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ORIGINAL PAPER

# Seasonal decline of offspring quality in the European starling *Sturnus vulgaris*: an immune challenge experiment

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**Abstract** In seasonally fluctuating environments, timing of reproduction is a crucial determinant of fitness. Studies of birds show that late breeding attempts generally result in offspring of lower reproductive value, with lower recruitment and long-term survival prospects. Several proximate mechanisms, including a seasonal decline of immune system functioning, may lead to a seasonal decline of offspring fitness. We investigated seasonal variation in offspring quality by subjecting first- and second-brood chicks of a sexually size dimorphic species, the European starling *Sturnus vulgaris*, to an immune challenge with a bacterial endotoxin (LPS), and evaluated their growth and physiological response in terms of total plasma antioxidant capacity (TAC), concentration of reactive oxygen metabolites and hematocrit. LPS challenge did not affect chick growth or oxidative status. However, hematocrit of second-brood chicks was higher in LPS chicks compared to controls. Body mass halfway through the rearing period (days 8–9 post-hatching), TAC and hematocrit were lower among second- vs. first-brood chicks. Interestingly, sexual dimorphism in body mass at days 8–9 post-hatching markedly differed between broods, first-brood males being 4.7% and second-brood males 22.7% heavier than their sisters, respectively. Pre-fledging mortality occurred among second-brood chicks only and was strongly female-biased. Our findings suggest that starling chicks, even if in poor conditions, are little affected by a bacterial challenge, at least in the short-term. Moreover, our study indicates that sex differences in body size, possibly mediated by sex-specific maternal investment in egg size, may heavily impact on pre-fledging survival in a different way in the course of the breeding season, resulting in sex-specific seasonal decline of offspring fitness. Finally, we suggest that levels of circulating antioxidants should be regarded among the proximate causes of the association between timing of fledging and long-term survival in avian species.

**Keywords** Antioxidants · Immune challenge · LPS · Oxidative stress · Seasonal variation · Sex-biased investment

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## Introduction

In seasonal environments, where ecological resources fluctuate, timing of reproduction is a major determinant of reproductive performance and fitness, since individuals that breed at the time of peak resource availability achieve greater fitness (Clutton-Brock 1988; Iwasa and Levin 1995). Natural selection on timing of breeding, acting via selection on the offspring, is therefore expected to be intense, and the fitness consequences of variation in timing of reproduction have been the subject of many studies, especially on birds, starting from the pioneering work by Lack (1947, 1954) (see also Klomp 1970; Perrins 1970). These studies mainly concern the relationships between fitness components, such as clutch size, fledging success, or survival/recruitment, and laying date, with some documenting hump-shaped relationships between fitness and laying date, while others highlighting linear declines of reproductive performance as the season progresses (e.g., Crick et al. 1993; Naef-Daenzer et al. 2001; Grüebler and Naef-Daenzer 2010). Whatever the shape of the seasonal fitness curve, there is general consensus that late nesting attempts result in low fitness returns (Crick et al. 1993). Two main ecological mechanisms have been advocated to explain a seasonal decline of reproductive success (review in Verhulst and Nilsson 2008). First, the 'parental quality hypothesis' posits that the low breeding output of late breeding individuals derives mainly from low-quality parents (e.g. younger and less experienced breeders, or parasitized birds) reproducing later (e.g. Rowe et al. 1994; Brinkhof et al. 1997; Møller et al. 2004). On the other hand, the 'breeding date hypothesis' posits that low breeding output of late breeding individuals is a consequence of a seasonal deterioration of environmental conditions, resulting in poor foraging success and thus nutritional constraints on offspring growth and condition (e.g. Parsons 1975; Brinkhof et al. 1993; Verboven and Verhulst 1996; Grüebler and Naef-Daenzer 2008). The two mechanisms are not mutually exclusive and may concur (e.g. parental body condition may decline in the course of the breeding season because resources deteriorates and this may in turn affect offspring fitness) to determine a seasonal decline in breeding performance and fitness, to the point that the two hypotheses are hardly distinguishable (Verhulst and Nilsson 2008). Moreover, genetic variation in timing of breeding may be maintained because seasonal clines in selection originate adaptive phenotypic clines, reinforced by assortative mating of early- and late-breeding individuals (Hendry and Day 2005). This may lead to reduced gene flow between early- and late-breeding individuals and genetic differentiation in relation to timing of breeding (Casagrande et al. 2006).

Many passerine birds lay two or more clutches per season (Cramp 1998) and thus offer the opportunity to investigate seasonal variation in offspring fitness among subsequent reproductive events within the same season while holding genetic variation in parental quality constant. Second clutches are often smaller (Cramp 1998) and produce low quality offspring with lower survival prospects (Hochachka 1990; Dubiec and Cichoñ 2001), though this effect may vary among years and species and depend on both pre- and post-fledging ecological conditions (Verboven and Visser 1998; Merino et al. 2000; Christe et al. 2001; Møller 2002; Grüebler and Naef-Daenzer 2008; López-Rull et al. 2011). In addition, offspring of some species may be sexually dimorphic already at the chick stage (Griffiths 1992; Badyaev 2002; Mainwaring et al. 2010), and male and female chicks may be differentially susceptible to seasonal deterioration of ecological conditions, either because of sex-specific susceptibility to harsh environments (Clutton-Brock et al. 1985; Griffiths 1992; Råberg et al. 2005; Bonisoli-Alquati et al. 2008) or to sex-related asymmetries in scrambling competition for access to parental resources (Uller 2006; Boncoraglio et al. 2008). In the first case, offspring of the larger sex (typically males) are predicted to achieve lower fitness, whereas in the second case, the opposite may be the case, as larger offspring may prevail in sibling competition vs. smaller offspring and enhance their share of food delivered by parents (Uller 2006).

Several, possibly interrelated, proximate mechanisms may account for the seasonal decline of offspring fitness. Although body size at fledging, partly reflecting nutritional conditions, positively predicted the probability of recruitment into the breeding population in studies of different passerine species (e.g. Hochachka and Smith 1991; Magrath 1991; Verboven and Visser 1998), hatching date was still found to predict recruitment irrespective of body size (Hochachka 1990; Verboven and Visser 1998). Moreover, several studies pointed out that immune responsiveness at the chick stage, implying a better ability to fend off parasites and pathogens, may also be an important predictor of long-term survival and recruitment, with even stronger effects than body size (Christe et al. 2001; Møller and Saino 2004; Moreno et al. 2005; López-Rull et al. 2011). Indeed, offspring immune responsiveness is a highly resource- and condition-dependent trait (Saino et al. 1997; Lochmiller and Deerenberg 2000; Norris and Evans 2000), and offspring in good immune conditions may thus survive better in the long term than those in poor immune conditions independently of body size per se (e.g. López-Rull et al. 2011). Several studies also reported that offspring immune responsiveness declines in the course of the breeding season, early-hatched offspring showing higher immunocompetence than late-hatched ones (Sorci et al. 1997; Dubiec and Cichoñ 2001; Wilk et al. 2006; López-Rull et al. 2011; but see Christe et al. 2001; Merino et al. 2000). Therefore, immune system functioning may qualify among the proximate factors causing a seasonal decline of offspring fitness.

In this study, we investigated seasonal variation of offspring quality in the sexually size dimorphic European starling (*Sturnus vulgaris*) by experimentally challenging the nestlings' immune system with a bacterial endotoxin (lypopolysaccharide (LPS) from *Escherichia coli* cell walls) and analysing their physiological and growth response, under the general expectation that high-quality nestlings in prime conditions should pay smaller costs for mounting an immune response compared to low-quality, poor condition, ones. We compared the effects of the immune challenge between first- and second-brood nestlings rather than early- and late-hatched ones, thereby controlling for variation in average genetic makeup of parents (see, e.g. Dubiec and Cichoñ 2001; Christe et al. 2001; Merino et al. 2000; López-Rull et al. 2011). LPS challenge has been often adopted to investigate the short-term effects of immune system activation by a bacterial endotoxin in the absence of the deleterious effects of the living pathogen (e.g. Bonneaud et al. 2003; Lee et al. 2005; Owen-Ashley et al. 2006; Romano et al. 2011). LPS is an inert, non-replicating antigen that induces a rapid inflammatory response, starting within a few hours after injection, triggering first a non-specific cell-mediated response that is followed by a humoral response and development of specific antibodies (Janeway and Travers 1999; Grindstaff et al. 2006).

As indicators of response to immune challenge, 24 h later (i.e. during the so-called acute phase response; Owen-Ashley and Wingfield 2007), we recorded total plasma antioxidant capacity (TAC), a measure of the overall capacity of tissues to resist attack by reactive oxygen species, plasma concentration of reactive oxygen metabolites (ROM), a marker of early oxidative damage (Costantini 2008; Monaghan et al. 2009), hematocrit and changes in growth. The innate inflammatory response induced by bacterial endotoxins does not come at no cost for the organism, as it is known to release free radicals that neutralise pathogens via their cytotoxic effects, but concomitantly damage molecules and cells (Halliwell and Gutteridge 1999; Bhattacharyya et al. 2004; Bertrand et al. 2006; Costantini 2008; Costantini and Møller 2009; Monaghan et al. 2009). These side effects of inflammation which may impose a limit to upregulation of the immune response are therefore expected to alter the oxidative status of the organism (Halliwell and Gutteridge 1999; Bhattacharyya et al. 2004; Bertrand et al. 2006; Costantini 2008; Costantini and Møller 2009; Monaghan et al. 2009). LPS is known to trigger secretion of pro-inflammatory cytokines by phagocytic cells that rapidly stimulate release of reactive oxygen and nitrogen species (Soszynski and Krajewska 2002; Bhattacharyya et al. 2004). Thus, LPS challenge may result in an increase of ROM and a decrease of TAC because circulating antioxidants (both endogenous and exogenous) may be used up to counter the side effects of the inflammatory response (Costantini 2008; Costantini and Møller 2009). Moreover, in birds, LPS is known to induce mass loss (Bonneaud et al. 2003; Bertrand et al. 2006) and to depress chick growth (Grindstaff 2008; Romano et al. 2011) either because of the direct energetic costs of mounting an inflammatory and immune response (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000) or of behavioural effects due to the induction of a characteristic 'sickness behaviour', resulting in reduced activity and food intake (Owen-Ashley et al. 2006). Finally, hematocrit, a measure of relative volume of erythrocytes over total blood volume, is a widely used condition index in avian studies, though its interpretation is not straightforward (review in Fair et al. 2007). However, both pathogen infection and changes in energetic condition and metabolism, such as those resulting from LPS challenge, may affect hematocrit values (Fair et al. 2007).

We predicted that second-brood chicks were in generally poorer condition than first-brood ones and therefore that the overall costs of the immune challenge (see Lochmiller and Deerenberg 2000) should be greater among second-brood chicks, resulting in slower growth among second- vs. first-brood LPS chicks compared to controls. We also formulated the general prediction that ROM should increase and TAC should decrease in LPS vs. control chicks, though we could not predict differences in oxidative response to LPS challenge between brood types. Finally, we investigated whether the effects of brood type and immune challenge on growth and physiology were sex-specific in a species where males are larger than females already at the chick stage (see "Results"; Chin et al. 2005). To our knowledge, this is one of the very few studies investigating variation in nestling quality (in terms of oxidative response to an immune challenge) between first and second broods in relation to sex.

## Materials and methods

### Field procedures and immune challenge

The study was carried out in a nestbox breeding population of starlings (74 nestboxes installed) near Ozzano Emilia (N Italy), during spring–summer 2010. The colony is located within a ca. 30-ha set-aside and naturalized area, surrounded by cornfields (>90% of the surface within a radius of 1 km); the neighbouring landscape also hosts several rural buildings with small orchards and a horse racecourse. Nestboxes were made of softwood panels (2 cm thick), with inside dimensions 15×15 cm (base)×45 cm (height) and entrance hole size diameter of 4.5 cm (distance of the hole from the base was 31 cm). Nestboxes were set up for the first time during early spring 2009. In 2010, occupancy rate (total number of nestboxes where at least one incubated clutch was laid over the entire season, i.e. including both first and second clutches) was 38%. In the study population, starlings

lay two clutches per season consisting of two to nine eggs (2009–2010, mean size of first clutch—5.1 (0.3 s.e.) eggs, $n$=26; second clutch—4.8 (0.1 s.e.) eggs, $n$=38). Mean laying dates of first and second clutches differ by more than 1 month and do not overlap (2009–2010; mean laying date, first clutches—13 April; second clutches—20 May). Among the clutches included in this experiment, first ones ($n$=8) were started between 6 and 19 April, while second ones ($n$=14) between 5 and 22 May. A few so-called intermediate clutches ($n$=3) (Pinxten et al. 1990) were excluded from the experiment. Only three nestboxes were occupied during both the first and the second brood, since most females likely changed nestbox between broods (see below; nestbox and mate changes between first and second broods occur frequently in starlings; Feare and Burham 1978). Nestboxes were not cleaned after fledging of first-brood chicks. As also reported in the literature (Cramp 1998), fledging success of second clutches (number of chicks fledged on clutch size) was lower than that of first clutches (2010, mean fledging success, first clucthes—0.60±0.10 s.e., $n$=12 clutches; second clutches—0.30±0.10 s.e., $n$=24 clutches). In the set of clutches included in the present experiment, all hatched chicks from first clutches successfully reached fledging age (19 days), whereas mortality occurred only among second-clutch chicks (see below and "Results"). Since most adult birds were not marked, we could not identify parental identity of many focal nestboxes. Therefore, we cannot exclude that some second clutches were very late first clutches, though this is unlikely given the high synchrony of both first and second clutches, well-spaced laying dates and exclusion of 'intermediate' clutches (see above). However, four females that were trapped and ringed at nestboxes while rearing first broods were retrapped while rearing the second clutch (one female in the same nestbox and the others in different nestboxes). Nestbox content was checked every 1–3 days during egg laying and every 1–2 days after hatching. At day 8 post-hatching, half of the chicks of each nest were subjected to an immune challenge with LPS, whereas the other half were subjected to a control treatment (in case of an odd number of nestlings, odd chicks were assigned at random to treatments). Fifty microlitres of a solution of 1 mg lyophilized LPS powder (026:B6 serotype, L8274, Sigma-Aldrich) diluted in 1 ml phosphate-buffered saline (PBS) was injected intraperitoneally. Since body mass of starling chicks at day 8 is ca. 45 g (46.47 g±1.10 s.e. in our sample of nestlings), the amount of LPS we chose to inject corresponds to ca. 1 mg/kg body mass, similarly to doses used in some previous studies (e.g. Alonso-Alvarez et al. 2004; Bertrand et al. 2006; Berthouly et al. 2008; Grindstaff 2008; Romano et al. 2011). Control nestlings were injected with the same amount of PBS only. Chick morphology [body mass, to the nearest 0.1 g with an electronic balance; tarsus length and length of the growing first primary feather (numbered descendantly; feather length hereafter) to the nearest 0.1 mm with dial calliper] was recorded on day 8 (before the immune challenge) and on day 9 (24 h after the immune challenge). In a subsample of birds, we also measured body mass at day 1 post-hatching ($n$=27 chicks from first broods and $n$=15 from second broods) that closely mirrors egg mass (Williams 1994; Krist 2011) and near fledging (17 days post-hatching; Chin et al. 2005; $n$=37 chicks from first broods and $n$=6 from second broods). We found that body mass at days 8–9 (mean value of measurements taken at both ages) strongly positively predicted body mass at days 16–17 (mean value of measurements taken at both ages) (first brood chicks: $r$=0.72, $P$<0.001, $n$=37; second brood chicks: $r$=0.81, $P$=0.054, $n$=6). Therefore, body mass at days 8–9 reliably reflects that at the end of the nestling period. On day 9, a blood sample (ca. 150 μl) was drawn from the brachial vein into microhematocrit capillary tubes and kept cool until processing (within a few hours, see below).

## Sex determination and assay of plasma TAC and ROM concentration

Blood samples were centrifuged at 11,500 rpm for 10 min (centrifuge radius 94 mm) and plasma separated from red blood cells (RBC). Hematocrit (proportion of RBC over total blood volume) was measured on capillary tubes with a ruler (nearest 0.5 mm). Plasma and RBC were then stored at −80°C until analyses.

Molecular sexing was performed using the method originally developed by Griffiths et al. (1998). We amplified part of the W-linked avian CHD gene (CHD-W) in females and its non-W-linked homologue (CHD-Z) in both sexes using polymerase chain reaction (see Griffiths et al. 1998 for details of procedure). All nestlings subject to this procedure were successfully sexed.

The plasma antioxidant barrier includes both exogenous (e.g. ascorbate, tocopherols, carotenoids) and endogenous (e.g. uric acid, enzymes) compounds (Costantini 2008; Monaghan et al. 2009). Plasma TAC was measured using the OXY-Adsorbent test (Diacron, Grosseto, Italy). This test uses a colorimetric determination to quantify the ability of the plasma antioxidant barrier to cope with the oxidant action of hypochlorous acid (HClO). Briefly, plasma (5 μl) was diluted 1:100 with distilled water. A 5-μl aliquot of the diluted plasma was added to 200 μl of a titred HClO solution. The solution was gently mixed and incubated for 10 min at 37°C. At the end of the incubation time, 5 μl of an alkyl-substituted aromatic amine solubilized in a chromogenic mixture was added. Such amine is oxidized by the residual HClO and transformed in a pink-coloured derivative. The concentration of coloured complex is directly proportional to the HClO excess and inversely related to

the antioxidant capacity of tested plasma. The intensity of the coloured solution was measured at 492 nm using a photometer (Multiskan EX, Labsystem). One standard sample of known TAC and one blank sample (5 μl of distilled water) were processed and used as reference. Antioxidant capacity is expressed as millimolars of HClO neutralised.

ROM are early peroxidation products of the exposure of biological macromolecules (such as proteins, lipids and nucleic acids) to reactive oxygen species (ROS) (Costantini 2008; Monaghan et al. 2009). ROM are relatively more stable than ROS, and therefore, they can be conveniently detected and quantified (Costantini 2008; Monaghan et al. 2009). The plasma concentration of ROM (primarily hydroperoxides, ROOH) was measured by the d-ROMs test (Diacron, Grosseto, Italy). The plasma (10 μl) was diluted with 200 μl of a solution containing an acetate buffer (pH 4.8) and an alkyl-substituted aromatic amine solubilised in a chromogenic mixture. The solution was gently mixed and then incubated for 75 min at 37°C. During incubation, the acidic pH of the acetate buffer favoured the iron release from plasma proteins. This metal catalysed the cleavage of ROOH in two different free radicals. Such radicals are able to oxidize the alkyl-substituted aromatic amine solubilized in the chromogen producing a pink-coloured derivative whose colour intensity is directly proportional to the concentration of ROM. After incubation, the absorbance was read at 492 nm using a photometer (Multiskan EX, Labsystem). One standard sample and one blank sample (10 μl of distilled water) were processed and used as reference. The results of d-ROMs test are expressed as millimolars of $H_2O_2$ equivalents.

Repeatability of TAC and ROM measurements, as assessed by the intraclass correlation coefficient of 20 individuals that were assayed twice, was high and statistically significant in both cases (TAC: $R$=0.56, $F_{19,20}$=3.54, $P$=0.004; ROM: $R$=0.56, $F_{19,20}$=3.59, $P$=0.003). Intra- and inter-assay coefficients of variation were, respectively, as follows: TAC, 5.0% and 7.1%; ROM, 3.3% and 5.2%.

## Statistical analyses

Variation in chick phenotypic traits in relation to brood (first vs. second), immune challenge and sex was investigated by means of mixed models. For traits measured both before and after immune challenge (body mass, tarsus and feather length), we included both nestbox and chick identity as nested random effects and included age (day 8 or 9 post-hatching) as an additional fixed factor. For traits measured only after the immune challenge (hematocrit, TAC, ROM), we included nestbox identity as a random factor. Interactions (up to the highest level of complexity) were included in initial full models. Final models were obtained by removing from the full model in a single-step all non-significant interactions of a given order. However, if a statistically significant interaction emerged, all interactions of the same order (and those of inferior orders) were kept in the final model. With this procedure, we aimed at reducing the probability of committing type I errors due to multiple statistical tests, as occurs with traditional stepwise procedures (e.g. Whittingham et al. 2006). The above analyses were repeated for the subset of chicks that could be attributed to the same four mothers (see above) during each clutch, though with following differences in model specifications: (1) four-way interactions were not tested in models of morphological traits because sample size was too small, and (2) female identity was included as an additional random effect in all models.

The analysis of survival to fledging was conducted on second-brood chicks only (all first-brood chicks fledged successfully) by means of a binomial mixed model (dependent variable coded as survivor=1 and non-survivor=0) with nestbox identity as a random grouping factor and immune challenge, sex and hatch date as predictors. Interactions could not be tested in this model because the design was poorly saturated (among non-surviving chicks, all but one were females; see "Results") and complex models did not converge (details not shown for brevity). The model was not overdispersed, and no correction to standard errors was therefore applied (Zuur et al. 2009). Finally, we compared the phenotype of second-brood females (no first-brood chick died and only a single second-brood male died; see above and "Results") that survived vs. those that died before fledging with mixed models, where female phenotypic traits were included as dependent variables and survival to fledging as a fixed factor. In the models of female body mass, tarsus and feather length, we included nestbox and chick identity as nested random effects (to account for repeated measurement of the same chick in consecutive days, days 8 and 9 post-hatching), whereas in models of hematocrit, TAC and ROM, we included only nestbox identity as a random effect. All analyses were carried out with the MIXED and GLIMMIX procedures of SAS 9.1.3 (Littell et al. 2006). In Gaussian mixed models, degrees of freedom were estimated by the Kenward–Rogers method, which provides a conservative estimate of the denominator degrees of freedom (Littell et al. 2006). Overall, analyses were carried out on 41 chicks [19 males (9 control; 10 LPS-injected) and 22 females (10 controls; 12 LPS-injected)] from 8 first broods and 45 chicks [15 males (8 controls, 7 LPS-injected) and 30 females (13 controls, 17 LPS-injected)] from 14 second broods. The analyses carried out on the subset of chicks from the same mothers were based on 36 chicks (21 from first broods and 15 from second broods). Sample sizes may vary slightly between analyses because of missing data (due accidental reasons, such as blood sample loss or amount too small for biochemical analyses).

Information on sample sizes is also reported throughout the "Results" and in figure captions.

## Results

### Variation in oxidative status and hematocrit

The analyses based on the complete dataset showed that plasma TAC and ROM concentration were unaffected by immune challenge (Table 1), but TAC of second-brood chicks was significantly lower than that of first-brood ones (Table 1; Fig. 1). Hematocrit was significantly lower among second- than first-brood chicks (Table 1; Fig. 1). Moreover, hematocrit was affected by immune challenge among second- but not first-brood chicks, with second-brood LPS-chicks showing a significantly higher hematocrit than controls (Table 1; Fig. 1). All these findings were qualitatively unaltered when the analyses were repeated on the subset of chicks reared by the same mothers in both the first and second brood (Table 1).

### Variation in growth

Based on the complete dataset, models showed that, at days 8–9 post hatching, second-brood chicks were ca. 19% lighter than first-brood ones (Table 2; Fig. 2). Tarsus length was also significantly smaller among second-brood chicks, whereas feather length did not differ (Table 2; Fig. 3). Increase of body mass between day 8 and day 9 post-hatching differed between sexes in a brood order-specific way, as testified by the statistically significant three-way interaction between brood type, sex and age (Table 2): post hoc comparisons revealed that among first-brood chicks, males were non-significantly larger than females at both ages (day 8, $t$=0.37, $P$=0.71; day 9, $t$=1.85, $P$=0.068), and body mass of both sexes increased significantly between day 8 and day 9 (males, $t$=8.63, $P$<0.001; females, $t$=4.80, $P$<0.001), while among second-brood chicks males were markedly heavier than females at both ages (both $t$>4.08, $P$<0.001), but body mass did not increase significantly from day 8 to day 9 (males, $t$=0.89, $P$=0.38; females, $t$=1.12, $P$=0.27) (Fig. 2).

**Table 1** Mixed models of TAC, ROM and hematocrit of nestling starlings at day 9 (i.e. 24 h after the immune challenge) based on the entire dataset ($n$=86) or on the subset of chicks raised by the same mothers in both the first and the second brood ($n$=36)

| Variable | All chicks[a] | | | Same mothers[b] | | |
|---|---|---|---|---|---|---|
| | *F* | *df* | *P* | *F* | *df* | *P* |
| TAC | | | | | | |
| Brood | 22.28 | 1, 57.7 | < 0.001 | 15.37 | 1, 9.1 | 0.003 |
| Immune challenge | 1.72 | 1, 59.3 | 0.19 | 2.40 | 1, 27.0 | 0.13 |
| Sex | 0.28 | 1, 68.0 | 0.60 | 0.09 | 1, 29.8 | 0.77 |
| Dropped terms | | | | | | |
| Brood × immune challenge | 0.03 | 1, 56.9 | 0.86 | 0.19 | 1, 23.9 | 0.67 |
| Brood × sex | 0.01 | 1, 63.7 | 0.96 | 0.28 | 1, 27.2 | 0.60 |
| Immune challenge × sex | 0.11 | 1, 64.7 | 0.74 | 0.34 | 1, 26.2 | 0.56 |
| Brood × immune challenge × sex | 0.19 | 1, 64.6 | 0.66 | 0.26 | 1, 24.3 | 0.62 |
| ROM | | | | | | |
| Brood | 0.03 | 1, 48.5 | 0.86 | 0.05 | 1, 17.7 | 0.82 |
| Immune challenge | 0.36 | 1, 61.3 | 0.55 | 0.05 | 1, 25.0 | 0.82 |
| Sex | 0.02 | 1, 69.9 | 0.88 | 0.08 | 1, 28.1 | 0.78 |
| Dropped terms | | | | | | |
| Brood × immune challenge | 2.20 | 1, 58.9 | 0.14 | 1.91 | 1, 21.4 | 0.18 |
| Brood × sex | 2.60 | 1, 64.7 | 0.11 | 0.82 | 1, 24.1 | 0.37 |
| Immune challenge × sex | 1.40 | 1, 65.5 | 0.24 | 3.89 | 1, 22.4 | 0.06 |
| Brood × immune challenge × sex | 0.49 | 1, 65.0 | 0.49 | 0.36 | 1, 21.6 | 0.56 |
| Hematocrit | | | | | | |
| Brood | 14.28 | 1, 66.0 | 0.003 | 15.37 | 1, 8.2 | 0.004 |
| Immune challenge | 6.01 | 1, 50.9 | 0.018 | 6.38 | 1, 23.7 | 0.019 |
| Sex | 3.26 | 1, 60.5 | 0.08 | 3.13 | 1, 26.4 | 0.09 |
| Brood × immune challenge | 8.60 | 1, 51.8 | 0.005 | 7.70 | 1, 23.8 | 0.010 |
| Brood × sex | 1.66 | 1, 57.3 | 0.20 | 0.52 | 1, 26.4 | 0.48 |
| Immune challenge × sex | 0.08 | 1, 58.1 | 0.78 | 0.14 | 1, 26.0 | 0.71 |
| Dropped terms | | | | | | |
| Brood × immune challenge × sex | 2.79 | 1, 59.0 | 0.10 | 0.04 | 1, 23.6 | 0.84 |

See "Materials and methods" for details on model simplification procedures. Degrees of freedom were estimated by the Kenward–Rogers method

[a]Mixed models with nestbox identity as a random factor

[b]Mixed models with nestbox and mother identity as random factors

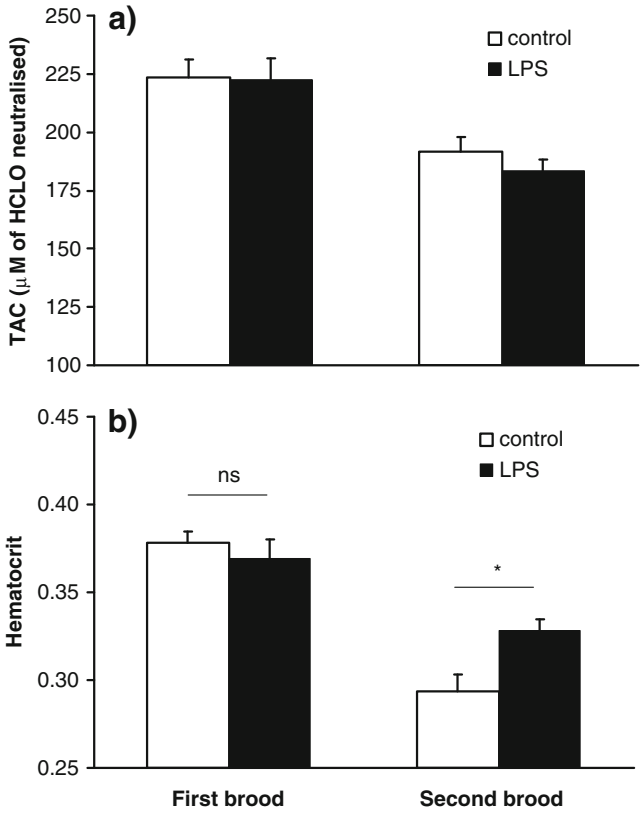


**Fig. 1** Mean (+ s.e.) values of total antioxidant capacity (*TAC*) and hematocrit (proportion of red blood cells) of first- and second-brood nestling starlings in relation to LPS challenge. Sample size is 40 chicks from first broods (19 controls and 21 LPS-injected) and 42 from second broods (19 controls and 23 LPS-injected); *ns* not significant ($P>0.05$); *$P<0.001$ at post hoc tests

Tarsus length of first-brood chicks was larger than that of second-brood ones (Table 2; Fig. 3). Female tarsi were smaller than male ones and especially so among chicks from second broods (Table 2; Fig. 3). Similarly, males had longer feathers than females among second- but not first-brood chicks (Table 2; Fig. 3). Furthermore, growth of wing feathers between day 8 and day 9 was faster among first- than among second-brood chicks (Table 2; Fig. 3). Immune challenge did not affect body mass, tarsus or feather length among either first- or second-brood chicks (Table 2).

The analyses carried out on the reduced set of chicks reared by the same mothers during the first and second brood were broadly supportive of the above findings (Table S1). For body mass, the three-way interaction between brood type, sex and age was only marginally non-significant ($F_{1,29.0}=3.18$, $P=0.085$), notwithstanding the much smaller sample size. Brood-specific sex dimorphism in body mass was confirmed (brood × sex interaction: $F_{1,26.2}=4.33$, $P=0.047$; Table S1). However, in the reduced dataset, tarsus length did not differ between first- and second-brood chicks, and there was no differential effect of brood type on sex dimorphism in tarsus length (Table S1). Feather growth was faster among first- vs. second-brood chicks, although at both ages wing feathers of second-brood chicks were significantly longer

**Table 2** Mixed models (with nestbox and chick identity as random factors) of body mass, tarsus length and feather length variation between day 8 and day 9 post-hatching in nestling starlings based on the entire dataset

| Variable | *F* | *df* | *P* |
|---|---|---|---|
| Body mass | | | |
| Brood | 16.77 | 1, 62.1 | <0.001 |
| Immune challenge | 0.54 | 1, 58.2 | 0.46 |
| Sex | 14.41 | 1, 67.6 | <0.001 |
| Age | 58.76 | 1, 74.8 | <0.001 |
| Brood × immune challenge | 0.23 | 1, 58.4 | 0.64 |
| Brood × sex | 5.15 | 1, 65.4 | 0.027 |
| Brood × age | 32.28 | 1, 74.8 | <0.001 |
| Immune challenge × sex | 0.38 | 1, 64.9 | 0.54 |
| Immune challenge × age | 2.95 | 1, 74.8 | 0.09 |
| Sex × age | 4.58 | 1, 74.8 | 0.036 |
| Brood × immune challenge × sex | 0.46 | 1, 64.9 | 0.50 |
| Brood × immune challenge × age | 2.46 | 1, 74.9 | 0.12 |
| Brood × sex × age | 4.38 | 1, 74.8 | 0.039 |
| Immune challenge × sex × age | 0.68 | 1, 74.8 | 0.61 |
| Tarsus length | | | |
| Brood | 10.13 | 1, 68.0 | 0.002 |
| Immune challenge | 1.17 | 1, 58.1 | 0.28 |
| Sex | 10.80 | 1, 66.4 | 0.002 |
| Age | 111.53 | 1, 77.2 | <0.001 |
| Brood × immune challenge | 1.17 | 1, 58.5 | 0.28 |
| Brood × sex | 4.60 | 1, 64.2 | 0.036 |
| Brood × age | 0.03 | 1, 77.2 | 0.86 |
| Immune challenge × sex | 0.05 | 1, 63.6 | 0.82 |
| Immune challenge × age | 1.13 | 1, 77.3 | 0.29 |
| Sex × age | 0.18 | 1, 77.1 | 0.67 |
| Feather length | | | |
| Brood | 0.03 | 1, 78.2 | 0.86 |
| Immune challenge | 0.09 | 1, 60.2 | 0.77 |
| Sex | 1.07 | 1, 64.8 | 0.30 |
| Age | 263.77 | 1, 77.5 | <0.001 |
| Brood × immune challenge | 0.02 | 1, 60.4 | 0.86 |
| Brood × sex | 3.87 | 1, 63.2 | 0.053 |
| Brood × age | 75.48 | 1, 77.5 | <0.001 |
| Immune challenge × sex | 0.17 | 1, 62.8 | 0.68 |
| Immune challenge × age | 0.81 | 1, 77.6 | 0.37 |
| Sex × age | 0.05 | 1, 77.4 | 0.83 |

Four-way interactions were not significant in any case and were removed from the models (all $P>0.49$). Three-way interactions were also not significant for models of tarsus and feather length (all $P>0.24$; see "Materials and methods" for details on model simplification procedures). Degrees of freedom were estimated by the Kenward–Rogers method

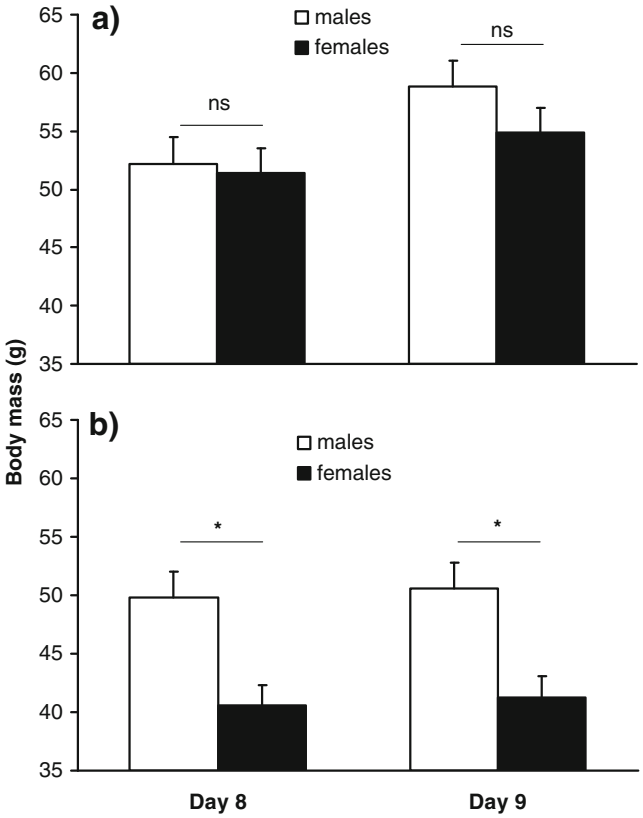


**Fig. 2** Mean (+ s.e.) values of body mass of **a** first- and **b** second-brood nestling starlings in relation to age and sex (values represent model-estimated means from the model shown in Table 2). Sample size is 41 chicks from first broods (19 males and 22 females) and 45 from second broods (15 males and 30 females); ns: not significant ($P>0.05$); *$P<0.001$ at post hoc tests

than those of first-brood ones (post hoc tests, $P<0.04$) (see main effect of brood in Table S1). Brood-specific sex dimorphism in feather length was not confirmed (Table S1), but this effect was weak also in the model based on the entire dataset (see Table 2). Minor discrepancies with respect to the results based on the entire dataset may reflect sampling effects due to the small sample size and will thus not be discussed further.

## Survival to fledging of second-brood chicks in relation to sex, immune challenge and phenotype

This analysis was performed only for second-brood chicks because all 41 chicks from first broods fledged successfully. On the other hand, 14 out of 45 (31%) chicks from second broods died before fledging (mean age of death was 12 days post-hatching, range 7–19). Among chicks that died before fledging, all but one were females. Thus, in a binomial mixed model with treatment, sex and hatching date as predictors and nestbox identity as a random factor, probability of surviving to fledging was predicted by chick sex, with males surviving better than females ($F_{1,28}=4.23$, $P=0.049$), whereas the effects of immune challenge and hatch date were non-significant ($F_{1,28}=0.01$, $P=0.91$ and $F_{1,28}=2.27$, $P=0.14$, respectively; interactions were not tested, see "Statistical analyses" for details). Body mass and tarsus length were significantly larger among surviving female chicks compared to non-surviving ones, whereas all other traits did not significantly differ between surviving and non-surviving females (Table 3).

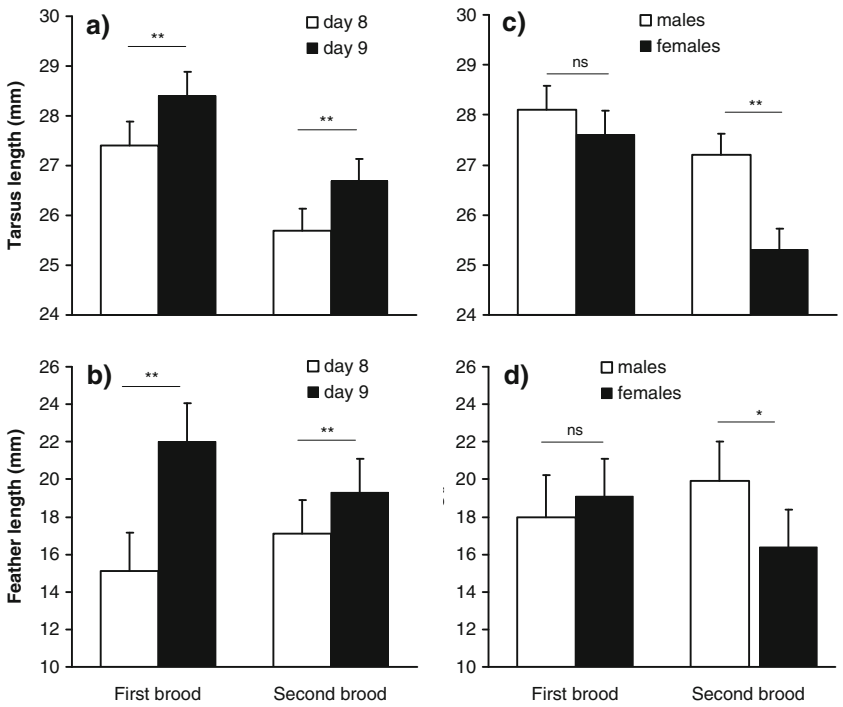


**Fig. 3** Mean (+ s.e.) values of tarsus and feather length of first- and second-brood nestling starlings in relation to age (*left column*, **a** and **b**) and sex (*right column*, **c** and **d**) (values represent model-estimated means from models listed in Table 2). Sample size is 41 chicks from first broods (19 males and 22 females) and 45 from second broods (15 males and 30 females); *ns* not significant ($P>0.05$); *$P<0.045$; **$P<0.001$ at post hoc tests

**Table 3** Phenotype (mean and s.e.) of second-brood females surviving ($n$=17) or not ($n$=11) to fledging

| Trait | Surviving | Non-surviving | $F$ | $df$ | $P$ |
|---|---|---|---|---|---|
| TAC | 181.90 (10.33) | 193.65 (12.59) | 0.56 | 1, 21.3 | 0.46 |
| ROM | 1.77 (0.17) | 1.50 (0.23) | 0.90 | 1, 15.2 | 0.36 |
| Hematocrit | 0.30 (0.01) | 0.31 (0.02) | 0.10 | 1, 22.4 | 0.75 |
| Body mass (g) | 45.13 (2.76) | 36.91 (2.80) | 6.99 | 1, 25.8 | 0.014 |
| Tarsus length (mm) | 26.49 (0.78) | 24.40 (0.81) | 4.92 | 1, 27.6 | 0.035 |
| Feather length (mm) | 19.50 (2.82) | 14.09 (2.85) | 3.86 | 1, 23.2 | 0.062 |

Values represent model-estimated parameters from mixed models with nestbox identity as a random factor (TAC, ROM, hematocrit) or mixed models with nestbox and chick identity as random factors (body mass, tarsus length, feather length; all measurements taken at days 8 and 9 post-hatching). Data from two females that died before day 8 were not available. Degrees of freedom were estimated by the Kenward–Rogers method

## Discussion

In this study, we examined seasonal variation in offspring quality by subjecting first- and second-brood starling chicks to an immune challenge with LPS and evaluated their growth and physiological response in terms of plasma TAC, ROM and hematocrit. LPS challenge did not affect growth or physiological condition of both first- and second-brood chicks of either sex, with the single exception of hematocrit, that was higher among second-brood (but not among first-brood) LPS chicks compared to controls. As expected, we found that second-brood chicks were in poorer condition than first-brood ones, and such differences were sex-specific for body mass at days 8–9 post-hatching. Importantly, the most relevant findings were qualitatively unaltered when the analyses were restricted to the subset of chicks reared by the same mothers during both the first and the second brood, strongly suggesting that any brood-specific pattern we detected on the entire population did not originate from genetic differences between parents occupying experimental nestboxes early and late in the season.

### Seasonal decline in condition and antioxidant defences

Chicks from first and second clutches showed remarkable differences in most of the condition indices we measured. Halfway through the rearing period, chicks from first broods were heavier than those of second broods and grew at a faster rate. Moreover, hematocrit and TAC were significantly lower among second-brood chicks. These findings indicate that the nutritional conditions of starling chicks worsened in the course of the season between first and second clutches (see "Introduction"; López-Rull et al. 2011) and that this may impair functioning of the antioxidant barrier (Monaghan et al. 2009).

Although the use of hematocrit as a condition index is widely debated (Fair et al. 2007), our finding that hematocrit of second-brood chicks was smaller than that of first-brood ones may suggest that the former were in poor nutritional state, had higher parasite burden or both, as shown by some previous studies of wild nestling birds (e.g. Richner et al. 1993; Merino and Potti 1998; Potti et al. 1999; Simon et al. 2005). The decline of TAC between first and second broods we observed corroborates recent reports by Costantini et al. (2010) and Norte et al. (2009) of a seasonal decline in the capacity to resist oxidative stress among nestling birds. Antioxidant defences have an important environmental component (Costantini and Dell'Omo 2006a; Rubolini et al. 2006; Norte et al. 2009) and may thus reliably reflect ecological conditions to which nestling birds are exposed to. Indeed, the relevance of the rearing environment in determining chick oxidative status was recently highlighted by a study of starlings showing that TAC was lower among experimentally enlarged broods compared to reduced ones, but only in a poor year in terms of ecological conditions (Bourgeon et al. 2011). In addition, ROM levels, though not affected by the harshness of within-brood competition, were 45% higher in a poor vs. a good year (Bourgeon et al. 2011).

Several mechanisms may concur to originate a reduced antioxidant capacity of second-brood chicks: for example, it may be a direct consequence of seasonal changes of antioxidant availability in nestling diet (Catoni et al. 2008), it may result from seasonal changes in maternal effects via egg quality mediated by a decline in parental phenotype (Rubolini et al. 2006; López-Rull et al. 2010), or from the observed nutritional deficiencies of second-brood nestlings (as indexed by their lower body mass) (see also Monaghan et al. 2009) due to seasonally deteriorating ecological conditions. In addition, a lower TAC among second-brood chicks may depend on a higher parasite load compared to first-brood ones (López-Rull et al. 2010).

### Brood- and sex-specific growth patterns and mortality

Male chicks were larger than female ones, but among second-brood chicks the extent of sexual dimorphism was more pronounced compared to first-brood ones. Halfway through the rearing period, males were 4.7% heavier than

females in first-brood chicks, but 22.7% heavier in second-brood ones. Thus, under poorer rearing conditions, size dimorphism in favour of the larger sex was increased (see Oddie 2000 for a similar finding in *Parus major*). This pattern may originate from seasonal and sex-specific maternal investment in egg mass. Indeed, an analysis of body mass at day 1 of age (that closely mirrors egg mass; see Krist 2011) of a subsample of 42 chicks (see "Materials and methods") revealed that body mass at hatching varied according to the combined effects of sex and brood (mixed model with nestbox identity as a random factor, $F_{1,28.5}$= 8.97, $P$=0.006), males being smaller than females among first-brood chicks (post hoc test, $t$=−2.24, $P$=0.034) whereas the opposite was the case among second-brood chicks ($t$=2.11, $P$=0.045). Thus, between day 1 and day 9, the size advantage of first-brood females at hatching weakened and males became larger, whereas males remained larger than females between day 1 and day 9 among second-brood chicks. An ontogenetic shift of sexual size dimorphism among first-brood chicks is in line with previous findings documenting larger female vs. male eggs in first clutches of the closely related *Sturnus unicolor* (Cordero et al. 2001).

Among altricial offspring, body size during the pre-fledging period, mostly mirroring hatch order, is an important determinant of the success in scrambling competition for access to food (e.g. Price and Ydenberg 1995; Slagsvold et al. 1995; Cotton et al. 1999; Oddie 2000). Thus, starling mothers may provide daughters with an early size advantage in first clutches in order to promote their competitiveness against larger sons and thus enhance their fitness prospects. Indeed, among first clutches, where females were larger than males at hatching, size dimorphism halfway through the rearing period was far less pronounced than among second-brood chicks. Moreover, pre-fledging mortality of second-brood chicks was strongly female-biased, and body size of females that survived to fledging age was larger than those not surviving, suggesting that a body size advantage may have significant fitness consequences even during the pre-fledging stage in this species. A sex-biased mortality during the pre-fledging stage has been repeatedly shown in several bird species (see reviews in Råberg et al. 2005 and Uller 2006), but to our knowledge, a difference in sex-specific survival to fledging between first and second broods has never been previously reported.

In starlings, a larger investment in female vs. male offspring early but not late in the season may be adaptive since probability of recruitment and breeding of females during their first breeding season after hatching may depend on fledging date, early fledging females having a higher probability of breeding when 1 year old, as shown in a study of *S. unicolor* by Cordero et al. (2001). On the other hand, for males *S. unicolor* that do not breed during their first breeding season after hatching, the probability of recruitment to the breeding population was only positively related to their body mass at hatching (Cordero et al. 2001). Our findings are therefore consistent with maternal effects via egg mass or composition favouring daughters early in the season, but males later on (Cordero et al. 2001).

The causes of female-biased mortality among poor quality second-brood chicks require further scrutiny. Individuals of the larger sex (usually males) are regarded as being more susceptible than those of the smaller sex (usually females) to harsh environmental circumstances because of their greater energetic requirements during growth (Clutton-Brock et al. 1985). On the other hand, asymmetries in competitive abilities due to sex differences in body size may counterbalance and even outweigh male energetic penalties (Uller 2006). The balance between these two opposing forces determining offspring fitness may be resolved by parental decisions that can favour either smaller or larger chicks depending on fitness payoffs. In the first case, which is typical of many passerine species with limited hatching asynchrony, parents tend to equalize competitive asymmetries by adopting a so-called brood survival strategy (Slagsvold et al. 1984), whereby parents reduce competitive gaps by preferentially feeding smaller, less competitive chicks that beg more vigorously (Bonisoli-Alquati et al. 2011). On the other hand, under unfavourable environmental circumstances, parents may reduce provisioning of small, poor quality chicks of low reproductive value and invest more into high quality chicks that may have higher chances of surviving to maturity, a strategy that may lead to brood reduction (Clark and Wilson 1981; Slagsvold et al. 1984). The latter is what seems to happen with second-brood females that are likely to be of low reproductive value because they are smaller than males at hatching and throughout rearing, and suffer high pre-fledging mortality. It would be interesting to disentangle whether female-biased mortality of second-brood chicks occurred via parental discrimination favouring male offspring or intense sibling competition favouring the larger males (e.g. Cotton et al. 1999).

## Effects of LPS challenge on offspring phenotype

LPS challenge had no detectable effects on offspring growth and physiology of first-brood chicks, though it affected hematocrit of second-brood chicks. The dose injected was similar to that used in previous studies where it was shown to cause a rapid negative effect on chick growth (Grindstaff 2008; Romano et al. 2011) (but see Berthouly et al. 2008). These studies were, however, based on a larger sample size as compared to ours, and a reduced power in our study may contribute to explaining non-significant results. Although we cannot exclude that negative effects on body mass emerged after day 9, this seems unlikely since LPS did not affect chick body mass a few days before fledging (days 16–

17) in the subsample of birds that was remeasured at that age (details not shown). Thus, assuming that the lack of detectable effects of LPS on chick traits is not a mere consequence of the relatively small sample size (though sample size was much larger than that of previous studies investigating the oxidative costs of immune response, see Costantini and Møller 2009), European starlings may be able to sustain the challenge imposed to the immune system by a bacterial endotoxin by paying a relatively small energetic cost (either direct or indirect, see "Introduction"), at least until fledging, even among nutritionally stressed second-brood chicks. We might only tentatively speculate about the possible causes of such an apparently minor effect of LPS challenge on starling chick fitness. For example, starlings are among the bird species showing the highest prevalence of *E. coli* (Morishita et al. 1999), and transgenerational priming of the offspring immune system by transmission of maternal antibodies towards *E. coli* via the eggs might at least partly buffer the costs of mounting an immune response, as shown by Grindstaff et al. (2006) and Grindstaff (2008) in both wild pied flycatchers (*Ficedula hypoleuca*) and captive Japanese quails (*Coturnix japonica*), respectively. In addition, the European starling is a colonial and cavity-nesting species, both conditions leading to a larger size of primary immune defence organs according to comparative evidence (Møller and Erritzøe 1996) and might have thus evolved a highly efficient system of defence against pathogen exposure (Møller and Erritzøe 1996; Møller et al. 2009). A previous study also suggested that the evolutionary and ecological history of a population, such as intense past selection for resistance to bacterial attacks in the present case, could play a role in the apparent lack of short-term response to LPS challenge (see, e.g. Lee et al. 2005 for lack of response in *Passer domesticus* vs. strong response in *Passer montanus*).

The increased hematocrit among second-brood LPS chicks is difficult to interpret (Fair et al. 2007) but may be a consequence of the rapid metabolic changes induced by LPS challenge (e.g. dehydration following a febrile state or variation in metabolic rate). A raise in hematocrit may thus be a sentinel of the higher maintenance costs of responding to LPS challenge among poor-condition second-brood chicks, whose hematocrit was also significantly lower than that of first-brood ones. This is in line with the prediction that the effect of immune challenge was stronger among poor-condition second-brood chicks compared to prime condition first-brood ones.

Some previous studies, including a meta-analysis, showed that immune challenge may affect oxidative status in avian species (Bertrand et al. 2006; Costantini and Dell'Omo 2006b; Costantini and Møller 2009). However, the meta-analysis highlighted significant heterogeneity in effect sizes, likely resulting from population-, dose- or antigen-specific differences (Costantini and Møller 2009) (see, e.g. Alonso-Alvarez et al. 2004; Horak et al. 2006 for studies not showing significant effects). Alternatively, a lack of response may result from compensatory (up)regulation of the antioxidant system, whose costs may be paid up later in life. However, the lack of effect of immune challenge on oxidative status markers is consistent with observed lack of effect on body growth, further corroborating the idea that immune challenge with the dose and LPS serotype we injected may not impose detectable costs to nestling starlings, at least in the short-term.

In conclusion, our study revealed a generalized seasonal decline in fitness-related traits of starling chicks, whose consequences were more severe for the smaller female offspring, suffering higher prefledging mortality than males in second broods. This larger seasonal decline of daughters' fitness may be mediated by seasonal variation in sex allocation by mothers. Furthermore, our study indicates that nestling starlings may be able to sustain an immune challenge, even if not in prime conditions, by paying a relatively small cost, possibly because they have evolved a highly efficient response to pathogen attacks. Finally, since antioxidant capacity is known to predict long-term survival (Bize et al. 2008; Saino et al. 2011), we suggest that a seasonal decline of antioxidant activity should be regarded among the proximate mechanisms generating a decline of long-term survival and recruitment prospects among late-season nestlings of bird species.

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**Ethical standards** This research was undertaken (capture and experimental treatments) under the combined prescriptions of Art. 4 (1) and Art. 7 (5) of the Italian law 157/1992, which regulates studies on wild bird species.

**Conflicts of interest** The authors declare that they have no conflict of interest.

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