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Early exposure to a bacterial endotoxin advances the onset of moult in the European starling

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In animals, events occurring early in life can have profound effects on subsequent life-history events. Early developmental stresses often produce negative long-lasting impacts, although positive effects of mild stressors have also been documented. Most studies of birds have investigated the effects of events occurring at early developmental stages on the timing of migration or reproduction, but little is known on the long-term effects of these early events on moulting and plumage quality. We exposed European starling *Sturnus vulgaris* nestlings to an immune challenge to assess the effects of a developmental stress on the timing of the first (post-juvenile) and second (post-breeding) complete annual moult, the length of the flight feathers, and the length and colouration of ornamental throat feathers. The nestlings were transferred to indoor aviaries before fledgling and kept in captivity until the end of post-breeding moult. Individuals treated with *Escherichia coli* lypopolysaccharide (LPS) started both moult cycles earlier compared to control siblings. Moult duration was unaffected by the immune challenge, but an advanced moult onset resulted in a longer moult duration. Moreover, female (but not male) throat feather colouration of LPS-injected individuals showed a reduced UV chroma. We argue that an early activation of the immune system caused by LPS may allow nestlings to better cope with post-fledging stresses and lead to an earlier moult onset. The effect of early LPS exposure was remarkably persistent, as it was still visible more than one year after the treatment, and highlighted the importance of early developmental stresses in shaping subsequent major life-history traits, including the timing of moult in birds.

A life-history event can be influenced by the current condition of an individual that, in turn, may be affected by conditions experienced during earlier life stages. Such 'silver spoon' effects, originating when individuals developing under favourable circumstances accrues fitness advantages later in life, are regarded as major factors shaping inter-individual variations in fitness in natural populations (Grafen 1988, Cockburn 1991). Although they are probably pervasive, the effects of early experiences on fitness traits are hardly quantifiable under natural conditions due to the difficulties involved in: 1) assessing an instantaneous response to altered environmental conditions, as animals may immediately compensate for a stress (Metcalfe and Monaghan 2001); 2) following individuals over different life-history events; and 3) detecting the impact of early conditions on subsequent stages of life, as such effects often fade with time.

Most of the studies documenting the effects from early conditions refer to impacts on fitness caused by nutritional deficiencies, parasite infestations, or extreme temperatures during development (Buchanan et al. 2003, Gluckman et al. 2005, Ardia et al. 2010, Walker et al. 2013). Any perturbation during development may have deleterious consequences on growth, immune system, and the neural functions of juvenile individuals, which may then impair the subsequent phases of annual and/or life cycle events (Lindström 1999, Lummaa and Clutton-Brock 2002, Monaghan 2008). However, it has been suggested that mild adverse developmental conditions may also have positive effects during adulthood (Crino and Breuner 2015). Under this scenario, an early developmental stress can be regarded as a force that shapes the phenotype of a growing juvenile in order to best suit the environmental conditions that may be faced during adulthood (Dantzer et al. 2013). Several examples of long-lasting negative impacts from early stressors have been documented in birds, including increased adult metabolic rate (Criscuolo et al. 2008), limited capacities to store fat prior to migration (Mitchell et al. 2011), reduced immunocompetence (Rubolini et al. 2005), and reduced song performance (Buchanan et al. 2003). However, it has also been shown that nestling zebra finches *Taeniopygia guttata* supplemented with oral corticosterone increased their reproductive success in the first breeding season (Crino et al. 2014).

Surprisingly, to our knowledge, the effects of an early stressor on the timing of moult, a major life-history event in the avian life cycle (Payne 1972), have been poorly investigated so far. Since moulting feathers requires a considerable amount of time and energy (Dietz et al. 1992), moult generally does not overlap with any other phase of the avian life cycle (Ginn and Melville 1983). Hence, individuals tend to adjust their moult schedule according to the timing of other life-history events, as well as to their current condition. For example, birds performing prolonged parental care or that were born from late clutches tend to delay the onset of their moult (Bojarinova et al. 1999, Morrison et al. 2015). In a recent study, female barn swallows *Hirundo rustica* were forced to regrow one tail feather during the breeding period, and their regrowth rate was lower when the original feather was removed after clutch initiation (Saino et al. 2014). The same study also showed that regrowth rate and replacement feather length were negatively correlated to clutch size. A delayed moult onset is usually associated with a faster moult progression that allows for, at least partly, a compensation for the delayed moult onset and the ability to cope with the seasonal constraints imposed by reproduction and, in migratory species, migration schedule. However, such adjustments are costly: fast moulting may indeed result into poor quality flight feathers (Dawson 2004, Serra et al. 2010), and/or less coloured plumage ornaments (Griggio et al. 2009, Vágási et al. 2010).

Among early developmental stressors, there is good evidence that nest ectoparasites and bacterial infections produce harmful effects on nestlings, which may allocate their resources through a trade-off between immunity and growth (Christe et al. 1996, Saino et al. 1997, Cantarero et al. 2013). However, little is known about the long-lasting effects of these early developmental stressors on the subsequent life-history stages. In a previous study on starlings *Sturnus vulgaris*, we showed that nestlings grown in nests deprived of ectoparasites advanced their moult onset compared to nestlings fledged from naturally infested nests (Pirrello et al. 2015).

Various studies show that birds undergo several hormonal and behavioural alterations when exposed to lypopolysaccharide (LPS), an endotoxin extracted from the outer membrane of Gram-negative bacteria that elicits an immune response in the absence of a living pathogen (Bonneaud et al. 2003, Munoz et al. 2010, Casagrande et al. 2015). In the barn swallow, for example, nestlings injected with LPS showed a reduced growth rate, an increased occurrence of fault bars in growing feathers (Romano et al. 2011), and a lower breeding success (expressed as the ratio between paired and recruited males) in the following breeding season (Romano et al. 2014). In the house sparrow *Passer domesticus*, adult birds injected with LPS during the post-breeding moult showed a slower moult speed than controls (Moreno-Rueda 2010). In two studies on adult pied flycatchers *Ficedula hypoleuca*, LPS-injected males (Sanz et al. 2004) and females (Ilmonen et al. 2000) showed delayed moult onset and inhibited tail feather regrowth, respectively. Adult starlings injected with LPS reduced their song rate when exposed to a standard diet, but not when supplemented with carotenoids (Casagrande et al. 2015).

In a previous study we showed that LPS treatment did not affect nestling starling growth nor their oxidative status (Serra et al. 2012). We then decided to keep part of these starlings in captivity to investigate the long-term effects of early LPS exposure. We investigated the effects of LPS challenge on the timing of the first and second complete annual moult, on the length of wing and tail feathers (i.e. the nine long primaries and the outermost rectrix), and on the quality of ornamental throat feathers, as assessed by their colouration and length. At the end of the breeding season, both juvenile and adult starlings undergo a complete moult that lasts approximately 100 d (Pirrello et al. 2015) and that produces a melanin-based plumage with iridescent structural colours spanning from green to purple. The ultraviolet colouration of the throat feathers is sex-dependent (Cuthill et al. 1999) and is a cue used by females to rank males, as females prefer males showing throat feathers with higher UV chroma (Bennett et al. 1997). Despite starlings not paying any apparent cost for mounting an immune response against LPS at the nestling stage (Serra et al. 2012), we may expect costs associated with the early immune challenge to arise later in their life cycle. We therefore predicted that birds subjected to LPS would show a delayed moult onset, faster and shorter moults, thereby producing lower quality throat ornamental feathers (lower UV colouration, Doutrelant et al. 2012), and shorter wing and tail feathers compared to controls.

Material and methods

Field procedures and immune challenge

The starlings used in this experiment originated from a breeding colony situated in northern Italy (Ozzano Emilia, Bologna, 44°28′N, 11°29′E) during the 2010 breeding season. The colony consisted of 74 nest boxes of softwood panels (2 cm thick) with inside dimensions of 15 × 15 cm (base) × 45 cm (height) and an entrance hole size with a diameter of 4.5 cm (the distance of the hole from the base was 31 cm). Nest boxes were set up for the first time during early spring in 2009. In the study population, starlings laid two clutches per season consisting of two to nine eggs (Serra et al. 2012). Although we had information on repeated laying for only a minority of clutches, in keeping with our previous analysis we kept the distinction between the first- and second-clutch period (Serra et al. 2012). The mean laying date of clutches laid during the first and second clutch periods differed by more than 1 month and there was no overlap in egg laying dates (Serra et al. 2012). In the period 2009–2010, the mean clutch size was 5.1 ± 0.3 SE eggs in 26 first clutches, and 4.8 ± 0.1 SE eggs in 38 second clutches. In 2010, the nest box content was checked every 1–3 d during egg laying and every 1–2 d after hatching. First clutches (n = 8) were laid between 6 and 19 April, while the second ones (n = 14) were between 5 and 22 May. A few so-called intermediate clutches (n = 3) (Pinxten et al. 1990) were excluded from the experiment. The hatching date of a clutch was identified as the day of hatching of the first egg. However, the hatching date of each individual was taken into account for the analyses on moulting. At hatching, each nestling was marked with a coloured plastic tape on the left tarsus, and after a

week the right tarsus was ringed with an individual metal ring. At day 8 post-hatching (considering the hatching date of the clutch), half of the nestlings in a brood were subjected to an immune challenge with *Escherichia coli* LPS, whereas the remaining siblings were subjected to a control treatment. For example, in a brood containing four nestlings, two of them were injected with LPS and the remaining ones were used as control. In case of an odd number of nestlings, odd chicks were assigned at random to either treatment. Fifty µl of a solution of 1 mg lyophilized LPS powder (026:B6 serotype, L8274, Sigma-Aldrich) diluted in 1 ml phosphate-buffered saline (PBS) were injected intraperitoneally. Since the body mass of starling chicks at day 8 was 46.89 g ± 1.55 SE in LPS-injected (min 29.6 g, max 56.4 g) and 45.98 g ± 1.59 SE in control (min 30.1 g, max 60.2 g) individuals ($t_{50} = -0.41$, $p = 0.69$), the amount of LPS we chose to inject corresponds to ca 1 mg kg^{-1} body mass, similarly to doses used in some previous studies (Grindstaff et al. 2006, Berthouly et al. 2008, Romano et al. 2011), and that should induce a strong physiological response in small passerines (Sköld-Chiriac et al. 2015). Control siblings were injected with the same amount of PBS only.

On day 9, a blood sample of ca 150 µl was drawn from the brachial vein into microhematocrit capillary tubes and kept cool until processing. All the nestlings were successfully sexed using the method originally developed by Griffiths et al. (1998), that we have already described in a previous study on the same sample of individuals (Serra et al. 2012).

Pre-fledging mortality did not differ between LPS-injected and control siblings (Serra et al. 2012). In addition, body mass at days 15–18 did not differ between experimental groups (LMM of nestling body mass, effect of treatment: $F_{1,14} = 1.53$, $p = 0.24$). A total of 29 nestlings (15 LPS-injected and 14 control siblings from 10 and 9 nests, respectively) were randomly distributed among four indoor aviaries of 200 × 80 (base) × 200 cm (height) 2–3 d before fledging and kept in captivity for more than one year, i.e. until the end of post-breeding moult. Males (7 first-brood and 4 second-brood nestlings) and females (13 first-brood and 5 second-brood) were kept in separate aviaries, while preventing visual contact between the sexes. At this age (18 d), the nestling starlings are readily capable of feeding autonomously and can be maintained in the absence of the parents. The birds were provided with food and water ad libitum and kept under natural daylight. When moved to the aviaries, all the starlings were treated according to the manufacturer's instructions for coccidia (Aviochina, Sulfaquinoxaline 20%, doses of 1.7 ml l^{-1}), intestinal pathogenic bacteria (Humatin, Paromomycin 2.5 g, doses of 12 ml l^{-1}), and nematodes (Panacur, Fenbendazole 10%, doses of 2 ml l^{-1}). Each product was diluted in water and given ad libitum to the starlings for five days. The entire treatment consisted of two consecutive cycles of 15 d. All birds were kept in the aviaries for two complete moult cycles, i.e. during the post-juvenile (2010) and post-breeding (2011) moults, and were not allowed to reproduce during their first breeding season. During the post-juvenile moult, 4 birds (3 LPS and 1 control) died, whereas at the end of 2010, one LPS male escaped and one control female died. In summer 2012, all the remaining birds (n = 23) were released.

Moult recording

The timing and duration of the primary feather moult was assumed to be a proxy for the complete moult (body and flight feathers), because in passerines the moult of primary feathers generally extends over the entire moult period (Jenni and Winkler 1994). The moult progression of the nine long primary feathers was checked weekly. The vestigial 10th primary feather was excluded from the analyses. All the birds were individually captured from the aviaries to score their moult progression: old feathers were scored 0, new feathers 5, and growing feathers from 1 to 4 depending on their stage of growth (Ashmole 1962). The checking date when the first primary feather (= the innermost) was found to be shed, which is the first feather that starlings lose at the beginning of moult (Ginn and Melville 1983), was used to define the starting date of each individual's moult. Specifically, we subtracted six days from this checking date to obtain the starting date of moult, which therefore corresponds to the day after the previous checking date. The moult was regarded as completed on the day when all the primary feathers were fully grown. Because of the temporal delay of the hatching date between first and second broods (ca 1 month), a temporal delay in the starting date of the post-juvenile moult was also expected. On the other hand, the starting date of the post-breeding moult that occurs on average one year after hatching was expected to hardly be influenced by the hatching date. In order to compare the timing of moult between first- and second-brood birds in both their post-juvenile and post-breeding moult, we obtained a moult rank as follows. For each of the three temporally well-separated moult periods, i.e. the post-juvenile moult of first-brood individuals (starting between 4 and 17 June 2010), the post-juvenile moult of second-brood individuals (starting between 8 and 29 July 2010), and the post-breeding moult (starting between 21 May and 17 June 2011), we scored the day when the first individual started to moult as the day 1. The moult onset of the other individuals within each moult period was then ranked on the basis of this day (i.e. a first-brood individual that started moulting 3 d after the first-moulting individual in that moult period was ranked as day 4; a second-brood individual that started moulting 10 d after the first-moulting individual in that moult period was ranked as day 11).

Feather measurements

At the end of both moult cycles, from each individual we plucked the outermost right rectrix feather from the tail and ten feathers from the throat in correspondence to the left carpal joint. We measured the length of these feathers with a caliper with an accuracy of 0.1 mm. We also measured the length of the nine long primary feathers from the insertion point in the wing to the feather tip with a ruler with an accuracy of 0.5 mm. The sum of the length of the nine long primary feathers (hereafter referred to as 'total primary length') was assumed to reflect the individual effort in producing the new plumage. The throat feathers from each individual were collected in plastic envelopes and kept in the dark until the spectral analyses were performed.

Spectral analyses of throat feathers

We measured the spectral reflectance of the ten individual throat feathers and then averaged the resulting spectra for each individual. We followed this method because of its higher repeatability compared to that obtained by measuring overlapping feathers (Meadows et al. 2011).

The spectral reflectance was measured with an Ocean Optics S2000 spectrometer. We considered only the wavelengths between 300 and 700 nm, as they correspond to the visible spectrum of birds that includes the UV component. Each measurement was done in a dark room to avoid any disturbance from the ambient light. The light optical-fiber probe was held at a 90 degree angle at a distance of 5 mm from the feather surface through a PVC tube mounted on the ferrule tip. The reflectance spectra were recorded with the Spectrasuite OOIBase32 software with standards set to a WS-2 white standard and dark before each measurement session. Meanwhile, we took the measurement of an individual throat feather, and the spectrometer automatically averaged five consecutive readings of the same location. Hence, the resulting spectrum for each individual corresponded to the average of 50 spectra.

In the European starling, females prefer males displaying throat feathers with higher reflectance in the ultraviolet range (Bennett et al. 1997). Hence, we calculated the proportion of reflectance between 300 and 400 nm with respect to total reflectance (UV chroma = $R_{300–400}/R_{300–700}$) (Griggio et al. 2010).

Statistical analyses

Linear mixed models were used to test the effect of the immune challenge, sex, and moult cycle on the timing of moult (onset and duration), the colouration of the throat feathers, the mean length of the throat feathers, the total primary length (sum of the length of the nine long primaries), and the length of the outermost rectrix feather. Immune challenge (0 = control, 1 = LPS), sex (1 = male, 2 = female), and moult cycle (1 = post-juvenile moult, 2 = post-breeding moult) were considered as explanatory fixed factors. The two-way interaction terms were initially included in the model and were subsequently dropped if not statistically significant ($p > 0.05$). Bird identity nested within brood identity was included as random intercept effect to account for repeated measures on the same individual in the two moult cycles, and for the non-independence of data from individuals fledged from the same brood, respectively. In the model of moult onset, we also included the individual hatching date as a fixed-effect covariate, because birds that hatch later are expected to begin the moult at a younger age (Bojarinova et al. 1999). In the model of moult duration, the moult starting date was included as a covariate, because birds whose moult starts later usually moult faster (Morrison et al. 2015). Finally, we tested for an effect of body size on total primary length by including tarsus length as a covariate in the model; however, since the effect of this covariate was not significant ($p = 0.60$) we removed it from the final model.

All the analyses were performed using the software R 3.0.3 (R Development Core Team). Mixed models were fitted using the 'lme' function of the R package 'nlme' (Pinheiro and Bates 2000). Post hoc pairwise comparisons were computed using the 'lsmeans' function of the R package 'lsmeans' (Lenth and Herv 2015). Since moult onset showed significant heteroscedasticity between moult cycles (Bartlett's test, $p = 0.009$), we included in the mixed models a variance structure which allowed for different variances according to moult cycle (Zuur et al. 2009). All other checking for homoscedasticity (Bartlett tests), normality of residuals (Shapiro–Wilk tests), and the plot of residuals against predicted values and against each explanatory fixed factor suggested an acceptable fit of all models (details not shown).

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.c3g3b> (Pirrello et al. 2016).

Results

Onset and duration of moult

LPS-injected birds started to moult earlier than control individuals during both post-juvenile and post-breeding moult, respectively (Table 1, Fig. 1a, b). The effect of the immune challenge on the moult onset was also statistically significant when running models on each moult cycle separately (effect of the immune challenge; post-juvenile: $–4.0 \pm 1.8$ SE, $F = 4.88$, $DF = 1, 14$, $p = 0.044$; post-breeding: $–7.4 \pm 3.2$ SE, $F = 5.21$, $DF = 1, 10$, $p = 0.046$), whereas the effect of sex was statistically significant only in the post-breeding moult (effect of the sex; post-juvenile: 2.1 ± 2.0 SE, $F = 1.10$, $DF = 1, 14$, $p = 0.31$; post-breeding: 8.7 ± 3.7 SE, $F = 5.61$, $DF = 1, 10$, $p = 0.039$). The onset of the post-breeding moult was more delayed on average than the onset of the post-juvenile moult (Table 1, Fig. 1a). Hatching date did not predict differences in the moult onset between moult periods, and this suggests that first and second brood birds started both moults rather synchronously (Table 1). The two-way interaction between moult cycle and hatching date was not statistically significant and was removed from the final model (details not shown).

Moult duration was unaffected by the immune challenge and did not differ between the sexes in both moult cycles (Table 1). Moult duration was significantly associated with the starting date of moult: as expected, birds that either fledged or began moulting later performed a shorter moult (i.e. they accelerated their moult rate). After statistically controlling for moult starting date, the post-juvenile moult was significantly longer than the post-breeding moult (moult duration; post-juvenile: 99.9 ± 1.4 SE; post-breeding: 95.5 ± 1.5 SE; Table 1).

Feather length

The immune challenge did not affect the length of throat feathers nor the length of the outermost rectrix, but differentially influenced the total primary length according to sex as revealed by the significant interaction between immune challenge and sex (Table 1). In particular, control males produced longer primary feathers than both LPS-injected (post hoc tests, $p = 0.049$) and control females ($p = 0.015$). Although the difference was not statistically significant (post

Table 1. Mixed models of the effects of the immune challenge, sex, and moult cycle on the onset and duration of moult, on the UV chroma and length of the throat feathers, on the total primary length, and on the length of the outermost rectrix. See Material and methods for details on the statistical procedures. Estimates are differences between LPS-injected vs control birds, females vs males, and post-breeding vs post-juvenile moult.

Variables	Estimate ± SE	DF	F	p
Moult onset				
Immune challenge	–4.6 ± 1.6	1, 14	8.09	**0.013**
Sex	3.2 ± 1.9	1, 14	2.87	0.11
Moult cycle	9.4 ± 2.0	1, 22	21.5	**<0.001**
Hatching date	–0.11 ± 0.06	1, 14	4.02	0.06
Moult duration				
Immune challenge	–0.5 ± 2.1	1, 12	0.05	0.83
Sex	–0.3 ± 2.2	1, 12	0.02	0.90
Moult cycle	–4.4 ± 2.0	1, 21	4.66	**0.043**
Moult starting date	–0.4 ± 0.1	1, 21	23.2	**<0.001**
Length of throat feathers				
Immune challenge	–0.6 ± 0.5	1, 12	1.39	0.26
Sex	–1.2 ± 0.5	1, 12	5.48	**0.037**
Moult cycle	3.6 ± 0.4	1, 21	78.9	**<0.001**
Total primary length				
Immune challenge	–2.3 ± 0.9	1, 11	6.40	**0.028**
Sex	–2.9 ± 0.8	1, 11	13.9	**0.003**
Moult cycle	0.6 ± 0.1	1, 19	19.6	**<0.001**
Sex × immune challenge[a]		1, 11	6.92	**0.023**
Length of the outermost rectrix				
Immune challenge	0.1 ± 0.5	1, 12	0.03	0.86
Sex	0.3 ± 0.5	1, 12	0.22	0.65
Moult cycle	0.8 ± 0.2	1, 22	13.3	**0.001**
UV chroma				
Immune challenge	0.011 ± 0.008	1, 9	1.70	0.22
Sex	0.010 ± 0.006	1, 9	2.56	0.14
Moult cycle	0.030 ± 0.003	1, 22	95.8	**<0.001**
Sex × immune challenge[b]		1, 9	7.59	**0.022**

[a]Control males have longer primaries than LPS (post hoc tests, p = 0.049) and control females (p = 0.015). Differences between experimental groups are shown in Fig. 2a.
[b]Control females have higher UV chroma than LPS females (post hoc tests, p = 0.017). Differences between experimental groups are shown in Fig. 2b.

hoc tests, p = 0.11), control males showed on average longer primary feathers than LPS-injected males (Fig. 2a).

The mean length of throat feathers, the total primary length, and the length of the outermost rectrix significantly increased from the first to the second moult cycle, i.e. the feathers produced during the post-breeding moult were longer than those produced during the post-juvenile moult (Table 1, Fig. 2a).

Throat feathers colouration

The UV chroma of throat feathers significantly increased from the first to the second moult cycle (Table 1, Fig. 2b). The immune challenge had a differential effect on UV chroma according to sex as indicated by the significant interaction between immune challenge and sex (Table 1). Specifically, the throat feathers of LPS-injected females showed a significantly lower UV chroma compared to control females (post hoc tests, p = 0.017), while no significant difference in the UV chroma of throat feathers emerged between LPS-injected and control males (p = 0.57) (Fig. 2b).

Discussion

We showed that an immune challenge at the nestling stage had a positive effect on the temporal pattern of the first (post-juvenile) and second (post-breeding) complete annual moult in the European starling. Contrary to predictions, the moult onset of birds injected with LPS was significantly earlier than that of control siblings. The duration of moult was unaffected by the immune challenge, but was positively associated with the starting date of the moult, as individuals that fledged and began moulting earlier showed a prolonged moult. In addition, LPS treatment decreased the UV chroma of throat feathers in females only.

In our study, an early exposure to LPS, despite not having any detectable effect on nestling phenotype during development (Serra et al. 2012), may have enforced the nestlings' immune system and positively influenced their response to adverse environmental condition. At the nestling stage, for example, the pathogenic microorganisms that live in the nest material may have detrimental consequences for nestling development. Bird parents usually limit the proliferation of parasites and pathogenic bacteria in their nest through sanitation behaviour (Clayton et al. 2010), such as the addition of green material or aromatic plants with antibacterial properties (Clark and Mason 1985, Mennerat et al. 2009). Bacterial infections may also be transmitted by ectoparasites (Shewen 1980). Our starling colony was highly infested by nest ectoparasites whose negative effects on the offspring have already been reported in several bird species (Christe 1996, Tomás et al. 2008, Cantarero et al. 2013). The dominant

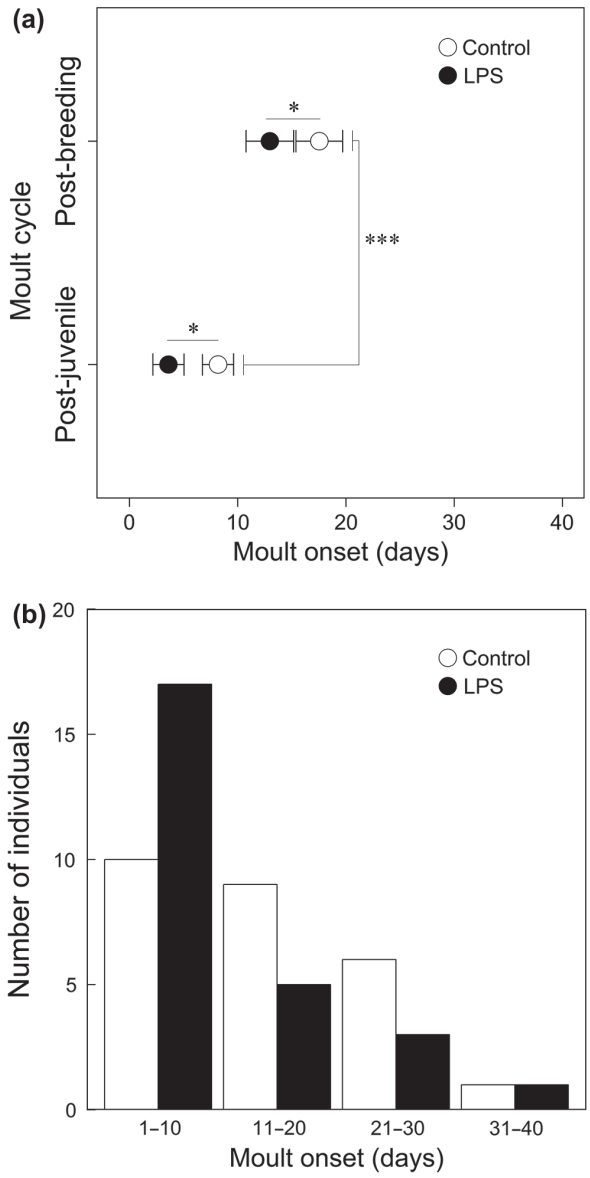

Figure 1. Onset of the (a) post-juvenile and post-breeding moult of individuals injected with either an endotoxin (LPS) or a saline solution (Control) during the nestling stage. In (b) the number of LPS-injected and control individuals divided by classes of moult onset are shown. The moult onset is calculated as days from the onset of moult of the first individual during each of three distinct moult periods (moult of first-brood individuals and second-brood individuals in the post-juvenile and post-breeding moult; see Materials and methods for details). The dots show the estimated marginal means ± SE from the mixed model shown in Table 1. Asterisks denote significant difference (* $p < 0.05$, *** $p < 0.001$).

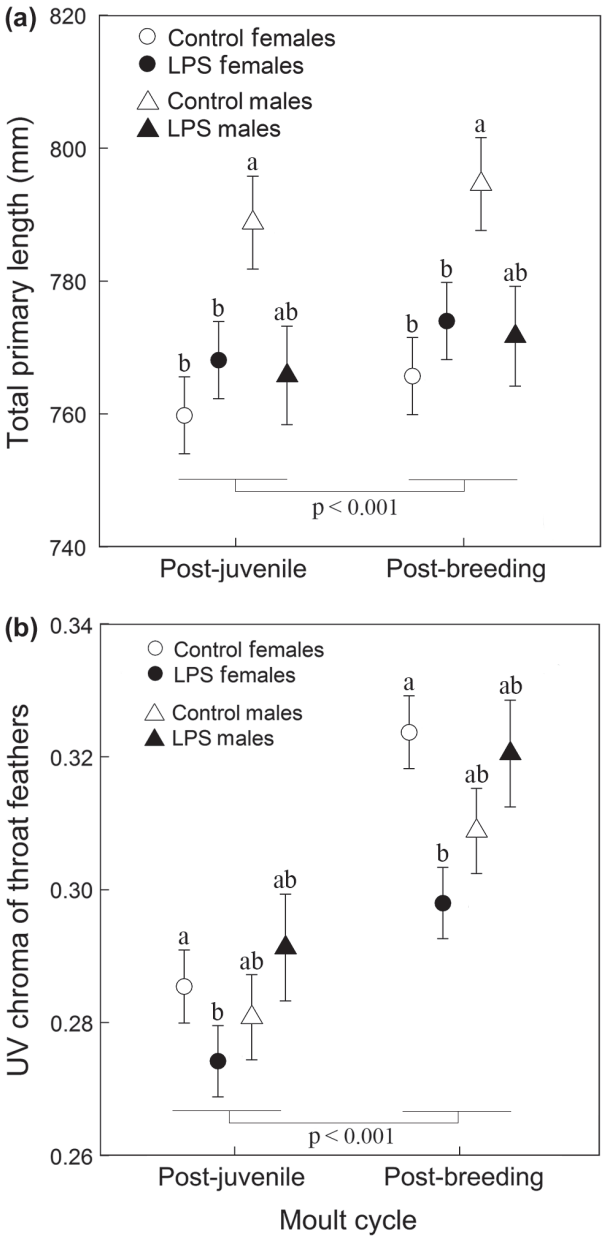

Figure 2. (a) Total primary length (sum of the length of the nine long primaries) and (b) UV chroma of the throat feathers produced in the post-juvenile and post-breeding moult in males (triangles) and females (circles) of the LPS-injected (black) and control (white) groups. The symbols show the estimated marginal means ± SE from the mixed model shown in Table 1. Different letters denote statistically significant ($p < 0.05$) differences at post hoc tests.

ectoparasite species in starling nests is the hematophagous fly *Carnus hemapterus* (Liker et al. 2001). It is plausible that this fly acts as a vector of viral and bacterial infections that are transmitted via the blood meals (Thomas and Shutler 2001). As a consequence, an enforced immune system may have improved the condition of newly fledged individuals, which could therefore anticipate the energetically costly process of moulting. Earlier moulting may improve feather quality and entail potential benefits for survival and reproductive success (Nilsson and Svensson 1996). Furthermore in migratory species, an earlier timing of moult allows individuals to perform a more efficient fuel accumulation, possibly leading to an earlier departure for migration or allowing migration with larger fuel stores (Lindström et al. 1994, Rubolini et al. 2002). Specifically, an earlier end of moult is expected to translate into an earlier onset of migration. This is corroborated by the observations that moult duration did not differ between groups and that the date of moult completion was (non-significantly) earlier in LPS compared to controls by ca 4 d (LMM of date of moult completion,

effect of treatment: –4.2 ± 2.6 SE, F = 2.59, DF = 1, 11, p = 0.136; other details not shown for brevity).

Many studies reported negative fitness effects of early developmental stressors (see Introduction), though a few documented positive effects (Moret and Siva-Jothy 2003). Others showed long-term impacts that are difficult to assess either positively or negatively (Andrews et al. 2015, O'Hagan et al. 2015, Kim et al. 2016). Among the studies reporting long-lasting positive effects, one showed that the larvae of the mealworm beetles *Tenebrio molitor* exposed to LPS increased their resistance against a fungal parasite at the adult stage (Moret and Siva-Jothy 2003). In contrast, male three-spined sticklebacks *Gasterosteus aculeatus* developed under harsh conditions increased their investment in carotenoid-based sexual signals which entails positive effects on fitness, but also showed a fast senescence rate (Kim et al. 2016). In starlings, an increased sibling competition, despite not having any detectable effect on nestling growth, caused alterations in flight performance and feeding behaviour later in life (Andrews et al. 2015, O'Hagan et al. 2015). In our study, the costs of early immune activation associated with LPS exposure were not detectable at the nestling stage (Serra et al. 2012), and the earlier onset of moult in LPS-injected individuals may be regarded as a beneficial effect caused by the early immune stimulation, although possible negative effects also occurred.

Our study also highlighted that the onset of moult was earlier in males vs females, as already shown in other bird species (Newton and Rothery 2005, Flinks et al. 2008, Serra et al. 2010). Hence, we provided evidence that in a species with biparental care, even if reproduction is inhibited, there are likely intrinsic factors (e.g. hormones) that lead to sexual difference in moult onset. Such a difference is likely to reflect an adaptive differentiation of moult timing between the sexes, whose difference in hormonal cycles (Dawson 1997) may lead to differential effects on timing and onset of moult. Moreover, we showed a significant sex difference in total primary length in the control group only, which suggests that LPS may have caused negative consequences on males but not on females in spite of inducing an earlier moult onset in males.

We found interesting age-related changes in the ornamental feather quality. In fact, the UV chroma of throat feathers, a cue used by females to rank potential partners (Bennett et al. 1997), was higher in plumages produce in the post-breeding moult. Hence, the immune challenge had a possible negative effect on the structural coloration of throat feathers in females only, as LPS-injected females showed a lower UV chroma than controls, while the UV chroma of male throat feathers appeared less susceptible to LPS. It is not clear whether differences in the UV chroma of throat feathers have any fitness consequence in females. We can only speculate on the reasons why the immune challenge had an (apparently) negative effect on the structural colouration only in females. The sex difference may be the result of a compensation in males for an ornamental trait whose expression probably has greater fitness consequences for males than for females. Indeed, LPS-injected males produced slightly shorter primary feathers (although the difference was not statistically significant) than control males, so we could hypothesise that males subjected to the immune challenge traded-off primary feather growth with throat feather quality. However, we found a statistically significant positive correlation between total primary length and UV chroma in LPS-injected males (LMM, F = 58.1, DF = 1, 2, p = 0.017) but not in control ones (LMM, F = 0.12, DF = 1, 4, p = 0.75). This further suggests that our immune challenge did not represent a cost for starlings, at least not for males, and that they instead obtained potential fitness advantages due to having an early immune activation at the nestling stage in terms of earlier moult onset.

The effect of our immune challenge on moult onset may also be explained by hormetic effects. Hormetic responses occur when potentially toxic agents exert positive fitness effects at low to intermediate doses, while lowering fitness at higher doses (Stebbing 1982, Costantini et al. 2010). The exposure to low doses of a toxin, instead of being detrimental for an organism, may increase its ability to cope with exposure to higher doses of the toxin that may occur later in life (Costantini et al. 2010). Additional studies whereby larger LPS doses are used and result in clearly negative fitness effects are required to provide evidence of hormetic responses.

In conclusion, an early immune challenge that did not negatively affect the nestling phenotype (Serra et al. 2012) lead to an early onset of the post-juvenile and post-breeding moult, possibly providing fitness advantages in LPS-injected individuals. Male throat feathers, playing an important role in mate choice, were not affected by LPS treatment either in the post-juvenile or in the post-breeding moult. Our immune challenge decreased the UV chroma of throat feathers of females, and whether this entails negative fitness consequences requires further study. Overall, our study corroborates the idea that early developmental stressors (i.e. exposure to a toxin) can have long-lasting effects on offspring performance, some of which can be positive, rather than negative (Crino et al. 2014).

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