore needed.

4.3. No evidence for inter-sexual differences in stress level and immunity

In the current study, neither nutritional stress, nor stress-induced immunosuppression were sex-specific. Hatching rank-related differences in levels of corticosterone and cell-mediated immunity affected male and female chicks in a similar way. These findings are not in agreement with previous studies on dimorphic bird species. For instance, female (the larger sex) subordinate kestrel chicks were shown to circulate higher levels of stress proteins (Hsp60) than males (Martínez-Padilla et al., 2004), and inter-sexual differences in CMI were reported in nestlings of several species including great tits (*Parus major*; Tschirren et al., 2003) and eurasian kestrels (Fargallo et al., 2002). Among plausible hypotheses that might explain this discrepancy, one could be that requirements for growth and hence physiological strategies, were similar between sexes (but see Fargallo et al., 2002). Interestingly, the lack of sexual dimorphism reported in collared dove chicks might be consistent with this hypothesis.

5. Conclusion

Overall, our results show that hatching asynchrony in collared dove imposes a specific pattern of chick growth, level of corticosterone (CORT) and cell-mediated immunity (CMI). They underline the importance of chick condition on CORT and CMI, but also suggest that reduced CMI in late-born chicks might be a strategy to save resources for growth. However, the physiological mechanisms lowering CMI, and particularly the role of corticosterone, remains unclear and requires further experiments to be elucidated. Further work is also needed to investigate whether the increase in CORT and decreased condition and CMI, reported in the current study, are transient effects, or ones which may linger into adulthood.

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