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Condition dependence of sexually selected signals in the European Starling (*Sturnus vulgaris*)

Condizione dipendenza dei segnali selezionati sessualmente in Storno (*Sturnus vulgaris*)

Direttore della Scuola: Prof. Giuseppe Zanotti

Coordinatore d'indirizzo: Prof. Andrea Augusto Pilastro

Supervisore: Prof. Andrea Augusto Pilastro

Dottorando: Simone Pirrello

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List of manuscripts

The thesis includes one published paper (manuscript 1) and six unpublished papers (manuscripts 2-7)

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

Lorenzo Serra, Simone Pirrello, Manuela Caprioli, Matteo Griggio, Alessandro Andreotti, Andrea Romano, Andrea Pilastro, Nicola Saino, Roberto Sacchi, Paolo Galeotti, Mauro Fasola, Fernando Spina, Diego Rubolini

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2. **Early exposure to a bacterial endotoxin advances moult timing in the European starling**

Simone Pirrello, Andrea Pilastro, Alessandro Andreotti, Fernando Spina, Diego Rubolini, Andrea Romano, Nicola Saino, Matteo Griggio, Lorenzo Serra

3. **The removal of nest ectoparasites increases the parental effort during the incubation period in the European starling**

Simone Pirrello, Andrea Pilastro, Caterina Suzzi, Alessandro Andreotti, Lorenzo Serra

4. **Nest ectoparasites decrease the nestling begging intensity in the European starling *Sturnus vulgaris***

Simone Pirrello, Diego Rubolini, Andrea Pilastro, Matteo Pozzato, Nicola Saino, Lorenzo Serra

5. **Nest-dwelling ectoparasites influence the start and duration of the first pre-basic moult in the European starling (*Sturnus vulgaris*)**

Simone Pirrello, Andrea Pilastro, Lorenzo Serra

*-- **Submitted to Journal of Avian Biology** --*

6. **A supplement of carotenoids during the post-juvenile moult increases the preening time of male European starlings in winter**

Simone Pirrello, Andrea Pilastro, Lorenzo Serra

7. **Male juvenile condition influences female choice in the European starling *Sturnus vulgaris***

Simone Pirrello, Andrea Pilastro, Andrea Brunelli, Lorenzo Serra

Abstract

Since Darwin, evolutionary biologists envisaged various hypotheses to explain the advantages for males to exhibit exaggerated secondary sexual traits, also known as ornaments. One of these hypotheses proposes that male ornaments may signal to the females the benefits they can get by mating, and that female preference for ornamented males is one of the main selecting forces explaining the evolution of secondary sexual traits. The evolutionary scenario becomes more complex if we consider that in many species males bear multiple ornaments. Why multiple ornaments should evolve? Why and how do females base their choice among different male traits? Different ornaments could signal different aspects of male quality (=benefits). For example, individual condition at different stages of male's life may by reflected by the expression of different ornaments, or diverse ornaments could respond differently to a similar stress experienced during a male life-history event due, for example, to a different response to carry-over effects. Condition dependence of male ornaments has been supported by a large number of studies on sexual secondary traits, yet whether and how different stresses at different life-history stages affect the expression of multiple ornaments has been investigated in a more limited number of cases. In my PhD project I used a passerine bird, the European starling (*Sturnus vulgaris*), as a model species given that males bear multiple secondary sexual traits. In this species it has been shown that females prefer males with long throat feathers that have high UV reflectance, that produce a complex song and, although with less empirical support, with more brightly colours on the beak. During my PhD I attempted to investigate the mechanisms that underlie the expression of male ornaments by experimentally manipulating individual condition during two energy-demanding life-history events, i.e. during nestling development and during the post-juvenile moult. Male attractiveness was directly evaluated in a female choice experiment. The first experimental manipulation aimed at investigating the effect of an immune challenge on growth and physiological responses on nestlings that were naturally infested with ectoparasites (manuscript 1), and to assess the temporal pattern of their first- and second-year moult (manuscript 2). The second experimental manipulation was used to test the effect of removing the stress caused by the nest ectoparasites on parental investment during the incubation

(manuscript 3), and on parent-offspring communication during the nestling stage (manuscript 4). I then examined the temporal pattern of the post-juvenile moult in males from ectoparasite-free and naturally infested nests (manuscript 5). A third experimental manipulation consisted of supplementing with or depriving of carotenoids the diet of males in the course of their post-juvenile moult. I further tested the effect of nest ectoparasites and diet i) on the preening activity of males during winter, i.e. from the end of the moult to the start of the breeding period (manuscript 6), ii) on the expression of male ornaments and iii) on female preference (manuscript 7). My results suggest that an early stress, i.e. endotoxin or ectoparasites, does not significantly affect the nestling growth. Nestlings whose immune system was challenged with an endotoxin (LPS) showed similar antioxidant capacity and oxidative damage to those measured on control nestlings, whereas hematocrit was higher in second-brood LPS-nestlings than controls. Nestlings in ectoparasite-free nests produced a more conspicuous postural begging than that produced by nestlings in naturally infested nests. Parents were also sensitive to nest ectoparasites because they increased the time spent at the nest during the incubation period, although the provisioning effort during the nestling period was not different among deparasitized and control broods. Moult was advanced in birds injected with LPS and in those grown without nest ectoparasites, as they were probably in better condition than their counterparts and could therefore anticipate such costly life-history event. Birds from ectoparasite-free nests moulted over a longer period than controls, whereas moult duration of LPS and control birds was similar. The supplement of carotenoids during moult resulted in an increase of time invested in preening their plumage a few months after the end of the moult, and showed an increased yellow colouration of the beak in the following breeding season. As result, males that were grown in ectoparasite-free nests and were supplemented with carotenoids during the following moult were preferred by females in the mate choice experiment.

In conclusion, the results of my PhD project provided an experimental evidence of carry-over effects in starlings. Males that experienced different stresses during the early stages of their life were less attractive for the females in their first breeding season. While I found a different colouration of the beak

among birds from ectoparasite-free and control nests, the throat feathers were of similar length and colouration. This difference in attractiveness could be due to song characteristics during mate choice tests. Any attempt to individually record male song unfortunately failed and the effect of early stresses on song could not be assessed directly. Despite this, the resulting female preference for males that experienced favourable juvenile conditions suggests that male ornaments are considered as a whole during mate choice, and their expression is probably influenced by carry-over effects. These findings seem therefore in agreement with the redundant signal hypothesis, which suggests that female integrate the information of all male ornaments to assess the mate quality.

General introduction

The theory of evolution through natural selection proposed by Charles Darwin in 1859 has been largely recognized as the force which explains much of the natural phenomena. Natural selection explains why within a population some individuals survive better than others. For this reason, this theory does not explain, or even contrasts, the evolutionary advantage to bear exaggerated and conspicuous structures that apparently reduce survival. In the book "*The Descent of Man, and Selection in Relation to Sex"* (1871) Darwin introduced the notion of sexual selection, which integrated the theory of natural selection, as the process that drives the evolution of secondary sexual traits (ornaments and armaments) as they increase the likelihood to mate and hence to reproduce. Fitness ultimately depends on the number of descendants produced by an individual. Antlers, bright colourations, songs, elaborated displays, odours are examples of secondary selected traits that enhance access to mates and reproduction, and they can be used in intra-sexual competition or in inter-sexual selection, or in both (Berglund et al. 1996).

In most animals, male is usually the sex that competes to have access to the other sex (female), because of the initial asymmetry in reproductive investment associated with anisogamy (Bateman 1948). When males cannot control directly the females and male-male contests do not solely determine male mating success, exaggerated, arbitrary male ornaments have evolved under female choice (Andersson 1994). Why females do not choose their mate indiscriminately and instead tend to prefer males expressing the most elaborated sexual traits is one of the most intriguing evolutionary question since Darwin. Among the several hypothesis proposed, females exert their mate preference by evaluating the male ornaments whose evolution depends upon three different processes, i.e. a sensory exploitation process(Ryan and Rand 1990), Fisherian runway process (Fisher 1930), and the handicap's hypothesis (Zahavi 1975). Clearly these hypotheses are not mutually exclusive. The sensory exploitation hypothesis predicts that a male trait is preferred by females as its expression exploits pre-existing female preference (i.e. a naturally-selected female preference). The Fisherian process, also called "sexy sons" hypothesis, yields genes for female preference for a male

trait to be genetically correlated to genes for the expression of that trait. Such ornaments thus evolve solely because of their reproductive advantage of male offspring, and their expression is not associated to male qualities other than his attractiveness to females. The handicap's hypothesis, also called "good genes" hypothesis, posits that ornaments are costly to be produced and maintained. Their expression will therefore reflect male quality (i.e. his general condition) and females, by choosing the most ornamented males among the prospecting partners will select the male in better condition. This is because males in better condition pay a smaller unitary cost for ornamentation and can therefore produce larger ornaments (Getty 1998, 2006). Hamilton and Zuk specifically proposed that females can assess the degree of past or current parasitic infection of males through the quality of their ornaments (Hamilton and Zuk 1982). An individual's condition is ultimately determined by the resources available for maintenance and reproduction, which are correlated with his genetic quality and his capability to withstand the environmental challenges that occurred i) during development (i.e. before ornaments are expressed) (Spencer and MacDougall-Shackleton 2011), ii) at the time when the ornaments are produced (Hill 1991, Scheuber et al. 2004, Martín and López 2010), and iii) after their production if the ornaments are costly to maintain (Griggio et al. 2010a, Hill 2011). Therefore, condition-dependent ornaments could potentially convey different types of information to females on individual conditions of prospecting partners.

Often males bear multiple ornaments, which may reflect past or current individual conditions, depending on the nature and ontogeny of the ornaments (Andersson 1994, Hill 2011). For example, colour ornaments in birds are often produced in different and temporally distinct periods of a male's life: colour feathers are usually formed several months before the breeding season, and once completed they show a limited phenotypic flexibility (but see Ornborg et al. 2002, Zampiga et al. 2004, Delhey et al. 2007, Griggio et al. 2010). In contrast, the colouration of bare parts such as skin and beak is usually expressed just at the beginning of the breeding season and it is typically a dynamic trait whose expression is mainly related to current individual conditions (e.g. Faivre et al. 2003). Consequently, it has been suggested that females may rely on different

ornaments to evaluate past and current condition of prospective partners (Hill 1991, Scheuber et al. 2004).

Multiple signals hypotheses

There are five different hypotheses that aim to explain the evolutionary advantage for males to invest in multiple ornaments (Candolin 2003, Lozano 2009). Two alternative hypotheses, i.e. the multiple message hypothesis (MMH) and the redundant signal hypotheses (RSH), are based on the assumption that the evolution of multiple ornaments is driven by female choice. The MMH states that different ornaments either convey information regarding different aspects of male quality or reflect male condition at different time scales (Møller and Pomiankowski 1993, Scheuber et al. 2003, van Doorn and Weissing 2004, Freeman-Gallant et al. 2010). The RSH, also called the backup hypothesis, posits that each ornament provides only a partial piece of information regarding the overall condition of an individual; thus females should rely on multiple ornaments to obtain a more honest representation of male condition (e.g. Alonso-Alvarez and Galván 2011). The unreliable signal hypothesis (USH) proposes that males used multiple signals to exploit pre-existing female preference, and therefore their evolution is explained by a Fisherian runaway process (Møller and Pomiankowski 1993). The chase-away sexual selection hypothesis (Holland and Rice 1998) assumes an antagonistic evolution of the multiple signals, whereby, at an evolutionary scale, males continuously introduced new signals to enhance their attractiveness, while females continuously show resistance to formerly effective signals to elicit the evolution of new signals in males. The most recent hypothesis, i.e. the interference hypothesis (IH), suggests that males evolve new signals to interfere with female choice and, consequently, to reduce female selectivity. Despite males pay a relevant cost for ornamentation they favour the evolution of multiple honest signals (Lozano 2009).

Carry-over effects

Individual's life-history includes a series of temporally separated events. Each event requires a certain amount of energy and time to be accomplished. Whenever one event affects subsequent events, individuals undergo the so-called "carry-over effects" (Lindström 1999, Harrison et al. 2011). This key concept can be reformulated to say that any stress that occurs during the life of an individual is likely to produce downstream consequences. Carry-over effects contribute to explain a significant part of the fitness difference among individuals (Harrison et al. 2011), although they are rather difficult to be measured in the wild for the difficulty to follow an individual during his different life stages. The recent development of animal tracking technologies are helping to overcome this issue and to find out that early life-history stress can effectively influence future individual performance (e.g. Mitchell et al. 2011). Another issue concerning the assessment of carry-over effects lies in the fact that organisms might instantaneously withstand the stress that occur during their life through compensatory responses (Metcalfe and Monaghan 2001). These compensatory responses could make negligible the performance difference among individuals for a certain life-history event, while their costs could be paid later in life (Lindström 1999, Metcalfe and Monaghan 2001, Lummaa and Clutton-Brock 2002).

Multiple ornaments can be produced at different times of the annual cycle. If they are condition dependent, their expression is linked to individual condition at the time when the ornaments are produced. Individual condition at any moment of organism's life, however, might be affected by individual condition experienced at earlier stages of life. An early stress could therefore influence the expression of ornaments (Spencer and MacDougall-Shackleton 2011). However, only recently the possibility that multiple ornaments reflect condition at different stages of male's life has started to receive attention (Walker et al. 2013).

Aim of the thesis

My PhD project aims at investigating which mechanisms underlie the expression of secondary sexual traits (SSTs) in the European starling (*Sturnus vulgaris*), that is a passerine bird in which males exhibit multiple ornaments whose expression is separated temporally and which are likely to be condition dependent. In the three years of my PhD project, I have conducted three experimental manipulations to investigate whether individual conditions during the main life-history events of males' life influence the expression of SSTs and the female choice. In particular, I performed two experimental manipulations at the nest and one during the post-juvenile moult to simulate three early stresses that could be experienced by males during two crucial phases of their development, which are likely to have consequences on their current and subsequent condition.

Collectively, I attempted to answer the following questions: are different SSTs influenced by stresses occurring at distinct life stages? Are they equally affected by early and current condition? Is the expression of SSTs influenced by carry-over effects due to early stresses? Is the female preference effectively influenced by such traits?

Study species

The European Starling is a facultative polygynous species that breeds in natural or artificial holes and cavities (Feare 1984). I used a nest-box breeding colony situated at Ozzano Emilia (Bologna), where starling pairs occupied the nest boxes for the first time in 2009. The arrival of the breeding pairs at the colony generally occurred from the end of February, but the nests started to be occupied from the end of March onward. The nests were abandoned around mid July. Female starlings lay 3-8 eggs in sequence, usually separated one another of about 24h. Eggs are incubated mainly by females, even though males can contribute to preserve the egg temperature when females leave temporally the nest. Hatching occurs 11-15 days after clutch completion, and nestlings fledge at about 21 days after hatching. Each breeding pair produces one, two, and exceptionally three depositions per season. A female that fails the first breeding

attempt at the beginning of the breeding season could produce a replacement clutch a few days after the failure of the first clutch. Polygyny can occur, and in these cases males contemporary attend more than a clutch.

Starling nests are infested by several ectoparasite species, but the dominant one is the carnid fly *Carnus hemapterus* that lives inside the nest material and feeds on blood of nestlings and adults (Liker et al. 2001), representing one major stress for growing nestlings and breeding adults. The presence of these flies in the nests can be indirectly detected from the secondary spottiness of eggshells (López-Rull et al. 2007). Starling eggs are initially immaculate blue-green due to a pigment, the biliverdin, a tetrapyrrolic bile pigment with antioxidant properties (Falchuk et al. 2002, Hanley et al. 2008). As the incubation progresses, the immaculate eggs of nests infested by *C. hemapterus* become progressively spotted with red-brownish small dots, resulting from the bites of ectoparasites on the incubating adults (López-Rull et al. 2007).

After the moult, the starling plumage is black iridescent. The structural colour component is produced by the superficial layer of keratin overlying a single, ordered layer of melanosomes (Hill and McGraw 2006). Juveniles (individuals born in the year) and adults (individuals born in previous years) carry out their moult in summer, where they renew the whole plumage. The new feathers have whitish tips that give to birds their spotted appearance (Feare 1984, Svensson 1992). During winter the spotted tips gradually wear away and by the start of the subsequent breeding season male starlings have a mainly black, glossy-looking plumage (Svensson 1992).

Males have multiple ornaments to attract females, that include length and UV colour of throat feathers (Bennett et al. 1997), complex song (Mountjoy and Lemon 1996), behavioural displays (Feare 1984), and probably odours (Amo et al. 2012). In the closely related Spotless starling (*Sturnus unicolor*), the yellow coloration of the beak reflects the amount of carotenoids and vitamin A circulating in plasma during the mating season, signalling to the females the physiological status of the prospecting males (Navarro et al. 2010). In the European starling the beak becomes brightly yellow in proximity to the breeding season. Whether beak colouration plays a role in female choice has not yet been demonstrated in this species.

Experimental design

In my experiments I used starlings that were born during three breeding seasons (2010, 2011, and 2012) in the nest box of a breeding colony situated in Northern Italy (Ozzano Emilia, Bologna, 44°28'N, 11°29'E). Nest boxes were installed in February and removed in September of each year. In 2010 a total of 74 nest boxes were installed, whereas in 2011 and 2012 the number of nest boxes installed was, respectively, 45 and 38.

Nest treatments

In the breeding season 2010, we experimentally challenged the nestlings' immune system with a bacterial endotoxin (lypopolysaccharide (LPS) from Escherichia coli cell walls) to analyse its effect on their growth and on total plasma antioxidant capacity (TAC), concentration of reactive oxygen metabolites (ROM) and hematocrit. LPS is commonly used to challenge the immune system of birds, and it also induces hormonal and behavioural responses (Bonneaud et al. 2003, Wegmann et al. 2015). Occupancy rate (total number of nestboxes where at least one incubated clutch was laid over the entire season) was 38%. Nest boxes were checked every 1–3 days during egg laying and every 1–2 days after hatching. At day 8 post-hatching, half of the nestlings of each nest were subjected to an immune challenge with LPS, whereas the other half was 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 nestlings 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, Berthouly et al. 2008, Romano et al. 2011). Control nestlings were injected with the same amount of PBS only.

In the breeding seasons 2011 and 2012, we experimentally removed the nest ectoparasites from a group of randomly selected nests whereas unmanipulated, naturally infested nests were considered as controls. A total of 84 clutches have been attended by female starlings during the incubation stage. The progression of

egg deposition was checked daily. After clutch completion, 36 nests were randomly assigned to the ectoparasite-free group and 48 nests to the control group. The nest-dwelling ectoparasites were removed from the nests using an antiparasitic spray (Frontline spray, Fipronil 0.25 g, Merial – Tolose, France), whereas control nests were sprayed with tap water. The following procedure, repeated every three days from clutch completion to fledging, has been carried out: eggs or nestlings were removed from the nests before spraying one shot of antiparasitic solution (or water) onto the nest cup. When the nest material was completely dry (usually within three minutes), eggs or nestlings were returned to the nest. When nestlings were removed from the nests, they were kept into a cardboard box and accurately examined to estimate the total amount of *Carnus hemapterus* hosted by the brood. The mean number of *C. hemapterus* counted in the nest during the nestling phase was considered as an index of the mean ectoparasite load of the nest.

Housing of starlings

Among the nestlings born during the breeding season 2010, a total of 8 males and 16 females have been moved to aviaries 2-3 days before fledging. Each aviary measured 200 × 80 (base) × 200 cm (height). The shape of these aviaries is that recommended by a recent study (Asher et al. 2009). When moved to the aviaries, the starlings were treated against endoparasites (coccidia, bacteria, and fungi), and were provided with commercial food for insectivorous species and water *ad libitum*. These birds were used to test the effect of the immune challenge at the nestling stage on the temporal pattern of the first- and the second-year moult (see manuscript 2). During the mating season 2012, we used 14 females to test their preference for males that were subjected to different nest and moult conditions (see below for further details).

A total of 81 males (39 in 2011 and 42 in 2012, respectively) originated from ectoparasite-free and control nests were randomly distributed among four indoor aviaries 2-3 days before they left the nest, and they were provided with food and water *ad libitum*. When moved to the aviaries, the starlings were treated against endoparasites (coccidia, bacteria, and fungi), and provided with a carotenoid-deprived diet until the start of the moult. When the moult started, birds were

randomly assigned to a carotenoid-rich or to a carotenoid-free diet (see below) which was maintained until their post-juvenile moult was completed (about three months). At moult completion, all birds were fed the same diet containing carotenoids. The food consisted of a mixture of 50% TH White Extra and 50% of TH White Soft (Raggio di Sole, Piacenza - Italy) that contains a concentration of lutein between 0.9 and 1.2 mg per kg. The dietary carotenoid supplementation consisted of an additional 50 g Versele-Laga Yel lux [Oropharma, Deinze - Belgium; extracted from marigolds (*Tagetes erecta*) and containing 8 g of lutein per kg] per one kg of base food, a dosage previously used on starlings (Van Hout et al. 2011). The combined effect of the two treatments (insecticide/parasitized and carotenoid/non-carotenoid diet) produced four experimental groups: 1) I/C; 2) P/C; 3) I/NC; and 4) P/NC.

I measured the temporal pattern of moult (see manuscript 5 for further details), the time budget during the subsequent winter (see manuscript 6), the expression of the sexual traits and their relative attractiveness through a two-way choice chamber in the subsequent breeding season (see manuscript 7).

Extended abstracts of the manuscripts

Condition during development

(Manuscripts 1, 3, and 4)

In many animal species, adverse conditions during early development elicit a trade-off between immunity and growth (e.g. Soler et al. 2003, Chin et al. 2005, Stier et al. 2014), which in turn causes long-term effects and major fitness consequences during adulthood (Lindström 1999, Kruuk et al. 1999, Metcalfe and Monaghan 2001, Lummaa and Clutton-Brock 2002, Walling et al. 2007, Krause et al. 2009, Auer et al. 2012, Monaghan et al. 2012). Also in humans, poor development conditions have negative effects on development of foetus and infant because they induce a change in the growth trajectories (Gluckman and Hanson 2004) that could impair health at adulthood (Harrison and Baune 2014, Maniam et al. 2014).

In birds, a common early stress is caused by nest-dwelling ectoparasites which boost an immune response (Szép and Møller 1999) and affect nestling growth in various species (Christe 1996, Tomás et al. 2008, Martínez-de la Puente et al. 2011, Cantarero et al. 2013). Several studies have demonstrated that nestling growth is adversely affected by the abundance of ectoparasites in the nest (Christe 1996, Tomás et al. 2008, Cantarero et al. 2013) either directly (Martínez-de la Puente et al. 2011) or indirectly, because they influence the parental behaviour (Richner and Tripet 1999). Hence, nest ectoparasites cause a two-fold cost for nestlings as they affect the nestling current condition and decrease their value for parents which may respond by redirecting their energy to future breeding attempts (Christe et al. 1996) and self-maintenance (Martínez-de la Puente et al. 2010).

Trivers (Trivers 1974) suggested that young are in conflict over parental care with the offspring of the same brood and with those from subsequent broods, with offspring of the current brood (which would benefit from the maximum parental investment) seeking greater levels of parental investment than parents (which tend to maximize their overall reproductive success) should be selected to provide (Macnair and Parker 1979). In altricial birds, offspring are entirely

dependent on food provided by parents, which are solicited by morphological and behavioural begging displays (Kilner 2002). The begging behaviour is influenced by brood size (Leonard et al. 2000), offspring relatedness (Briskie et al. 1994, Boncoraglio and Saino 2008), age and sex of the nestlings (Teather 1992, Cotton et al. 1999, Saino et al. 2003, but see Whittingham et al. 2003). A recent study on pied flycatchers (*Ficedula hypoleuca*) has demonstrated that begging is also associated to the abundance of nest ectoparasites (Cantarero et al. 2013), whereas in Darwin's finches (*Geospiza fortis*) nestlings which were highly infested by blood-sucking parasites during the previous night produced less intensive begging the following day (O'Connor et al. 2014). Both studies, however, found a positive correlation between begging intensity and parental provisioning rates, as shown also in great tits (*Parus major*) (Christe et al. 1996). More generally, the intensity of begging is positively related to the hunger status of chicks (Cotton et al. 1999, Johnstone and Godfray 2002) which promotes the secretion of corticosterone (Kitaysky et al. 2001), the main avian stress hormone (Schmidt and Soma 2008). While the intensity of begging is not affected by yolk testosterone levels (Pilz et al. 2004), it is more pronounced in nestlings with high testosterone level during growth (Goodship and Buchanan 2007), and it has been showed that high levels of plasma testosterone and corticosterone cause an increase in begging displays (Schmidt and Soma 2008, López-Rull et al. 2011). Considering that nest-dwelling ectoparasites are one of the major stressors for nestlings, it can be thought that corticosterone levels should be positively associated with the abundance of ectoparasites, and thus with the begging of nestlings. However, the contrasting results provided so far (Cantarero et al. 2013, O'Connor et al. 2014) on the effect of nest ectoparasites on begging intensity deserve further investigations.

Along with the postural begging, the colouration of bare parts such as skin and mouth, and the colouration of some plumage patches concur to signal the nestling condition to parents (Jourdie et al. 2004, Bize et al. 2006, Galván et al. 2008, Griggio et al. 2009a, Jacob et al. 2011). For example, the orange-yellow colouration of the nestling mouth found in several bird species is positively correlated to the amount of circulating carotenoids (Ewen et al. 2008, Thorogood et al. 2008) and to the physiological condition of chicks (Hunt et al. 2003, Dugas

and Rosenthal 2010, but see Ewen et al. 2008). A bright ultraviolet colouration of the skin is correlated to nestling conditions and is associated with an increased parental effort (Jourdie et al. 2004, Bize et al. 2006). In addition, it has been demonstrated that the yellow colouration of the flanges and the ultraviolet colouration of the skin are involved in the begging displays of starling nestlings, and that parents use to adjust their investment in the current breeding attempt (Jacob and Heeb 2013). Since nest ectoparasites adversely affect nestling condition, with carry over effects on subsequent life-history stages, we predict that they also affect the expression of sexual secondary traits. It can also be predicted that this effect is stronger the earlier (i.e. temporally closer) a signals is produced.

Results

In the manuscript 1, we showed that nestlings whose naïve immune system was challenged with an endotoxin (LPS) did not grow at a different rate as compared to control nestlings. Instead, I found that their physiological parameters, namely TAC, ROM, and hematocrit, differed significantly between first and second broods. In particular, plasma TAC and ROM concentration were unaffected by the immune challenge, whereas TAC of second-brood nestlings was lower than that of first-brood ones. Hematocrit was significantly lower in second-brood nestlings, and was higher in LPS-nestlings than in controls. In contrast, LPS treatment had no effect on the hematocrit of first-brood nestlings.

In the manuscript 3, we present the results of a two-years study showing that the presence of nest ectoparasites caused a reduction of the time spent by parents in the nests. As result, ectoparasite-free broods were attended longer by parents and fledged a larger number of nestlings. We therefore hypothesized that nestlings from ectoparasite-free nests received a direct advantage, i.e. the absence of nest ectoparasites, and an indirect benefit, i.e. a greater provisioning effort from parents. In the manuscript 4 we show that nestlings from ectoparasite-free nests exhibited a more intensive postural begging than nestlings from naturally infested nests, whereas the visual components of begging were unaltered, along with hematocrit levels and immune response. While parents increased their

provisioning effort in accord to an increased postural begging, they were not influenced by the removal of nest ectoparasites.

Effect of an early stress on moult

(Manuscripts 2 and 5)

Moult is one of the most costly events for birds (Payne 1972, Dietz et al. 1992), thereby it does usually not overlap with other costly life-history events such as reproduction and migration (Barta et al. 2008). A male's ability to acquire adequate energy resources during moult shapes the quality of the resulting plumage, the quality of his feather ornaments and eventually his sexual attractiveness (Pap et al. 2008). Moult is even more challenging for freshly fledged birds, in particular in those cases in which it starts within a few weeks after fledging (Jenni and Winkler 1994), when their ability to feed autonomously is not completely achieved.

In case of limited resources or poor condition, individuals can delay the start of their moult and increase the number of concurrently growing feathers, although this negatively affects the quality of flight feathers (Badyaev and Duckworth 2003, Dawson 2004, Serra et al. 2010), and plumage ornaments (Serra et al. 2007).

In several cases, birds must rely on both food obtained during moult and on previously stored reserves to fulfil the energetic requirements of the moult (Fox et al. 2009, Fox and King 2011). Freshly fledged birds unable to satisfy their nutritional needs may face a decline in conditions (Metcalfe and Monaghan 2001, McGraw et al. 2002, Searcy et al. 2004, Krause et al. 2009, Harrison et al. 2011) and therefore perform an impaired post-juvenile moult (Badyaev and Duckworth 2003). Hence, although nutrition during moult is expected to have the strongest effect on the expression of feather signals (and hence on male mating success), its effect may be amplified by nestling conditions either additively or multiplicatively.

Carotenoids are considered to be a key component of animal diet, as they can only be obtained from food (Goodwin 1980), and insufficient carotenoid content in the diet has detrimental effects to the organism, in particular during

moult (McGraw et al. 2011). As expected, the effect of carotenoid limitation is particularly pronounced in those species that exhibit yellow/red carotenoid-based SSTs (e.g. Blount et al. 2003b, Biard et al. 2006). Indeed, carotenoid-pigmented feathers honestly signal the nutritional status of the bird during moult (Hill and Montgomerie 1994, Hill 2000, Navara and Hill 2003). However, little is known on the effect of carotenoids on the structural coloration of plumages. The only study conducted so far suggests that in blue tits (*Cyanistes caeruleus*) carotenoid supplementation does not affect the UV/blue feathers (Peters et al. 2011). In several bird species, carotenoids are also found in the beaks, whose yellow colouration signals short-term changes in individual conditions and is used in female choice (Faivre et al. 2003, Navarro et al. 2010, Rosenthal et al. 2012). In adult spotless starlings, the yellow colouration of the beak reflects the plasma concentration of carotenoids during the mating period (Navarro et al. 2010). However, it is likely that the expression of the yellow beak colouration in the following breeding season could be influenced by the assumption of carotenoids early in life (Blount et al. 2003a).

Individual condition during the post-juvenile moult is expected to be influenced also by individual condition at the nestling stage. We previously cited several studies that have demonstrated how nest-dwelling ectoparasites have a negative effect on nestling growth. However, there is less information on downstream effects caused by the nest ectoparasites on individual condition during adulthood. The lack of effects of nest-dwelling ectoparasites on nestlings is particularly surprising, because the nestling stage is a crucial period in the lifetime of a bird, and the stresses that occur at this stage can have detrimental effects later in life (Hochachka and Smith 1991, Holveck et al. 2008, Krause et al. 2009, Walker et al. 2013), such as during moult.

Results

In the manuscript 2, we measured the temporal pattern of the first- and the second-year moult of male and female nestlings that were injected with an endotoxin (LPS-treated) or with a saline solution (control) at the nest (see manuscript 1). First-year moult tended to be advanced, whereas second-year moult was significantly anticipated, in LPS-treated as compared to control

individuals. This anticipation appears in contrast with predictions, as LPS injection should negatively affect individual condition and this should cause a delay in the moult start as compared to controls. An explanation to this unexpected result is that the boosting of the immune system caused by LPS injection may have better prepared these nestlings to future bacterial infections possibly associated with ectoparasite bites as compared to their control counterparts. Indeed, in our LPS experiment all nests were heavily infested by ectoparasites (see the other manuscripts) which are important vectors of bacterial infections (Benskin et al. 2009). This hypothesis would need be tested in a 2x2 factorial experiment in which the effects of LPS injection on moult are compared in relation to the ectoparasite load.

In the manuscript 5, we have tested the effect of nest ectoparasite on first-year moult timing and on spectral properties of the throat plumage. We found that birds fledged from ectoparasite-free nests anticipated the onset of the moult and completed the moult over a longer period as compared to birds fledged from naturally infested nests. Despite these differences in moult onset and duration, the spectral reflectance of the resulting plumages was unaffected by the nest treatment (see also manuscript 7).

Our studies therefore provide support to the general idea that moult is a costly activity, whose timing could be used as an index of individual condition.

Maintenance of the ornaments

(Manuscript 6)

Survival and reproduction are influenced by the individual investment in preserving the good state of the integuments (e.g. skin, scales, plumage, pelage). Social grooming in primates is perhaps the most famous example of this activity, which has a twofold function: hygiene and enforcement of social bonds. Far from being limited to primates, grooming has been reported in numerous *taxa* such as, for example, fishes (Hobson 1971), reptiles (Russell and Rosenberg 1981), mammals (Ferkin and Leonard 2004), and birds (Walther 2003). These types of maintenance behaviours are energy-demanding and time-consuming (Croll and

McLaren 1993), and thereby the good state of the integument is likely to reflect individual condition.

Birds invest considerable time and energy in preening, a suite of behaviours aimed at cleaning (dirt and ectoparasites), arranging, oiling, and ordering the feathers (Cotgreave and Clayton 1994, Walther and Clayton 2005, Clayton et al. 2010, Waite et al. 2012). Such behaviours are primarily devoted to maintain the thermoregulatory and ornamental functions of the plumage (Zampiga et al. 2004, Lenouvel et al. 2009, Clayton et al. 2010). Recently, it has been suggested that preening conveys also public information to other group members and potential mates (Danchin et al. 2004), although there is contrasting evidence that such behaviours are associated to individual condition (Yorinks and Atkinson 2000, Surmacki and Hill 2013). To date, only two studies tested whether preening is condition-dependent. A study on juvenile apapanes (*Himatione sanguinea*), a honeycreeper living in the island of Hawaii, showed that individuals infected with malaria spent significantly less time preening when compared to healthy birds (Yorinks and Atkinson 2000). In contrast, a study on the American goldfinch (*Spinus tristis*) failed to find a positive correlation between coccidian infestation and preening rate (Surmacki and Hill 2013). However, these studies are carried in captive birds, whereas the effect of infections on wild birds could be stronger as they have limited access to food and may be time-limited because of the trade-off with other activities, such as foraging and vigilance against predators (Redpath 1988, Cucco and Malacarne 1997).

As shown in previous studies (Zampiga et al. 2004, Lenouvel et al. 2009), the consequences of a reduced investment in preening, due to poor individual conditions, should result in the lower expression of plumage ornaments in the following breeding season. In manuscript 6 we investigated the effect of dietary carotenoid availability during moult on preening time in male starlings during the months following the end of their first moult.

Results

As expected, we found that males provided with carotenoids during the post-juvenile moult subsequently spent more time in preening as compared to males deprived of carotenoids during the moult. This result suggests that males

supplemented with carotenoids were in better condition. The lack of carotenoids in the diet, in contrast, did not influence plumage colouration, which is determined by structural colours (ordered layers of keratin and melanin), suggesting that this male ornament is not sensitive to this type of stress.

Carry-over effects on male ornaments and female preference

(Manuscript 7)

The expression of condition-dependent ornaments may be differently influenced by individual condition at different life stages occurring before (such as during development) or at the time that the ornaments are expressed (see general introduction). However, in the case of carry-over effects, the different stages of an individual's life are also likely to be linked one to the other, so that past condition would influence current condition. As a result, the expression of male ornaments are predicted to be influenced by the individual condition experienced during the different life-history events in a complex way which may be difficult to predict because it will depend on the stresses experienced at different life stages, by their interactive effect on current and future condition, and by the specific mechanism regulating a trait expression. Empirical evidence on carry-over effects in the expression of sexual traits are still scarce (Blount et al. 2003a), in particular in the case of multiple ornaments.

Results

In the manuscript 7 we showed the effect of two temporally distinct stresses, i.e. nest ectoparasites and availability of dietary carotenoids during the post-juvenile moult, on the expression of multiple ornaments in male starlings. Furthermore, we tested the effect of these two early stresses on male attractiveness to females during the subsequent breeding season using a two-way choice chamber. We showed that beak colouration was influenced by the carotenoid supplementation, whereas the throat feather colouration was unaffected by both the early stresses. As consequence, however, females showed

a clear preference for males that fledged from ectoparasite-free nests and that received a carotenoid-rich diet during the course of the moult.

General conclusions

During my PhD project I have provided evidence that the stresses that occur during early life-history events (i.e. at the nest or within the first six months of life) in male European starlings influence the expression of SSTs and male sexual attractiveness. My results support the general idea that the nestling stage represents a crucial period in the lifetime of a bird and that is highly influenced by parents-offspring communication. A stress which occurs at the nest (i.e. within the first three weeks after hatching) could potentially influence an individual's fitness by 1) modifying the parental investment in current breeding attempts, 2) affecting the subsequent moult pattern: the removal of nest ectoparasites and the activation of the immune system determined by LPS injection had a positive effect on nestling condition as it can be deduced by their anticipated moult start. Furthermore, nestlings from ectoparasite-free nests also showed a longer moult duration. The stresses occurring at the nestling stage, however, did not produce a measurable effect on the expression of SSTs. By contrast, the dietary carotenoid treatment during the moult had positive downstream effects on both plumage maintenance (moulting birds supplemented with carotenoids invested more time in preening their plumages during winter) and yellow beak colouration measured during the following breeding season.

Although some of the SSTs we measured were apparently unaffected by our experimental manipulations, the results of our female choice experiment indicate that females are apparently more efficient in detecting males that experienced the most favourable juvenile conditions, i.e. nests without ectoparasites and availability of carotenoids during moult. These results demonstrate that studies on multiple signals that are solely based on the direct measurement of the SSTs may actually underestimate the effect of different stresses on the overall male attractiveness. From this point of view, the inclusion of a direct measure of male attractiveness trough female choice experiments is highly recommended. These results do not allow to firmly distinguish between the alternative hypotheses explaining the evolution of multiple traits, although they strongly suggest that females tend to integrate the information from multiple ornaments, possibly in a multiplicative manner, when they come to choose their mate. It has to be noted,

however, that these hypotheses are not mutually exclusive and multiple signals may be constituted by traits that convey distinct information on different stresses and other that convey a redundant signal. Indeed, considering that all SSTs are condition dependent but often determined by different underlying physiological processes and genetic machineries, it is unlikely that they are perfectly equally affected by any stress (redundant signal hypothesis) or that some of them are totally unaffected by stresses (multiple signal hypothesis). Whatever the underlying evolutionary model, it is worthwhile noting that early stresses (such as nest ectoparasites) that did not evidently affected nestling parameters such as growth rate, body mass at fledging, skin and flange colouration, nonetheless significantly affected male performance about one year later. These results clearly indicate that carry-over effects can multiply the effects of a stress over the time. One of our predictions was that the temporally closer a stress is to the time when an ornament is produced or maintained, the stronger is expected to be the effect on the ornament. Our results, however, do not support this prediction, and instead suggest that early stress can affect later expressed signals and highlight the importance of carry-over effects in the expression of sexually selected traits.

MANUSCRIPTS

MANUSCRIPT 1

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ORIGINAL PAPER

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

Lorenzo Serra · Simone Pirrello · Manuela Caprioli · Matteo Griggio · Alessandro Andreotti · Andrea Romano · Andrea Pilastro · Nicola Saino · Roberto Sacchi · Paolo Galeotti · Mauro Fasola · Fernando Spina · Diego Rubolini

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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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L. Serra · S. Pirrello · A. Andreotti · F. Spina
Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA),
via Ca' Fornacetta 9,
40064 Ozzano Emilia, Bologna, Italy

M. Caprioli · A. Romano · N. Saino · D. Rubolini (✉)
Dipartimento di Biologia, Università degli Studi di Milano,
via Celoria 26,
20133 Milan, Italy
e-mail: diego.rubolini@unimi.it

M. Griggio
Konrad Lorenz Institute of Ethology, Department of Integrative Biology and Evolution, University of Veterinary Medicine,
Savoyenstraße 1a,
1160 Vienna, Austria

S. Pirrello · A. Pilastro
Dipartimento di Biologia, Università degli Studi di Padova,
via U. Bassi 58/B,
35131 Padua, Italy

R. Sacchi · P. Galeotti · M. Fasola
Dipartimento di Scienze della Terra e dell'Ambiente,
Università degli Studi di Pavia,
via Ferrata 1,
27100 Pavia, Italy

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

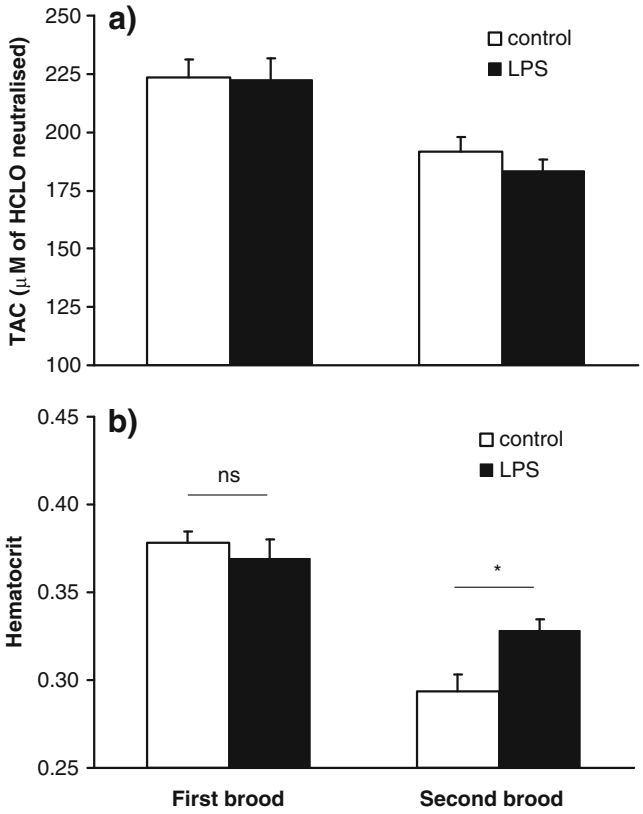

Fig. 1 Mean (+ s.e.) values of total antioxidant capacity (*TAC*) and hematocrit (proportion of red blood cells) of first- and second-brood nestling starlings in relation to LPS challenge. Sample size is 40 chicks from first broods (19 controls and 21 LPS-injected) and 42 from second broods (19 controls 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

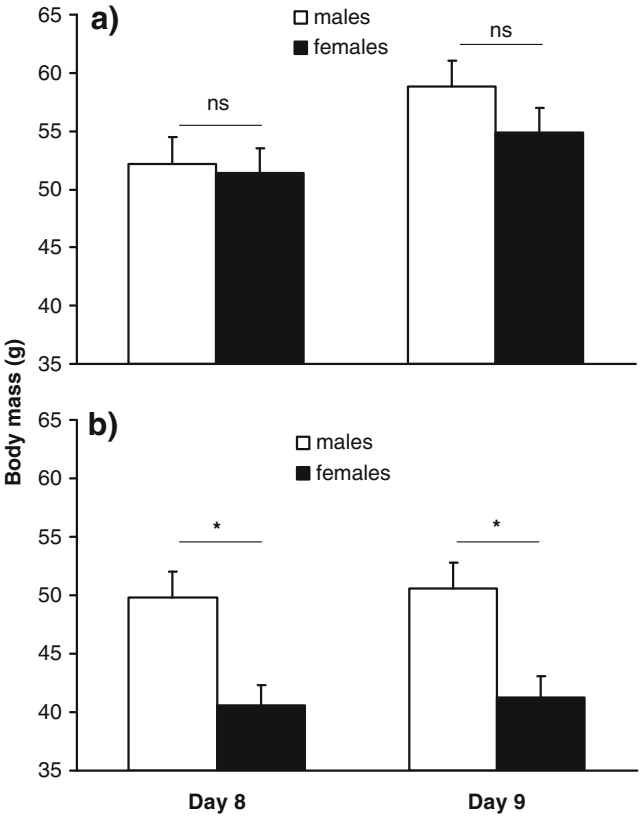

Fig. 2 Mean (+ s.e.) values of body mass of **a** first- and **b** second-brood nestling starlings in relation to age and sex (values represent model-estimated means from the model shown in Table 2). Sample size is 41 chicks from first broods (19 males and 22 females) and 45 from second broods (15 males 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).

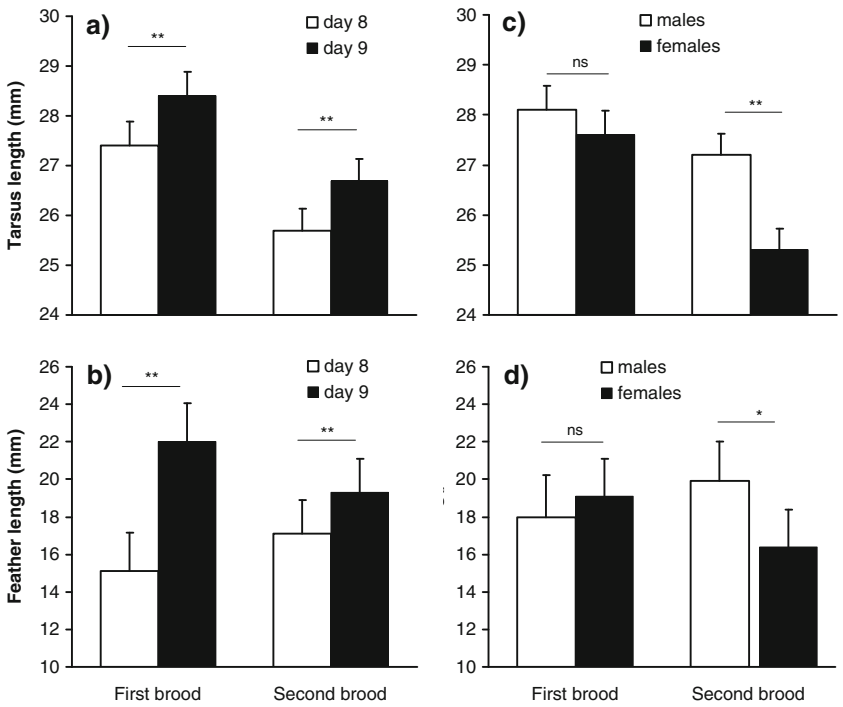

Fig. 3 Mean (+ s.e.) values of tarsus and feather length of first- and second-brood nestling starlings in relation to age (*left column*, **a** and **b**) and sex (*right column*, **c** and **d**) (values represent model-estimated means from models listed in Table 2). Sample size is 41 chicks from first 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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MANUSCRIPT 2

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

Simone Pirrello[a,b], Andrea Pilastro[b], Alessandro Andreotti[a], Fernando Spina[a], Diego Rubolini[c], Andrea Romano[c], Nicola Saino[c], Matteo Griggio[b], Lorenzo Serra[a]

[a]*Istituto Superiore per la Protezione e la Ricerca Ambientale, 40064, Ozzano Emilia, Bologna, Italy*
[b]*Dipartimento di Biologia, Università degli Studi di Padova, 35121, Padova, Italy*
[c]*Dipartimento di Bioscienze, Università degli Studi di Milano, 20133, Milano, Italy*

Abstract

Early stresses often produce carry-over effects that can potentially influence the life-history trajectories of an organism. However, there are still scant experimental studies on carry-over effects in birds. In this study, we used an immune challenge at the nestling phase to investigate its effects on the first- and second-year moult, and the relative plumage quality in the European starling (*Sturnus vulgaris*). Birds whose nestling's immune system was challenged with a bacterial endotoxin (lypopolysaccharide (LPS) from *Escherichia coli* cell walls) weakly anticipated the first post-juvenile moult and significantly anticipated the second-year moult as compared to birds treated with a saline solution. Moult duration was unaffected by the immune challenge but strongly influenced by the starting date of the moult, given that delayed moult start was positively correlated to shorter moult. The throat feather colouration, which is a sexually selected trait in starlings, and that is directly related to moult duration, did not differ among groups in either the years. Our study therefore provide an experimental evidence that an early immune challenge yields to carry-over effects which influence the temporal pattern of moult at one year from the stress. This result supports the idea that various costly events in the lifetime of an organism could be potentially influenced by an earlier stress.

Introduction

A life-history event can be influenced not only by current individual condition but also by individual conditions in previous life stages. These carry-over effects are quite common in nature, but so far there are scarce experimental evidences due to the difficulties to follow an individual

across its annual cycle (Harrison et al. 2011). In many cases, downstream effects are unlikely to be detected because individuals carry out compensation measures to withstand adverse current condition, such as a stress, that apparently do not impair instantaneous processes but that produce negative effects during future events of their life cycle (Metcalfe and Monaghan 2001). New technologies for tracking animals are providing support to overcome the difficulty to follow an individual during the course of its life (Mitchell et al. 2011, 2012), while the issue to perceive which processes are linked to past individual conditions still remains to be understood.

Moult is one of the most costly events for birds (Payne 1972), as it requires substantial amounts of energy (Dietz et al. 1992) that, in cases of limited resources, birds have to detract from other costly processes or behavioural activities (Siikamaki et al. 1994, Barta et al. 2008). Alternatively, in order to reduce overlapping their moult with other costly phases of their life cycle, low-condition individuals can delay the start of their moult and increase the number of concurrently growing feathers, although this negatively affects the feather quality (Badyaev and Duckworth 2003, Dawson 2004, Serra et al. 2010), and then the quality of their plumage ornaments (Serra et al. 2007). While there are evidence that deposition of pigments such as melanins and carotenoids in feathers is correlated to individual conditions (McGraw and Hill 2001, Jacquin et al. 2011, Minias et al. 2014), thereby prime individuals pay a minor unitary cost of laying pigments in feathers, it is still unclear whether iridescent plumages are condition-dependent (Peters et al. 2007).

In this study, we considered a group of European starlings (*Sturnus vulgaris*) that was used in a previous study to examine their responses to an immune challenge as nestlings (Serra et al. 2012). The immune system of a group of nestlings were challenged with lypopolysaccharide (LPS), an endotoxin extracted from the outer membrane of Gram-negative bacteria, whereas a group of control was treated with a saline solution (PBS). LPS is commonly used to elicit an immune response in the absence of a living pathogen and causes several hormonal and behavioural alterations in birds (Bonneaud et al. 2003, Owen and Moore 2006). However, our LPS treatment did not impair the nestling condition in terms of total plasma antioxidant capacity (TAC) and concentration of reactive oxygen metabolites (ROM). In contrast, hematocrit resulted higher among second-brood (but not among first-brood) LPS nestlings compared to control (see (Serra et al. 2012) for further details). Here we investigated whether the early immune challenge, despite weakly affecting the nestling condition, has the potential to produce downstream effects on the temporal pattern of the first- and the second-year moult, and thus to influence the quality of the resulting plumages in either the years. The European starling has a melanin-based plumage with iridescent structural colours that goes from green to purple, and both juveniles and adults perform a complete moult that in the middle latitudes it takes about three months (Manuscript 5). Previous studies on

the European starling have shown that this species can well tolerate a nutritional stress during moult (Bauer et al. 2011) and can rapidly compensate for an increased allostatic load by down-regulating the response to stresses (Kostelanetz et al. 2009).

Although we did not find an effect of LPS injection on nestling starlings (Serra et al. 2012), there are other studies that showed the negative effects of this bacterial infection on different bird species (Bonneaud et al. 2003, Owen and Moore 2006, Piault et al. 2008, Romano et al. 2011). For example, house sparrows (*Passer domesticus*) injected with LPS moulted slower than individuals injected with a saline solution, and this suggested a trade-off between immune response and moult (Moreno-Rueda 2010). In two studies on the Pied flycatcher (*Ficedula hypoleuca*) the immune system of males (Sanz et al. 2004) and females (Ilmonen et al. 2000) was challenged with LPS during the breeding season. Females inhibited the growth of tail feathers that were experimentally plucked, whereas males delayed the moult start. We therefore predicted that starlings subjected to LPS as nestlings, despite not showing an instantaneous response in terms of some physiological parameters, would have suffered the cost of the immune activation later in life, for example delaying the moult start. An alternative hypothesis could be that the costs associated with mounting an instantaneous response to an early immune challenge would have prepared LPS nestlings to future costly events, with the consequence that LPS birds advanced the moult start. A more general prediction was that birds which started the moult earlier are likely to moult over a longer period and therefore produced plumages of better quality.

Materials and Methods

Field procedures and immune challenge

The starlings used in this experiment originated in the 2010 breeding season from nest boxes situated in Northern Italy (Ozzano Emilia, Bologna, 44°28'N, 11°29'E). From the middle of March to the end of the breeding season (June-July), we checked the nest boxes daily for the progression of egg deposition.

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.

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

Pre-fledging mortality was not different among LPS (8 dead out of 46 hatched) and control (24 dead out of 89 hatched) nestlings ($\chi^2 = 1.05$, P = 0.30). A total of 24 nestlings (8 males and 16 females) were randomly distributed among three indoor aviaries 2-3 days before they left the nest. At this age birds become readily capable of feeding autonomously and can be maintained in the absence of the parents. Each aviary measured 200 × 80 (base) × 200 cm (height), and birds were provided with food and water *ad libitum*. When moved to the aviaries, all of the starlings were treated against endoparasites (coccidia, bacteria, and fungi).

Timing of moult

The timing and duration of the primary moult was assumed as a proxy of the complete moult (body and wing feathers). The moult progression of the 9 long primary feathers was checked weekly. The starting date of the moult was considered as the previous checking date to the checking date when the first primary feather (=the innermost) was found shed. The moult was considered completed at the checking date when all the primary feathers were found fully grown.

In 2010 we considered the variable "start of moult" as the time lag from hatching to the start of the moult. At the end of the moult from each individual we plucked five feathers from the right side (looking at the underpart of the bird) of the throat in the correspondence of the carpal joint, which were housed in individual plastic envelopes and kept in the dark until the spectral analyses were performed.

Spectral analyses

We measured the spectral reflectance of individual throat feathers and then averaged for each individual. We decided to follow this method because of the higher repeatability of this procedure compared to that obtained overlapping the feathers (Meadows et al. 2011).

The spectral reflectance of each feather was measured with an Ocean Optics S2000 spectrometer and then filtered to maintain only the wavelengths between 300 and 700 nm that correspond to the visible spectra of birds. Each measurement occurred in a dark room, with no artificial light that might affect the spectral shape. The light optical-fiber probe was held at 90 degree and at a distance of 5 mm from the feather surface through a PVC tube mounted on the ferrule tip, which also excluded any noise from the ambient light. Reflectance spectra were recorded with the Spectrasuite OOIBase32 software, with standards set to a WS-2 white standard and dark before each measurement session. The spectrometer automatically averaged five consecutive readings of the same location, while we took a single measurement from each feather.

The mean reflectance corresponded to the average value of reflectance in the 300-700 nm range and was regarded as an index of feather and beak brightness (Hill and McGraw 2006). Given that in the starling female mate preference is influenced by the ultraviolet range of spectra (Bennett et al. 1997), we also calculated the UV chroma of throat feathers as 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. 2010b). The spectrum of throat feathers has two peaks respectively in the UV (300-400 nm) and green (500-600 nm) ranges, so we considered the UV and green brightness separately.

The investigated range of wavelengths (300-700 nm) was first divided into 40 classes of 10 nm, and then the mean brightness of the whole spectrum was subtracted from the mean brightness of each class. This standardisation eliminates the strong correlation between the first PCA score and brightness (Cuthill et al. 1999). We therefore performed a principal component analysis (PCA) to analyze the spectrum of throat feathers. We only considered the first and second PCA scores, since combined they explained more than 80% of the total spectral variance. Pearson's correlations were used to investigate the co-variation between PCA scores and colour parameters.

Statistical analyses

All data were normally distributed, so we used parametric statistics. We used Linear Mixed Models (LLMs) to test the effect of immune challenge and sex on the temporal pattern of moult (start and duration) and on the throat feathers colouration. Immune challenge (coded as 1 for LPS birds, and 0 for controls), sex (coded as 1 for males and 2 for females), and their interaction were considered as explanatory factors in the initial model but the interaction term was subsequently removed as predictor because never significant. Nest identity was included in the models as a random intercept effect to control for the non independence of data from individuals fledged from the same nest. We carried out separate analyses for the first and the second moult. In the LMM where first year moult start was the dependent variable we included the hatching date as the

covariate, given that birds which hatch later in the breeding season begin the moult later. In the LMMs which considered the moult duration, the starting date of the moult was included as the covariate, considering that birds whose moult begin later usually perform a shorter moult.

Statistical analyses were carried out using the software R 3.0.3 (R Development Core Team). All of the tests were two-tailed and significant when $P < 0.05$.

Results

Timing of moult

The immune challenge at the nestling phase allowed birds to advance the start of the second-year moult of about one week (Tab. 1, Fig. 1). This anticipation in LPS birds was found also in the first-year moult, although the difference was not significant as compared to control birds. Females postponed the start of the second-year moult of about 9 days than males. In both the years, moult duration was unaffected by the immune challenge and did not differ among sexes, whereas it was mainly explained by the starting date of the moult because, as predicted, birds that began moulting later carried out shorter moult (Tab. 1).

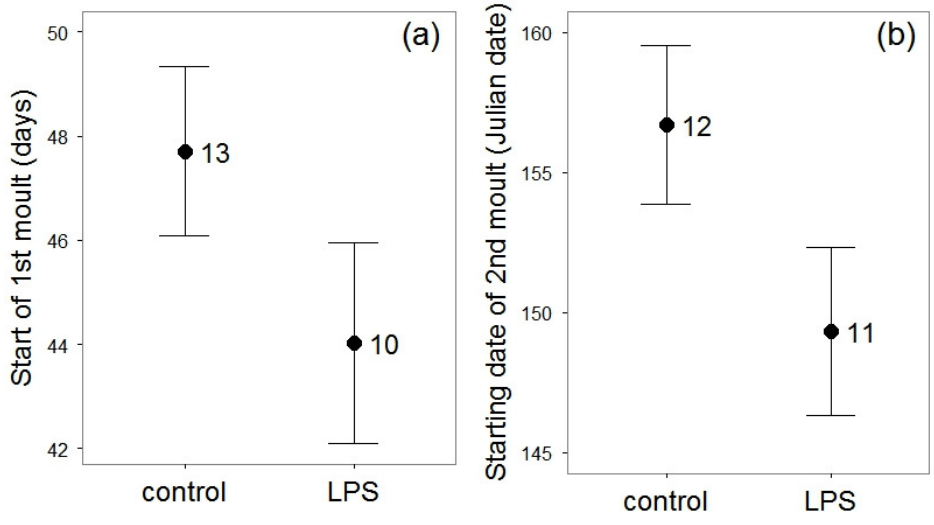


Fig. 1 – Start of the first moult (a) and the second moult (b) of starlings injected with an endotoxin (LPS) or with a saline solution (control) during the nestling stage. The start of the first year moult stands for the days from hatching to the start of the moult. The start of the second year moult is indicated as the Julian date (1 January = 1). The bars show the marginal means ± SE, whereas the values are the sample sizes. The starting date of the first moult in one LPS bird, and the starting date of the second year moult in one control bird were unknown.

Variables	Estimate ± SE	d.f.	F	P
Start of 1st moult				
Immune challenge	-3.7 ± 2.2	1, 15.4	2.87	0.11
Sex	1.3 ± 2.5	1, 18.2	0.28	0.60
Hatching date	-0.3 ± 0.1	1, 9.4	22.9	**< 0.001**
Duration of 1st moult				
Immune challenge	0.3 ± 2.6	1, 19	0.02	0.89
Sex	1.2 ± 2.9	1, 19	0.17	0.68
Starting date of 1st moult	-0.2 ± 0.1	1, 19	7.23	**0.015**
PC1 1st plumage				
Immune challenge	0.2 ± 0.3	1, 20	0.55	0.47
Sex	-1.6 ± 0.3	1, 20	31.7	**< 0.001**
PC2 1st plumage				
Immune challenge	-0.3 ± 0.4	1, 20	0.60	0.45
Sex	-0.7 ± 0.4	1, 20	2.8	0.11
PC3 1st plumage				
Immune challenge	-0.5 ± 0.4	1, 15.7	1.71	0.21
Sex	-0.3 ± 0.4	1, 18.9	0.56	0.46
Starting date of 2nd moult				
Immune challenge	-7.4 ± 3.2	1, 15.6	5.21	**0.037**
Sex	8.7 ± 3.7	1, 18.4	5.61	**0.029**
Duration of 2nd moult				
Immune challenge	-1.5 ± 3.1	1, 19	0.23	0.63
Sex	-0.7 ± 3.1	1, 19	0.04	0.83
Starting date of 2nd moult	-0.5 ± 0.2	1, 19	10.1	**0.005**
PC1 2nd plumage				
Immune challenge	-0.3 ± 0.4	1, 20	0.60	0.44
Sex	-0.7 ± 0.4	1, 20	2.75	0.11
PC2 2nd plumage				
Immune challenge	-0.01 ± 0.30	1, 12.6	0.002	0.96
Sex	-0.7 ± 0.4	1, 15.7	3.38	0.09
PC3 2nd plumage				
Immune challenge	-0.5 ± 0.4	1, 13.7	1.66	0.22
Sex	0.4 ± 0.4	1, 18.4	0.72	0.41

Tab. 1 - Linear mixed models of the effects of the immune challenge on moult start, moult duration and plumage colouration of the first and second year. See Materials and Methods for details on statistical procedures.

Plumage colouration

The principal component analysis provided three PC scores which explained 96% of the total spectral variance. Person's correlations between the PC scores, ultraviolet chroma and brightness showed that the first PC score is positively correlated to brightness (first plumage: Pearson's correlation = 0.66, P = 0.001; second plumage: Pearson's correlation = 0.69, P < 0.001) whereas the third PC scores is positively correlated to UV chroma (first plumage: Pearson's correlation = 0.90,

P < 0.001; second plumage: Pearson's correlation = 0.64, P = 0.001). The second PC score was negatively correlated to brightness in first plumages (Pearson's correlation = -0.59, P = 0.003) while it showed a non-significant positively correlation to brightness in second plumages (Pearson's correlation = 0.38, P = 0.07).

The throat feathers colouration did not differ between LPS and control birds, whereas first-year females showed darker throat feathers than males (Tab. 1). This sex-difference, despite not significant, remained in the second-year plumage.

Discussion

We experimentally demonstrated that an early immune challenge that apparently did not influence the nestling condition, produced carry-over effects on the first- and second-year moult, given that birds injected with LPS showed a significant anticipation of the second-year moult and a non-significant anticipation of the first-year moult. Our results suggest that the immune challenge at the nestling stage could have enforced the immune system of birds that, in turn, improved their general condition as they were allowed to advance the costly event of moult. Moult duration, in contrast, resulted unaffected by the immune challenge whereas it was directly related to the starting date of the moult, as individuals that moulted over longer periods were those that start moulting later. Similar moult durations could be the ultimate factors that prevented to find a significant difference of the throat feather coloration among LPS and control birds.

In this study, we considered a breeding colony of starling which is highly infested by nest ectoparasites (Manuscript 4), whose negative effects on nestling growth has been demonstrated in several bird species (Christe 1996, Tomás et al. 2008, Martínez-de la Puente et al. 2011, Cantarero et al. 2013). There is also evidence that nest ectoparasites boost an immune response in nestlings (Szép and Møller 1999), thereby the nestling resources could be trade-off between their growth and immunity. The dominant ectoparasite species in starling nests is the fly *Carnus hemapterus* which mainly feeds on blood of adults and nestlings (Liker et al. 2001). This hematophagus fly not only affects the nestling condition by sucking the blood, but due to its bite it might transmit to the host a complex of bacteria and virus that could contribute to additively impaired the nestling health state. As a consequence, an immune challenge at the halfway through the somatic growth of nestlings could stimulate their naïve immune system, and despite a potential negative impact on nestling development (that we did not find), it could improve the immune performance of the bird in the long term. This prediction seems in agreement with our results as LPS nestlings, despite not showing any significant effect on some hematological parameters (i.e. TAC, ROM, and hematocrit),

probably paid an instantaneous cost to mount an immune response at the halfway through their growth that, to the other hand, it is likely to have increased their resistance against bacteria and virus during the final phase of the rearing period. Considering that the infestation of nest ectoparasites increased over the rearing period the likelihood to be infected by pathogens is higher in older nestlings. The immune enforcement is thus expected to make LPS nestlings more resistant against pathogens in the period of the maximum ectoparasite infestation. To the other hand, control birds, which did not suffer the cost associated to mounting an immune response at the halfway through their growth, were more affected by pathogens during the last phase of the nestling stage. This hypothesis is supported by the fact that nestling body mass at fledging was not different among LPS and control nestlings (Serra et al. 2012). These compensatory responses that LPS and control nestlings performed in temporally distinct phases of their growth, i.e. at the halfway through the rearing period in LPS nestlings and at the end of the rearing period in control nestlings, were probably more costly for control birds as they weakly delayed the first moult and significantly postponed the start of the second-year moult. Admittedly, it is not possible to assess whether the cost associated to mounting an immune response is higher than that associated to contrast pathogens, also considering that such responses have been occurred at different nestling ages. We can hypothesized that although the immune challenge induced an energy expenditure for nestlings, it also produced a long-term advantage for the immune system and the general conditions of birds that could better withstand the subsequent life-history events. By contrast, the costs paid by control nestlings to combat pathogens and the inherent stress in the last phase of the rearing period were not equally linked to advantages during adulthood. Our results could be also explained by hormetic effects, whereby an early mild stress, instead of being detrimental for the organism, can increase the capacity to withstand future stresses in life (Costantini et al. 2010). To confirm this hypothesis, we should investigate whether the dose of LPS injected to nestlings (ca. 1 mg/kg body mass) could represent a mild stress or not, although the results provided so far seem in agreement with this hypothesis.

Often moult duration and plumage quality are positively correlated, as prolonged moult generally corresponded to better plumages (Serra et al. 2007, Vágási et al. 2010). In this study, we showed that the immune challenge influenced the start of the moult but not moult duration. As a consequence, it is not surprising that plumage quality was not different among LPS and control birds, even though individual condition during the course of the moult, which might impair the feather renewal, was not considered in this study. Our findings are coherent with the results of a previous study on starlings, where we showed that the removal of nest ectoparasites allowed males to anticipate and prolong their post-juvenile moult, nonetheless, without an effect on plumage

quality (Manuscript 5). Both these studies suggest that the expression of plumage ornament in starlings is unaffected by early stresses or, alternatively, that iridescent colours are poorly detected through our methods of measuring the spectral shape. Indeed, it cannot be excluded that iridescence of feathers may vary accordingly to small variation of the angle of viewing which, on the contrary, could be better exploited by adults during the mate choice.

In conclusion, our results suggest that the immune activation during development is well tolerated by nestling starlings, which probably performed compensatory measures that prevented to observe an evident effect on individual conditions in the short-term. Contrary to the general prediction that an early stress could have harmful effects later in life, we found that starlings injected with a bacterial endotoxin at the nestling stage advanced the moult start, probably because of hormetic effects that, however, should be tested in future researches. Moreover, it is worthwhile to investigate whether other life-history stages could be positively influenced, even in those species where nestlings showed an immediate negative response to the bacterial infection.

MANUSCRIPT 3

The removal of nest ectoparasites increases the parental effort during the incubation period in the European starling

Simone Pirrello[a,b], Andrea Pilastro[b], Caterina Suzzi[b], Alessandro Andreotti[a], Lorenzo Serra[a]

[a] *Istituto Superiore per la Protezione e la Ricerca Ambientale, 40064, Ozzano Emilia BO, Italy*

[b] *Dipartimento di Biologia, Università di Padova, 35131, Padova, Italy*

Abstract

Breeding pairs adjust their investment in reproduction with respect to a series of factors such as relative partner's investment, environmental conditions, and communication with nestlings, which are likely to influence the quality of the breeding attempt. While there are evidence that nest ectoparasites are challenging for the breeding pairs during the nestling stage, poorly investigated is the effect of parasites on parental investment during the egg phase. In this two-years study, we showed the effect of nest ectoparasites on parental investment during the incubation period in European starling (*Sturnus vulgaris*). Parents spent more time in nests without ectoparasites than in nests with ectoparasites, even though it did not correspond to a different reproductive success among ectoparasite-free and naturally infested nests. Parental investment was positively correlated to fledgling rate, that is the number of fledged nestlings divided by the number of hatched nestlings. Our results suggest that nest ectoparasites affects the parental investment in reproduction already at the egg phase. However, adults may deal with other factors at the nestling stage such as offspring's signals and partner's investment that are likely to further influence their reproductive success.

Introduction

One of the key concepts in the life-history theory is the trade-off between parental investment in current reproduction and the future reproductive success of parents (Trivers 1972). Investment in the former represents a cost in terms of energy expenditure that generally has detrimental effects on parents' body condition and may impair their future survival and/or breeding performance (Nur 1988; Daan et al. 1996; Hanssen et al. 2005). Accordingly, both males and females adjust the amount of energy allocated towards current and future offspring in order to maximise their reproductive success (Williams 1966). Offspring survival and future reproductive

success not only depend on the amount of parental investment, but also on the intrinsic quality (or condition) of the young. So, all things being equal, parents are expected to invest more in the offspring whose probability of surviving and reproducing is greater (Burley 1986). For these reasons, parents may be expected to use cues or signals to reflect nest, egg, chick and mate quality to properly assess the "value" of each breeding attempt and, consequently, adjust their investment in the current reproduction.

For example, adult birds vary their reproductive effort in relation to nestling signals (Kacelnik et al. 1995; Bize et al. 2006; de Ayala et al. 2007) and mate quality (de Lope et al. 1993; Saino et al. 2002; Saino et al. 2003; Rutstein et al. 2005; Horváthová et al. 2011). There is also evidence that parental investment is tuned according to egg and nest features, possibly as a result of a differential allocation strategy (e.g. (Christe et al. 1996; Soler et al. 1998; Moreno et al. 2004; Avilés et al. 2009)). For example, the nest parasite load negatively affects the nestling growth (Christe et al. 1996; Martínez-de la Puente et al. 2010), and parents respond by either increasing their provisioning rates (Christe et al. 1996) or reducing their efforts in the current reproduction (Bukacinski and Bukacinska 2000; Avilés et al. 2009). Clearly, ectoparasites in the nest are likely to reduce nestling fitness, because they face a trade-off between bodily growth and immune system development (Merino 2010). Recent studies have shown that nest ectoparasites influence the parents' behaviour during the nestling stage, probably not only as a result of the communication between offspring and adults (Cantarero et al. 2013), but also because of the direct negative impact on adults (Martínez-de la Puente et al. 2011). However, there are still scarce information on the effect of the nest ectoparasites on the parental investment during the incubation period.

In the present study, we used the European starling (*Sturnus vulgaris*) as a model species to investigate whether the removal of nest ectoparasites during the incubation period could influence the parental investment. We randomly assigned the nests to two experimental groups: 1) nests treated with antiparasitic spray (ectoparasite-free nests) and 2) nests treated with water spray (controls). We then measured the proportion of time spent by males and females in the nest during the egg phase. Both starling parents contribute to incubating the eggs and feeding the chicks (Tinbergen 1981; Feare 1984; Wright et al. 1998; Komdeur et al. 2002). We predicted that parents would differentially adjust their reproductive investment on the basis of the presence of ectoparasites in the nest. Nests without ectoparasites should be more attended by parents, because they would be perceived of better quality and/or involve a minor stress for adults. As a consequence, it is likely that a higher proportion of time spent by parents in the nest would result in a greater hatching success of the clutches deprived of ectoparasites.

Materials and Methods

Study area

The experimental study was carried out in Ozzano Emilia (Bologna), Italy (44°28'N, 11°29'E), at the Istituto Superiore per la Protezione e la Ricerca Ambientale (ISPRA) during the breeding seasons 2011 and 2012. We installed 45 nest boxes (in 2011) and 38 nest boxes (in 2012) on the roofs of two buildings at a height of approximately 15 m and randomly orientated with respect to cardinal points. The nest boxes breeding colony was first installed in 2009, and starlings occupied this colony every year from March to July. Every year the nests were naturally infested by different ectoparasite species, and the dominant ones was the fly *Carnus hemapterus* which mainly feeds on the blood of nestling and adult starlings (Liker et al. 2001).

The first egg was laid on 31st March in 2011 and on 6th April in 2012. The starling pairs produced on average 5 eggs (min 2, max 7) that were incubated mainly by females, whereas males partly contributed to reduce the heat loss of the eggs when females left their nests. The breeding adults were sexed on the basis of morphological traits (Svensson 1992) and marked with individual combinations of coloured rings, which allowed us to identify the male and the female at a distance.

Experimental design

At clutch completion, we randomly assigned each nest to ectoparasite-free or control groups. Parasite-free nests (15 in 2011, and 21 in 2012) were obtained by treating the nest material with an antiparasitic spray (Frontline spray, Fipronil 0.25 g, Merial – Tolose, France) that eliminated all the ectoparasites from the nest, whereas control nests (20 in 2011, and 28 in 2012) were treated with tap water, thereby their ectoparasite loads were unaltered. Each treatment consisted of removing all of the eggs from the nest and then spraying one shot of antiparasitic solution (or water) onto the nest cup. When the nest material was completely dry (usually within three minutes), the eggs were returned to the nest. Both treatments were repeated every three days until fledging, so that during the incubation phase (in about 12 days) it corresponded to 4 treatments.

The nest ectoparasite load was estimated only during the nestling stage, given that during the egg phase it could be underestimated because the ectoparasites feed on incubating adults, so they rapidly move into the nest material when adults fly away from the nest. In contrast, during the nestling stage, the ectoparasites hosted by nestlings are easier to be detected and can be considered as a good index of the overall ectoparasite load of the nest. Hence, we considered the nest ectoparasite load as the mean number of ectoparasites counted during the treatments.

The parental investment during the egg phase was measured with 4 nest observations of 50 minutes in 2011 and of 60 minutes in 2012. The different observation time among years was due to a technical constrain in 2011 that was overcome in 2012. In order to account for this constrain, we considered the parental investment in the clutch as the proportion of time spent by parents inside the nest with regard to the total recording time. The observations were carried out with the technique of digiscoping using two Swarovski 80HD telescopes and two Nikon P6000 cameras. The recording equipments were settled at least 15 m away from the nest boxes, and no evidence of disturbance to the breeding pairs was detected. Observations were carried out in four different periods of the day: 07:00-09:00; 09:00-11:00; 16:00-18:00; and 18:00-20:00. No more than one observation per day was conducted in each nest, and overall recordings covered all the four daily periods in randomised order during incubation.

Statistical analyses

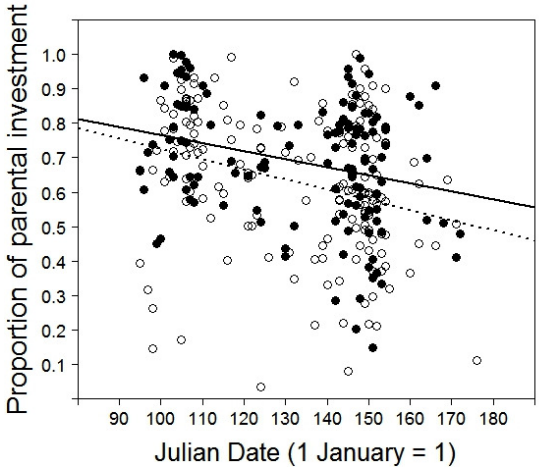
The proportion of time in the nest with regard to the overall recording time was arcsin-square root transformed. The proportion of time spent by parents (MF_{time}) and by females (F_{time}) in the nest were normally distributed after the arcsin-square root transformation, thus they were used as dependent variables in two linear mixed models (LMMs) where treatment (2 levels: ectoparasite-free, control) was the explanatory factor. Observation date was included as the covariate to account for the change in parental investment that naturally occurs across the breeding season, i.e. later clutches are often of lower quality and usually less attended by parents. Nest identity was included in the model as the random intercept factor to account for repeated testing of the same nest. The proportion of time spent by males in the nest (M_{time}) showed a zero-inflated distribution. We therefore tested the effect of the antiparasitic treatment on M_{time} through a two-steps procedure. First, we re-coded the video-recordings of males to a binomial distribution where each video-recording was coded as '1' or '0' if males, respectively, visited or did not visit the nest. We then used a mixed model assuming a binomial distribution (GLMM) to test whether the videos in which males visited the nest ($M_{0/1}$) were different among ectoparasite-free and control nests. Treatment was therefore included in the model as predictor, and nest identity was considered as the random intercept effect to account for repeated testing of the same nest. In the subsequent step, we considered only the videos in which males visited the nest to investigate whether the proportion of time spent by males in the nest ($M_{time(1)}$) was different among nest treatments. This subset of data was normally distributed so we could apply a LMM where $M_{time(1)}$ was the dependent variable, treatment was the fixed factor and nest identity was the random intercept effect.

The clutch size, the number of hatched eggs and the number of fledglings were used to calculate the following reproductive parameters: hatching rate (HR, number of hatched eggs divided by clutch size), fledgling rate (FR, number of nestlings fledged divided by number of nestlings hatched), and clutch success (CS, number of nestlings fledged divided by clutch size). These variables were normally distributed, thus they were used as dependent variables in three mixed models to test whether they differed among ectoparasite-free and control nests.

Mixed models were carried out using the library *lmerTest* within the software R 3.0.3 (R Development Core Team).

Results

The proportion of time spent by parents in the nest (MF_{time}) was significantly higher in ectoparasite-free than control nests (effect of the antiparasitic treatment: 0.07 ± 0.03 SE, d.f. = 1, 77.6, t = 2.49, P = 0.015; Fig. 1). The proportion of time that females spent in the nests (F_{time}), which is the sex that often invest more energy in the breeding attempt, was not different among ectoparasite-free and control nests (antiparasitic treatment: 0.04 ± 0.03 SE, d.f. = 1, 77.3, t = 1.18, P = 0.24). The videos in which males visited the nest ($M_{0/1}$) were not different among nest treatments (antiparasitic treatment: 0.3 ± 0.4 SE, z = 0.82, P = 0.41). Also considering only the videos in which males visited the nests, the proportion of time ($M_{time(1)}$) that they spent in nests without ectoparasites was similar to the time spent by males in nests with ectoparasites (antiparasitic treatment: 0.08 ± 0.06 SE, d.f. = 1, 39.5, t = 1.41, P = 0.17).



Tab. 1 - Proportion of time spent by parents in the nest during the incubation period. Nests treated with the insecticide are shown with black dots (the corresponding regression line is solid black), whereas nests naturally infested with ectoparasites are shown with white dots (dashed regression line).

Hatching rate (HR), fledgling rate (FR), and clutch success (CS) did not differ among ectoparasite-free and control nests ($P > 0.5$). The proportion of time spent by parents in the nest was positively correlated to FR (Pearson's correlation: $t = 2.7$, d.f. $= 73$, $P = 0.009$), thus to the proportion of nestlings which fledged with respect to the brood size.

Discussion

In this two-years study, we investigated the effect of the nest ectoparasites on the proportion of time that starling parents spent in the nest during the incubation period. Nest ectoparasites are one major stress for breeding pairs, and while there is evidence of their negative effect on nestling growth and on parent-offspring communication (e.g. (Møller 1993; Cantarero et al. 2013) but see (Tripet et al. 2002), it is unclear whether they could influence the parental investment during the incubation period. We demonstrated that in the European starling the removal of nest ectoparasites increased the proportion of time that parents spent in the nest during the incubation stage, although the contribution of each partner was not significantly different among nest treatments.

The parental investment in reproduction can be influenced by various factors, such as nest quality (Gwinner and Berger 2005), begging of chicks (Jacob et al. 2011), allocation of the other partner (Wright and Cuthill 1990), which are likely to determine the reproductive investment of the breeding pair. During the incubation period the adults rely on a reduced number of cues to assess the quality of the reproductive attempt, and their allocation in the current reproduction could change significantly if they evaluate that costs outweigh the benefits. Considering the differential investment in reproduction between the sexes (Kokko and Jennions 2008), we should expect a differential response among males and females to nest condition. Male starlings are facultative polygynous, and thereby should be more sensitive to the quality of each reproductive attempt as they could adjust their overall investment in order to allocate more resources in the clutch or brood that provides the maximum fitness return (Smith et al. 1995). Our study showed that females invested more time in the nest during the incubation period, whereas males often did not visit the nest at all. This implies that the female contribution was greater than that of males. Indeed, considering the proportion of time spent by females (F_{time}) and males (M_{time}) in the nests with respect to the overall time (MF_{time}), females stayed in the nests 92% of the overall time. Nonetheless, the proportion of time spent by females in the nest did not differ significantly among ectoparasite-free and control nests. Females therefore invested a similar amount of energy among nest treatments, in support to the idea that they are less susceptible to nest ectoparasites and, more in general, to nest condition once they started to incubate their eggs. Clearly this assumption does

not exclude that females will pay the costs associated with attending the ectoparasite-load clutches later in life with possible negative consequences for their survival. Males visited the ectoparasite-free nests in 32% of videos (N = 130) and the control nests in 27% of videos (N = 173), but this difference, along with the proportion of time they spent in the nests of both treatments ($M_{time(1)}$), was not significant.

Males and females tended to stay longer in nests without ectoparasites than in nests with ectoparasites, but their overall contribution was significantly different among treatments. It remains unclear whether the adults stayed longer in nests without ectoparasites because they perceived the higher quality of these nests or because of the absence of the stress caused by the ectoparasites. Whatever the reason for spending more time in ectoparasite-free nests, we showed that irrespective to the nest treatments the greater was the time in the nests the higher was the fledgling rate, i.e. the number of nestlings fledged with respect to the number of nestlings hatched. Fledgling rate is a reproductive parameter which should be mainly influenced by the parental investment during the nestling period, considering that it accounts for the number of nestlings that the breeding pair raised till fledging. Parents that spent more time in the nests during the egg phase are likely to have invested also more energy in raising the chicks. It is quite surprising, however, that the other reproductive parameters (hatching rate and clutch success), which are referred to, respectively, the number of nestlings hatched and the number of nestlings fledged with respect to clutch size were not associated to the time spent by parents in the nests during the incubation period. This result is difficult to be explained given that it seems in contrast with the other findings. It was more likely that a greater parental investment during the incubation period would have yielded to a larger amount of nestlings hatched and, if the parental allocation was also greater during the nestling period, as suggested by the FR, to a greater number of nestlings fledged. Contrary to prediction, the number of nestlings hatched and the number of nestlings fledged with respect to clutch size were not correlated to parental investment during the incubation period. A possible explanation could be that female starlings often are involved in extra-pair copulations (Pinxten and Eens 1997), and thereby they lay eggs which belong to different partners. If one or more partners were unfertile, a few eggs will remain unhatched and their proportion should be positively associated to the frequency of extra-pair copulations. Consequently, clutch size is probably an unreliable cue to assess the effectiveness of the parental investment in a polygynous species with extra-pair copulations. As a consequence, the reproductive parameters that are calculated on the basis of the clutch size (HR and CS) could be also unreliable.

In conclusion, we showed that the presence of nest ectoparasites represents a stress for the breeding pairs during the incubation period, and therefore that is one of the main factors that

influence the fitness of adults. We also showed that a greater proportion of time spent in the nest, whatever the nest ectoparasite load, was directly related to the fledgling rate, which is a reproductive parameter that is strongly influenced by the overall parental investment in reproduction. Studies on parental allocation in reproduction could therefore rely on the proportion of time spent by parents in the nests during the incubation period as a proxy of the overall investment in the reproductive attempt.

MANUSCRIPT 4

Nest ectoparasites decrease the nestling begging intensity in the European starling *Sturnus vulgaris*

Simone Pirrello[a,b], Diego Rubolini[c], Andrea Pilastro[b], Matteo Pozzato[b], Nicola Saino[c], Lorenzo Serra[a]

[a]*Istituto Superiore per la Protezione e la Ricerca Ambientale, 40064, Ozzano Emilia, Bologna, Italy*
[b]*Dipartimento di Biologia, Università degli Studi di Padova, 35121, Padova, Italy*
[c]*Dipartimento di Bioscienze, Università degli Studi di Milano, 20133, Milano, Italy*

Abstract

In altricial birds, offspring solicit food provisioning through multiple signals that shape the allocation decisions of parents. These conspicuous displays have evolved under a conflict of interests, where offspring seek more resources than parents should be selected to provide. Parent-offspring communication can be further complicated by different stresses, such as predation risk, limited food or environmental condition. In this study, we investigated the effect of removing the stress caused by nest ectoparasites on the postural and visual components of begging in the European starling (*Sturnus vulgaris*). Visual and postural components of begging and parental efforts were measured at two nestling ages (at 4 and 10 days). The removal of nest ectoparasites increased the postural begging intensity but did not influence the parental provisioning effort. The presence of nest ectoparasites did not affect body mass, hematocrit level, and the cell-mediated immune response of nestlings. Flange and skin colouration, although were not influenced by nest ectoparasites, resulted associated to body mass. Flange brightness was positively correlated to PHA response. Intensity of postural begging increased in older nestlings and parents responded by increasing their provisioning frequency. Parents visited more often 4 days broods with brighter flange colouration, and 10 days broods with more UV and yellow saturated flange. A greater number of fed nestlings during each parent's visit were observed in broods with more UV saturated skin. Our results suggest that parental allocation decisions in the European starling are poorly affected by the nest ectoparasites, but are influenced by the multiple components of begging.

Introduction

Parent-offspring communication is a complex and dynamic event that is regulated by several factors. There is a conflict of interests between young and parents, as the former often require more resources than the latter are selected to provide (Williams 1966). Begging of nestling birds is a multimodal signal where postural, visual, and acoustic components have evolved to solicit parental care (Godfray 1995; Kilner 2002; Jacob et al. 2011). Nestling solicitations are aimed at obtaining a greater share of limiting resources (Godfray 1995) or as signals of need that parents use to adjust their allocation strategies and maximize their fitness return (Trivers 1974; Johnstone and Godfray 2002).

The postural components of begging are influenced by brood size (Leonard et al. 2000), offspring relatedness (Briskie et al. 1994; Boncoraglio and Saino 2008), age and sex of nestlings (Teather 1992; Cotton et al. 1999; Saino et al. 2003), but see (Whittingham et al. 2003). The intensity of postural begging reflects the hunger status of chicks (Cotton et al. 1999; Johnstone and Godfray 2002). Along with the postural begging, the visual components of begging, i.e. skin and mouth colourations, convey different information to parents (Jourdie et al. 2004; Bize et al. 2006; Jacob et al. 2011). For example, the orange-yellow colouration of the nestling mouth is positively correlated to the amount of circulating carotenoids (Ewen et al. 2008; Thorogood et al. 2008) and to the physiological condition of nestlings (Hunt et al. 2003; Dugas and Rosenthal 2010), but see (Ewen et al. 2008). Skin with high ultraviolet reflectance results in prime condition nestlings and increases the parental effort during the current breeding attempt (Jourdie et al. 2004; Bize et al. 2006). In addition, it has been demonstrated that the flange yellow colouration and the skin ultraviolet colouration are dynamic components of begging in nestling starlings that parents use to adjust their investment in the current breeding attempt (Jacob and Heeb 2013).

Three main models could explain how the begging of nestlings affects parental provisioning strategies (Mock et al. 2011). In the first model, parents have a total control on allocation decisions, whereby they adjust their provisioning on the basis of the signals perceived from nestlings. The other two models ("scramble begging model" and "sibling negotiation model") predict a different key assumption, i.e. the allocation decisions are mainly affected by the competition among nestlings whereas parents face only a passive choice. Environmental conditions play a determinant role in parental allocation strategies, as recently shown in two passerine species, Alpine swift (*Apus melba*) and European starling (*Sturnus vulgaris*), where parents preferred to feed nestlings with low signal intensities as the breeding season progressed (Bize et al. 2006)

Many experimental studies showed that the communication between parents and nestlings can be influenced by the ectoparasites hosted by their nests (Christe et al. 1996; Richner and Tripet 1999; O'Connor et al. 2010; Cantarero et al. 2013; O'Connor et al. 2014). Nest-dwelling ectoparasites have been shown to affect the individual conditions within the brood, as infested nestlings are solicited to boost an immune response (Szép and Møller 1999) and increase their growth effort (Christe 1996; Tomás et al. 2008; Martínez-de la Puente et al. 2011; Cantarero et al. 2013). A recent study on pied flycatchers (*Ficedula hypoleuca*) showed that begging was associated to the abundance of nest ectoparasites (Cantarero et al. 2013), whereas in Darwin's finches (*Geospiza fortis*) nestlings that were highly infested by blood-sucking parasites during the previous night produced less intensive begging the day following (O'Connor et al. 2014). Both studies, however, found a positive correlation between begging intensity and parental provisioning rates, as also shown in great tits (*Parus major*) (Christe et al. 1996). Parents could compensate for the nest ectoparasites by grooming nestlings and investing time in nest sanitation activities (Cantarero et al. 2013). Nest ectoparasites therefore represent one of the major stresses for both nestlings and parents. Although various studies examined the effects of ectoparasites on the nestlings and the breeding pairs, it was poorly investigated the direct effect of ectoparasites on the different components of begging and the relative responses of parents.

In this study, we tested the effect of removing the ectoparasites from the European starling nests on the multiple components of begging and on parental provisioning. A group of nests was treated with a water-based antiparasitic spray to remove the ectoparasites from the nest material whereas a group of control nests was treated with a water spray. We measured the visual and postural components of begging, the number of parental visits per hour, and the number of fed nestlings during each visit in two different phases of the nestling stage, i.e. at 4 and 10 days. We considered the nestling body mass, the hematocrit levels, and the immune response as indexes of nestling condition.

Nestling starlings infested by ectoparasites are expected to be weaker than their ectoparasite-free counterparts as the stress caused by the ectoparasites should yield nestlings to high weight loss, mainly due to blood sucking parasites (Fessl et al. 2006), and to a reduced sleep time (O'Connor et al. 2010). Considering the contrasting results provided so far on begging in parasitized nestlings (Christe et al. 1996; Cantarero et al. 2013; O'Connor et al. 2014), we aimed to investigate whether parasite removal could influence the conspicuousness of begging in nestling starlings.

The visual components of begging, i.e. flange and skin colourations, are condition –dependent traits in nestling starlings (Jacob et al. 2011) so they were expected to change accordingly to nestling condition. It has been shown that flange carotenoid of nestling starlings is a dynamic trait

that is positively correlated to nestling body mass (Jacob et al. 2011; Jacob and Heeb 2013). We therefore predicted that nestlings from ectoparasite-free broods should present more saturated flange carotenoids as they were expected to have greater body mass than naturally infested nestlings.

Parents could respond to the nest ectoparasites either increasing their visit frequency at the nest, as shown in blue tits *Cyanistes caeruleus* and great tits (Christe et al. 1996; Tripet and Richner 1997; Hurtrez-Bousses et al. 1998), or increasing the number of nestlings provided with food during each visit, as recently found in Darwin's finches (O'Connor et al. 2014).

Materials and Methods

Study area and field procedures

In February 2012, we installed 38 nest boxes in Northern Italy (Ozzano Emilia, Bologna, 44°28'N, 11°29'E) on the roofs of two buildings at a height of approximately 15 m and randomly orientated with respect to cardinal points. The nest boxes were made of softwood panels (2 cm thick) with inside dimensions of 15 × 15 cm (base) × 45 cm (height) and an entrance hole size diameter of 4.5 cm (the distance of the hole from the base was 31 cm). The roof of the nest boxes could be opened to check the progression of the breeding attempts. Each nest box was provided with a transparent plastic box with dimensions of 13 x 7 cm (base) x 4 cm (height) that was fixed to the inner side of the roof and where the digital infrared camera (Mini Vehicle DVR CR-01, China) was positioned for recording. The infrared camera was collocated in the plastic box just before starting the recording and was removed soon after the recording ended, whereas a black cardboard was kept in the plastic box to simulate the presence of the infrared camera in the remaining time of the breeding event.

The nest boxes were checked daily during the period 15:00-18:00 to control for progression of egg deposition. We used 29 breeding events that occurred in 24 nest boxes. Two consecutive depositions were observed in 5 nest boxes which, however, were cleaned and sanitized between the two breeding attempts. After clutch completion, we randomly assigned each nest to the treated or control group. Laying date (mean ± SE = 142 ± 4 Julian day in treated nests, and 136 ± 6 Julian day in control nests; t-test = 0.8, P = 0.5), clutch size (mean ± SE = 5.1 ± 0.2 eggs in treated nests, and 4.9 ± 0.3 eggs in control nests; t-test = 0.4, P = 0.7) and brood size at 4 days (mean ± SE = 3.9 ± 0.2 nestlings in treated nests, and 4.1 ± 0.3 nestlings in control nests; t-test = -0.6, P = 0.5) and at 10 days (mean ± SE = 3.8 ± 0.2 nestlings in treated nests, and 3.8 ± 0.4 nestlings in control nests; t-test

= 0.03, P = 0.97) did not differ among nests in treated and control group. We experimentally removed the ectoparasites from 16 nests (deparasitised) and considered another group of 13 naturally infested nests as control. The ectoparasites were removed using an antiparasitic spray (Frontline spray, Fipronil 0.25 g, Merial – Tolose, France), whereas the control clutches were sprayed with tap water. Each treatment consisted of removing the eggs or chicks from the nest and then spraying one shot of antiparasitic solution or water onto the nest cup. When the nest material was completely dry (usually within three minutes), the eggs or chicks were returned to the nest. Both treatments were repeated every three days until fledging. Different ectoparasite species were observed within the nest boxes, e.g. fleas and mites, but the dominant species was the fly *Carnus hemapterus* which mainly feeds on the blood of nestling starlings (Liker et al. 2001), and can thus be considered as one of the major stresses for growing nestlings. During the nest treatment we counted the total amount of *Carnus hemapterus* hosted by the brood to assess the effectiveness of the antiparasitic treatment. We considered the mean ectoparasite load of the nest as the mean number of *C. hemapterus* counted in the nest during the nestling phase. The antiparasitic spray removed almost all the ectoparasites from the nests (mean ectoparasite load: 0.4 ± 0.3 SE in ectoparasite-free nests and 9.3 ± 1.3 SE in control nests, Kruskal-Wallis test: $\chi^2 = 19.7$, $P < 0.001$), and the difference between treatments was consistent over the entire nestling period.

The brood behaviour within each nest box was recorded in two trials when nestlings were, respectively, 4 and 10 days old, as these times correspond to two different phases of the nestling growth. At four days nestlings grow at a higher intensity than at 10 days, as their increase in body mass has its maximum slope (mean ± SE body mass at 4 days: 24.6 ± 0.8 g). At 10 days the nestlings have approximately reached the plateau of their body mass (mean ± SE body mass at 10 days: 52.2 ± 0.8 g) that was approximately the body mass at fledging which in the European starling occurs when nestlings are 19-21 days (mean ± SE body mass at fledging: 60.0 ± 0.8 g). During each trial, the nestlings were weighed next to the nest boxes with an electronic balance (accuracy of 0.1 g). In the plastic box on the nest roof we changed the black cardboard with the infrared camera that was supplied during the entire trial by an external battery that was hidden in proximity to the nest box, as well as the electric wire connecting the battery to the camera. We did not observe any effect of the presence of the battery and the electric wire on parents' behaviour during the course of the trials. The footages were carried out during the period 8:00-12:00 for an average time of 169 minutes (max 226, min 93). At the end of each trial, the infrared camera was removed from the nest and changed with the black cardboard, and the power supply devices (i.e. battery and wire) were removed from the proximity of the nest boxes. Afterward, half of the brood was moved to the laboratory for measuring nestling flange and skin colouration, whereas the other half of the brood

was left in the nest to avoid brood desertion. Nestlings were kept warm and at dark during the transport to the laboratory. After about 10 minutes the two halves of the brood were exchanged, and after 20 minutes from the end of the trial the entire brood was returned to the nest.

Hematocrit assay and immune response

At the end of the second trial, a blood sample was drawn from the brachial vein of each nestling into 3-4 micro-hematocrit capillary tubes. We used a Centric 150 centrifuge (Intercontinental Srl, Anzio, Italy) to centrifuge the blood samples at 11500 rpm for 10 min (centrifuge radius 94 mm) and to separate the plasma from the red blood cells. The hematocrit (proportion of red blood cells over total blood volume) was measured on capillary tubes with a dial caliper (accuracy of 0.1 mm).

The nestling immunocompetence was estimated with a PHA-induced skin swelling test (Tella et al. 2008). A subcutaneous injection of 0.2 mg of the mitogen phytohemagglutinin (PHA - Sigma, L-8754) diluted in 0.5 ml phosphate-buffered saline (PBS) was done to the right wing patagium of each nestling at the end of the second trial. The induced swelling at the site of injection was measured after 24h with a micrometer (Teclock SM-112, Japan, accuracy of 0.01 mm). The cell-mediated immunocompetence was assessed as the difference between the thickness of the patagium post- and pre-injection.

Visual components of begging

We measured the spectral reflectance of the flange and the skin of each nestling with an Ocean Optics S2000 spectrometer as these colourations represent the visual components of begging in nestling starlings (Jacob et al. 2011). Before each measurement session, the spectrometer was calibrated to a WS-2 white standard and to darkness, so the measurements were referred to these standards. At the end of the first trial, we measured the spectral reflectance of flange and skin, whereas at the end of the second trial we measured only the spectral reflectance of flange as the skin was totally covered by the feathers. The ambient light was always excluded with a black PVC tube mounted on the ferrule tip of the probe that held the probe tip at a distance of 5 mm from the surface. The probe was always held at 90 degree from the skin or flange. We took two measurements for the flange (to the right and left side) and four for the skin (head, back, throat, and breast), which were the averages of five consecutive measures taken automatically by the spectrometer. The final spectra of flange and skin was the mean spectra among, respectively, two and four different measurements.

The spectral reflectance was analysed using separate principal component analyses (Cuthill et al. 1999), i.e. two for flange (at 4 and at 10 days) and one for skin (at 4 days). The investigated range of wavelengths (300-700 nm) was first divided in 40 classes of 10 nm, then the mean brightness of the whole spectrum was subtracted from the mean brightness of each class. This standardization eliminates the strong correlation between the first PCA score and brightness (Cuthill et al. 1999). We therefore performed three PCAs to analyse the spectra of flange at 4 and 10 days and the spectrum of skin at 4 days. We considered two principal components for the flange and three for the skin spectra, that together accounted for 91.5% (flange at 4 days), 93.7% (flange at 10 days), and 92% (skin) of variance of the original spectra. The mean reflectance corresponds to the average value of reflectance in the investigated range and we regarded it as an index of brightness (Hill and McGraw 2006). Skin reflectance showed only one peak in correspondence of the ultraviolet part of the spectrum (300-400 nm), whereas flange reflectance had two peaks, one in the UV range and one in the visible part of the spectrum (400-700 nm). The peak in the visible part of the flange spectrum has been shown to reflect the carotenoid availability in nestlings of different species (e.g. (de Ayala et al. 2007; Thorogood et al. 2008). We calculated the UV chroma (UVC) of the flange and the skin as the proportion of reflectance between 300 and 400 nm with respect to total reflectance ($UVC = R_{300-400} / R_{300-700}$). We also calculated the carotenoid-chroma (CaroC) of the flange [$(R_{450-R700}) / R_{700}$], as it represents the most appropriate objective colorimetric parameter for quantifying the spectral purity (Hill and McGraw 2006). Pearson's correlations were used to investigate the co-variation between PCA scores and colour parameters.

Postural begging and parental provisioning

The video-recordings analyses were performed using VLC media player 2.1.3 (Free Software Foundation, Inc., Boston, MA). A video-recording was divided in begging events that corresponded to the times in which parents entered in the nests to provide food. During each begging event we scored the maximum begging intensity of all the nestlings using the scale proposed by Leonard et al. (Leonard et al. 2003). This begging scale considers the conspicuousness of the begging intensity and includes the following scores: 0 (head down, no gaping), 2 (head up, gaping, sitting on tarsi), 3 (same as 2, plus neck stretched upward), 4 (same as 3, but body lifted off tarsi), and 5 (same as 4, plus wings waving). Postural begging intensity for each begging event was evaluated as the mean of individual scores of all nestlings. During each begging event we also considered the number of nestlings that were fed by the parent (number of fed nestlings) and we calculated the number of parental visits per hour (parental provisioning rates).

Statistical analysis

The measures of nestling condition (body mass, hematocrit, and response to PHA test) were analysed with linear mixed models (LMMs). Body mass was included as dependent variable whereas treatment (coded as 1 for deparasitized and 0 for control) and nestling's age (4 and 10 days) were considered as fixed factors. Interaction between treatment and age was initially entered as fixed factor, but it was subsequently removed from the final model as not significant. Hematocrit and response to PHA test were included as dependent variable in two separate LMMs, where treatment (1 for deparasitized and 0 for control) and date were included as fixed factors. Nest identity was included as random intercept effect to account for repeated testing of the same brood at two ages.

The analyses of the visual component of begging, i.e. PC1 and PC2 of flange at 4 and 10 days; PC1, PC2, and PC3 of gape at 4 and 10 days; PC1, PC2, and PC3 of skin, were performed with linear mixed models with the dichotomous term "treatment" as predictor. Brood size and nestling body mass were included in the models as covariates, whereas nest identity was included as random intercept effect to account for repeated testing of the same brood at two ages.

Because we did not identify the nestlings during each begging event, in order to avoid pseudoreplication we considered the mean begging intensity of the brood during each begging event as dependent variable in a linear mixed model assuming a Gaussian distribution. Treatment (1 for deparasitized and 0 for control), nestling age (4 and 10 days), and the interaction between treatment and nestling age were included as predictors. Brood size was included in the model as the covariate because the number of nestlings is predicted to influence the begging intensity. Nest identity was included as random intercept effect to account for repeated testing of the same brood at two ages.

Parental provisioning rate was analysed with a mixed model assuming a Gaussian distribution where the antiparasitic treatment, the nestling age, and the interaction between antiparasitic treatment and nestling age were the predictors. Nest identity was included as random intercept model to account for repeated testing of the same brood at two ages. Brood size was included as the covariate because the frequency of parental visits should be related to the number of nestlings to feed.

The number of fed nestlings was analysed with a mixed model assuming a Poisson distribution with antiparasitic treatment and nestling age as explanatory factors. The interaction term between treatment and nestling age was initially included in the model but subsequently removed as not significant. Nest identity was entered as random intercept effect to account for repeated testing of the same brood at two ages. Brood size was included as the covariate as the

number of nestlings which were feed at each feeding event should be related to the number of nestlings in the nest.

Mixed models were carried out using the library *lmerTest* within the software R 3.0.3 (R Development Core Team). Differences between groups were explored by post hoc tests using the library *multcomp*. We adopted an information-theoretic approach, testing all linear combination of variables, and then using Akaike's Information Criterion (AIC) to select the best performing model.

Results

Effects of ectoparasite removal on nestling condition and nestling visual signals

All the measures associated to nestling condition were not significantly influenced by our experimental manipulation. Nestling body mass resulted unaffected by the antiparasitic treatment in either the trials (Tab. 1). Also the increase of body mass from 4 to 10 days was similar among treated and control nests (LMM, parasite removal: -28.2 ± 28.5, d.f. = 1, 23.2, t = -1.0, P = 0.33; brood size: -6.2 ± 18.1, d.f. = 1, 23.5, t = -0.34, P = 0.73), thereby nestlings grew at similar speeds in nests with or without ectoparasites.

Variables	**Fixed Effects**	**Estimate ± SE**	**d.f.**	**t**	**P**
Body mass	Treatment	-0.3 ± 15.6	1, 23.8	-0.02	0.99
(N = 199)	Age (4 or 10 days)	286.1 ± 8.8	1, 172.1	32.4	< 0.001
Hematocrit	Treatment	0.4 ± 1.5	1, 17.8	0.24	0.82
(N = 75)	Date	-0.13 ± 0.04	1, 17.7	-3.41	0.003
Immune response	Treatment	-0.5 ± 5.4	1, 18.9	-0.10	0.92
(N = 84)	Date	0.02 ± 0.14	1, 18.9	0.16	0.88

Tab. 1 – Linear mixed models of the effects of ectoparasite removal on nestling condition. Nest identity was included as random intercept model. The interaction term between fixed factors were initially included in the model but subsequently removed as not significant. See methods for further details on statistical procedures.

At 10 days, hematocrit levels and swelling responses to the PHA test were not different among nestlings in treated and control nests, whereas they were positively correlated to nestling body mass. Heavy nestlings showed high hematocrit levels (LMM with nest identity as random intercept; hematocrit: 8.3 ± 1.7, d.f. = 1, 41.4, t = 4.84, P < 0.001) and strong immune response

(LMM with nest identity as random intercept; PHA response: 2.9 ± 0.5, d.f. = 1, 81.6, t = 6.03, P < 0.001). Hematocrit levels, but not the swelling responses, decreased during the course of the breeding season (Tab. 1).

All the principal component scores of the flange and the skin spectrum were unaffected by the antiparasitic treatment (Tab. 2).

Variables	**Fixed effects**	**Estimate ± SE**	**d.f.**	**t**	**P**
Flange PC1 (4 days)	Treatment	-0.02 ± 0.30	1, 21.5	-0.063	0.95
	Brood size	-0.15 ± 0.19	1, 22.0	-0.776	0.45
	Body mass	-0.005 ± 0.001	1, 91.2	-2.989	0.004
Flange PC2 (4 days)	Treatment	0.06 ± 0.28	1, 21.5	0.206	0.84
	Brood size	-0.20 ± 0.18	1, 22.1	-1.151	0.26
	Body mass	-0.009 ± 0.002	1, 92.0	-4.955	< 0.001
Flange PC1 (10 days)	Treatment	-0.12 ± 0.29	1, 23.4	-0.394	0. 70
	Brood size	0.14 ± 0.19	1, 24.4	0.731	0.47
	Body mass	-0.003 ± 0.001	1, 93.6	-2.464	0.016
Flange PC2 (10 days)	Treatment	-0.04 ± 0.31	1, 23.2	-0.114	0.91
	Brood size	0.01 ± 0.20	1, 23.6	0.053	0.96
	Body mass	-0.002 ± 0.001	1, 93.5	-1.977	0.051
Skin PC1	Treatment	0.08 ± 0.33	1, 23.3	0.256	0.80
	Brood size	-0.21 ± 0.21	1, 23.5	-0.996	0.33
	Body mass	-0.008 ± 0.001	1, 85.3	-6.102	< 0.001
Skin PC2	Treatment	-0.44 ± 0.34	1, 23.5	-1.318	0.20
	Brood size	-0.33 ± 0.21	1, 23.8	-1.571	0.13
	Body mass	0.004 ± 0.002	1, 91.6	2.196	0.031
Skin PC3	Treatment	-0.12 ± 0.32	1, 22.9	-0.378	0.71
	Brood size	0.07 ± 0.21	1, 23.2	0.336	0.74
	Body mass	-0.001 ± 0.002	1, 92.5	-0.410	0.68

Tab. 2 – Linear mixed models of the effects of nest treatment, brood size, and nestling body mass on the principal components of the PCAs on the flange and the skin spectrum.

In either the nestling ages, Flange PC1 was negatively correlated to brightness whereas Flange PC2 was positively correlated to UVC and CaroC (Tab. 3). Flange PC1 of 4 and 10 days nestlings, and Flange PC2 of 4 days nestlings were also negatively correlated to nestling body mass

(Tab 3). As result, nestlings with lower body mass showed bright flange with low saturated UV and yellow colourations, especially during the first trial.

In 4 days nestlings, Skin PC1 resulted negatively correlated to UVC whereas Skin PC3 was negatively correlated to brightness (Tab. 3). Nestling body mass resulted correlated to Skin PC1 (negatively) and to Skin PC2 (positively) (Tab. 3). This indicates that during the first trial heavy nestlings showed more saturated skin in the ultraviolet part of the spectrum. The combination of flange and skin colouration yielded 4 days nestlings at low body mass to have more coloured flange and less coloured skin in the UV range.

Brightness was negatively correlated to UVC and CaroC, and UVC was positively correlated to CaroC (Tab. 3).

	B	UVC	CaroC
Brightness (B)			
UV Chroma (UVC)	-0.36*		
Carotenoid Chroma (CaroC)	-0.43*	0.78*	
Flange PC1 at 4 days	-0.63*	0.44*	0.30**
Flange PC2 at 4 days	0.44*	0.59*	0.41*
Flange PC1 at 10 days	-0.60*	0.53*	0.48*
Flange PC2 at 10 days	0.53*	0.76*	0.53*
Skin PC1	0.12	-0.46*	
Skin PC2	0.04	-0.13	
Skin PC3	-0.20**	0.09	

Tab. 3 – Pearson's correlations between the principal components of the PCAs and the colour parameters Brightness, UV chroma, and Carotenoid chroma of the flange and the skin spectra. * $P < 0.001$, ** $P < 0.05$

Correlation between physiological condition and nestling visual signals

Flange PC1 score at 10 days was negatively correlated to the swelling response to PHA test (Flange PC1: -0.021 ± 0.008, d.f. = 1, 78.6, $t = -2.67$, $P = 0.009$; Fig. 2). Since Flange PC1 at 10 days was negatively correlated to brightness and positively associated to UVC and CaroC, the prime immune system of nestlings resulted in flanges with high brightness, and low UV and yellow saturation. In contrast, Flange PC1 and Flange PC2 of 10 days nestlings did not result associated to hematocrit levels (Flange PC1: -0.05 ± 0.03, d.f. = 1, 56.4, $t = -1.65$, $P = 0.104$; Flange PC2: -0.04 ± 0.03, d.f. = 1, 65.4, $t = -1.63$, $P = 0.107$).

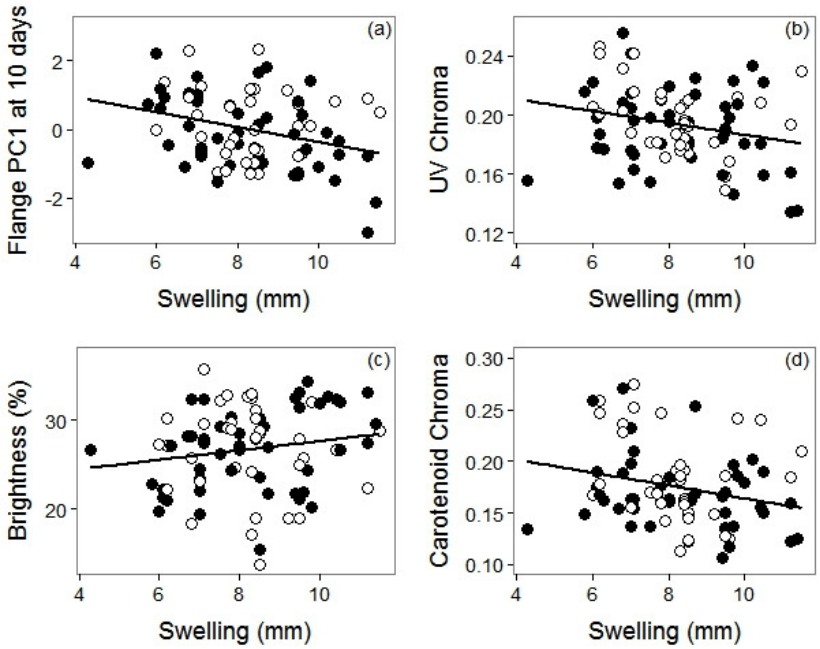


Fig. 2 – Correlation between the PHA response (skin swelling) and the first principal component score of the flange spectrum (a), UV Chroma (b), Brightness (c), and Carotenoid Chroma (d) of 10 days nestlings. Black dots = nestlings from deparasitized nests; white dots = nestlings from control nests.

Postural begging and parental provisioning

The mean intensity of postural begging was larger in deparasitized than in control broods, and positively related to brood size (Tab. 4). In broods with a larger number of offspring, begging was thus more intense compared to smaller broods. We found also a significant increase of postural begging intensity from 4 to 10 days nestlings. Nestlings from ectoparasite-free nests showed a more intensive postural begging at 4 days whereas at 10 days their begging resulted less conspicuous than that of nestlings from control nests (Fig. 3).

Parental provisioning rate, i.e. the number of visits per hour, did not differ significantly among treated and control nests (Tab. 4), whereas parents increased the visit frequency to broods from 4 days to 10 days nestlings. Parental provisioning rate was associated to mean begging intensity of 10 days nestlings (Pearson's correlation = -3.87, P = 0.001), while it was not correlated to begging intensity of 4 days nestlings (Pearson's correlation = -0.31, P = 0.76). At 10 days, nestlings that produced a higher begging intensity were visited less often by parents. During each visit, parents fed more nestlings during the first trial than during the second trial, whereas the antiparasitic treatment did not affect the number of fed nestlings during each visit (Tab. 4).

Variables	Fixed Effects	Estimate ± SE	d.f.	t / z	P
Postural begging intensity	Treatment	0.8 ± 0.3	1, 24.2	2.35	0.027
	Age (4 and 10 days)	2.2 ± 0.1	1, 1068	17.89	< 0.001
	Treatment * Age	-1.5 ± 0.1	1, 1160	-11.92	< 0.001
	Brood size	0.7 ± 0.1	1, 682	8.55	< 0.001
Parental provisioning rate	Treatment	3.8 ± 3.3	1, 32.3	1.12	0.27
	Age (4 and 10 days)	12.7 ± 3.0	1, 21.3	4.24	< 0.001
	Treatment * Age	-4.6 ± 3.3	1, 16.7	1.42	0.17
	Brood size	-1.7 ± 1.6	1, 30	1.07	0.30
Number of fed nestlings	Treatment	0.08 ± 0.08	-	1.05	0.30
	Age (4 and 10 days)	-0.10 ± 0.09	-	-1.14	0.25
	Treatment * Age	-0.01 ± 0.11	-	-0.10	0.92
	Brood size	0.03 ± 0.04	-	0.76	0.45

Tab. 4 – Mixed models of the effect of nest treatment, nestling age, and their interaction on the postural begging of nestlings, on parental provisioning rate (number of visits / h), and on number of fed nestlings during each visit. Brood size was included in the models as the covariate.

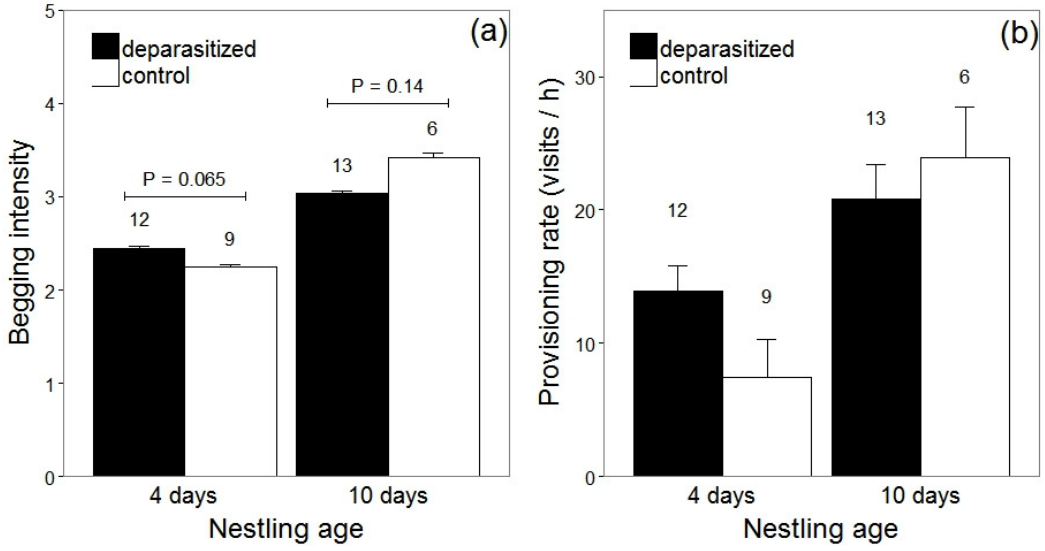


Fig. 3 – Intensity of postural begging (a) and parental provisioning rate (b) in deparasitized and control nests at the two nestling ages (4 and 10 days). Bars are mean values + SE. Above each bar is shown the number of broods, whereas the P-values is associated to Tukey post hoc test.

Covariation between visual signals and parental effort

In broods of 4 days nestlings, parental provisioning rate was positively related to Flange brightness, whereas in broods of 10 days nestlings parents visited more often broods with higher UV and Carotenoid Chroma (Fig. 4). While Skin colouration did not affect the frequency of parental visits, the number of fed nestlings was greater in broods with higher Skin UV Chroma (Fig. 5).

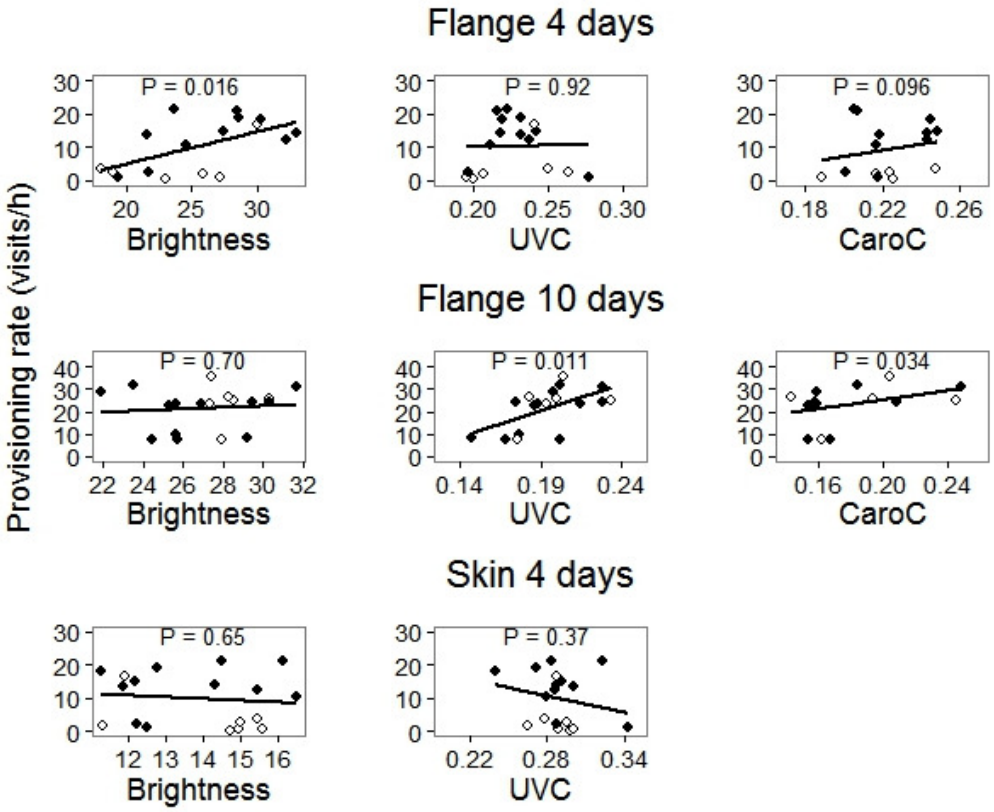


Fig. 4 – Correlation between provisioning rate (parental visits per hour) and nestling visual signals, i.e. brightness, ultraviolet chroma (UVC), and carotenoid chroma (CaroC). Black dots shown deparasitized nests whereas white dots shown control nests. P-value is associated to Pearson's correlation**.**

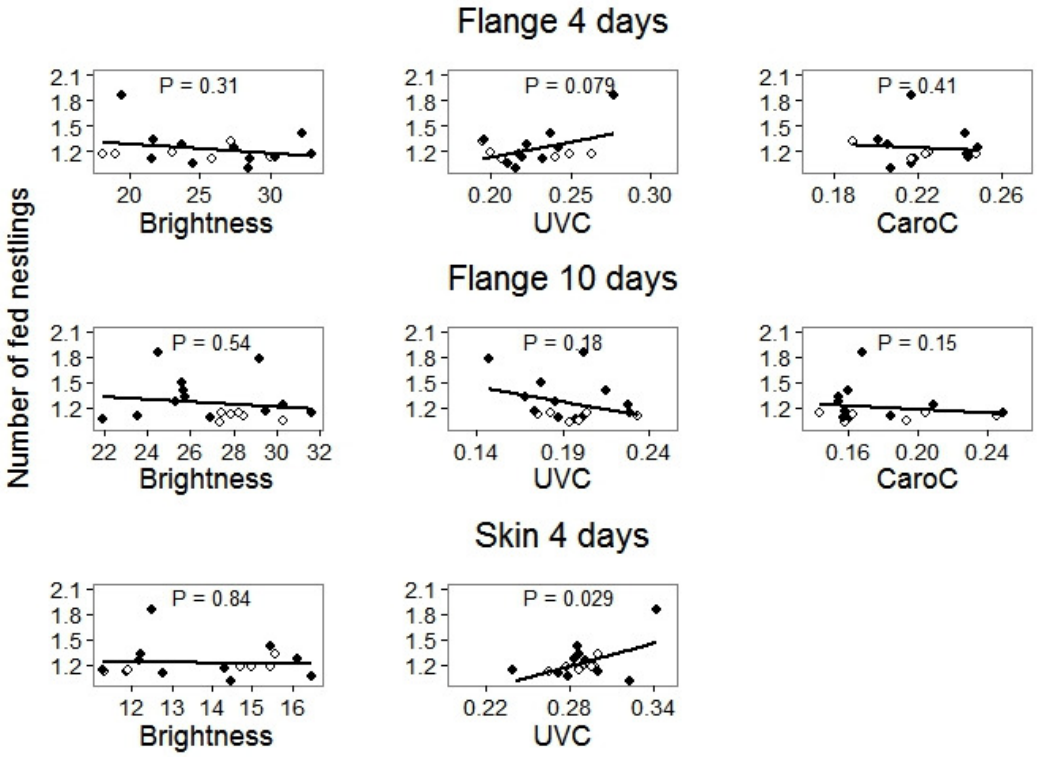


Fig. 5 – Correlation between the number of fed nestlings during each visit and nestling visual signals, i.e. brightness, ultraviolet chroma (UVC), and carotenoid chroma (CaroC). Black dots shown deparasitized nests whereas white dots shown control nests. P-value is associated to Pearson's correlation**.**

Discussion

In this study we showed that the postural begging of nestlings, but not the provisioning rate of parents, was significantly affected by the removal of nest ectoparasites. The removal of this early stress from the nests increased the conspicuousness of postural begging whereas it did not affect the visual components of begging. The visual component of begging, however, influenced the parental provisioning effort as parents increased the frequency of visits in proportion to Flange brightness of 4 days nestlings, and Flange UV chroma and yellow chroma of 10 days nestlings. Parents also increased the number of fed nestlings as the Skin UV chroma increased. We also showed that postural begging intensity increased as the nestlings became older. Parents responded to the increase of postural begging intensity by increasing the visit frequency.

Effects of ectoparasite removal on nestling condition and nestling visual signals

The removal of nest ectoparasites did not influence the condition and growth of nestlings. Body mass, hematocrit level and PHA response were similar among deparasitized and control nests. Other studies on European starlings showed that nestling body mass was not correlated to nest mite load (Gwinner et al. 2000; Gwinner and Berger 2005), even though PHA response and hematocrit level were lower in highly infested broods (Gwinner et al. 2000). In other cavity-nesting species, however, nestlings from parasitized nests showed lower body mass, growth speed, and hematocrit level than nestlings from deparasitized nests (Richner et al. 1993; Brommer et al. 2011; Cantarero et al. 2013). To the other hand, the non-significant effect of parasite removal on PHA response was also found in blue tits (Brommer et al. 2011). We also showed that hematocrit level of nestlings decreased during the course of the breeding season, that can reflect a decline in offspring quality within this starling population (Serra et al. 2012). Hematocrit level was also positively correlated to nestling body mass, that is in line with a previous study on starlings (Gwinner et al. 2000). The positive correlation between PHA response and nestling body mass was not tested in previous studies on starlings, whereas a recent review on the PHA response in relation to body mass in different bird species have found contrasting results (Tella et al. 2008). Hence, this is the first study that shows an increased PHA response in relation to an increased nestling body mass.

Flange and skin colourations, that are condition-dependent traits of the visual begging in nestling starlings (Jacob et al. 2011), resulted unaffected by the parasite removal but were associated to nestling body mass. These findings can be explained by the non-significant effect of parasite removal on nestling condition. Flange carotenoid chroma and Flange UV chroma were positively associated to nestling body mass, and their role as multiple signals in parent-offspring communication has been recently proposed (Jacob et al. 2011). Skin UV chroma, that is another signal used by parents to adjust their allocation decisions (Jourdie et al. 2004; Bize et al. 2006) was positively correlated to nestling body mass, that is in line with previous studies on starlings (Bize et al. 2006; Jacob et al. 2011).

Correlation between physiological condition and nestling visual signals

High ultraviolet and carotenoid chroma reflect the prime immune system of nestlings. In contrast, hematocrit level was not correlated to any visual components of begging. These results provide support to the general idea that various visual traits of begging in starlings could potentially signal to parents different aspects of nestling condition (Jacob et al. 2011). For example, nestling house sparrows (*Passer domesticus*) injected with corticosterone, which is the main avian stress hormone (Schmidt and Soma 2008), reduced their flange carotenoid colouration (Loiseau et al.

2008). Flange colouration therefore reflects offspring nutritional condition (Kilner and Davies 1998; Thorogood et al. 2008; Jacob and Heeb 2013).

Postural begging and parental provisioning

In response to parasite removal nestlings increased the mean begging intensity, while parents did not change their provisioning effort. From 4 days to 10 days nestlings, offspring increased the mean begging intensity, and were visited more often by parents. The effect of nest ectoparasites, despite not significant, tended to be stronger among 4 days nestlings, when ectoparasite-free broods showed a more intensive begging than controls, while the same stress did not affect the begging intensity of 10 days nestlings. This result can be explained by the more than doubled increase of body mass between the two nestling ages (22.4 ± 5 g at 4 days, and 51.0 ± 0.8 g at 10 days), and by the general idea that younger nestlings are more sensitive to ectoparasites than older nestlings, as shown in pied flycatchers (Cantarero et al. 2013). This key assumption should be particularly true in nestlings of small-sized passerine species (20-30 g), where the harmful effect of nest ectoparasites has been repeatedly demonstrated (Christe et al. 1996; Szép and Møller 1999; Tomás et al. 2008; Martínez-de la Puente et al. 2011; Cantarero et al. 2013). Parents responded to the increased begging intensity of older nestlings (ca. 2.2 begging score) by increasing their visit frequency. This is in line with previous studies (see references above), and suggest that also adult starlings adjust their allocation decisions on the basis of the postural begging intensity of their offspring.

Covariation between visual signals and parental effort

Various studies on starlings showed that parental effort is affected by the visual components of begging, such as skin and flange colouration (Jourdie et al. 2004; Bize et al. 2006). A recent study provided an experimental evidence that flange and skin colouration reflect different aspects of nestling condition, and therefore could potentially influence the allocation decisions of adults (Jacob et al. 2011). We therefore provide a support to this idea, as parental provisioning rate increased in proportion to Flange colouration, whereas the number of fed nestlings increased in proportion to Skin ultraviolet chroma.

Conclusions

Our results therefore provide an experimental evidence that in the European starling begging involves multiple signals which potentially convey different information to parents regarding the quality (begging as “signal of quality”) or the condition (begging as “signal of need”) of nestlings, and parents adjust their allocation decisions on the basis of such signals.

MANUSCRIPT 5

Nest-dwelling ectoparasites influence the start and duration of the first pre-basic moult in the European starling (*Sturnus vulgaris*)

Simone Pirrello[a,b], Andrea Pilastro[b], Lorenzo Serra[a]

[a] *Istituto Superiore per la Protezione e la Ricerca Ambientale, 40064, Ozzano Emilia BO, Italy*

[b] *Dipartimento di Biologia, Università di Padova, 35131, Padova, Italy*

Abstract

Nest-dwelling ectoparasites represent an early stressor for birds as they impair the development of nestlings, which can adaptively respond by adjusting their growth rate to current conditions. While nest ectoparasites produce long term effects on nesting adults, no study has examined if the nest ectoparasites have an impact on nestling moult pattern. Here, we investigated whether the presence of ectoparasites in the nest influences the start and duration of the first pre-basic moult in the European starling. We experimentally removed the nest-dwelling ectoparasites from a group of nests and used a group of unmanipulated (i.e. naturally infested) nests as the control. The moult began at an earlier age and lasted longer in birds from ectoparasite-free nests as compared to their control counterparts. The timing of moult was also affected by the hatching date (i.e. birds fledged later had shorter moults) and the brood size (i.e. birds in larger broods started their moult at an older age). We also provided evidence that the removal of nest ectoparasites influences the individual conditions during the course of the moult. In control birds, we observed a decrease in hematocrit levels, whereas birds from ectoparasite-free nests maintained unaltered hematocrit levels. Our study showed that the nest-dwelling ectoparasites adversely affect the timing of the moult and individual conditions in juvenile starlings with possible major consequences for their subsequent life-history events.

Introduction

Environmental stressors like parasites or food limitations not only affect the current condition of an individual, but particularly when they occur at early stages of life, they can have pervasive effects on subsequent life stages (Metcalfe and Monaghan 2001, Gluckman et al. 2005). It has been suggested that this occurs because individuals counterbalance their physiological deficits

with a compensatory growth effort which has negative effects later in life (Metcalfe and Monaghan 2001, Mitchell et al. 2011). In birds, for example, a common early stressor is the load of nest-dwelling ectoparasites which have been shown to trigger an immune response (Szép and Møller 1999) and increase the growth effort in nestlings of various cavity-nesting species (Christe et al. 1996, Tomás et al. 2008, Martínez-de la Puente et al. 2011, Cantarero et al. 2013).

Long term effects of nest-dwelling ectoparasites have been demonstrated on nesting adults (Møller 1993, Richner and Tripet 1999, Fitze et al. 2004), whereas there is less information on their long-term effects on nestlings. For example, nest ectoparasites caused adverse long term effects on nesting blue tits (*Parus caeruleus*) where nest infestations of hen fleas have reduced the return rate of parents as breeders the year following (Richner and Tripet 1999). In nesting barn swallows (*Hirundo rustica*), abundant mite loads in first clutches decreased the clutch size, anticipated the laying date, and reduced the frequency of second clutches within the same season (Møller 1993). On the contrary, a 4 year study on great tits (*Parus major*) failed to demonstrate a negative effect of nest-dwelling ectoparasites on the parents' future reproductive success (Fitze et al. 2004). The lack of effects of nest-dwelling ectoparasites on nestlings is surprising, because the nestling stage is a crucial period in the lifetime of a bird, and the stressors which occur at this stage can have detrimental effects during adulthood (Hochachka and Smith 1991, Holveck et al. 2008, Krause et al. 2009, Walker et al. 2013).

In the present study, we examined the effect of nest-dwelling ectoparasites on individual conditions and the timing of the primary moult in the European starling (*Sturnus vulgaris*) which is a species in which juveniles perform a complete pre-basic moult about 30-40 days after fledging (Jenni and Winkler 1994) (Dawson 2004). Moult is one of the most costly events for birds (Payne 1972), and it is even more challenging for freshly fledged birds as their moult often starts only a few weeks after fledging (Jenni and Winkler 1994). For this reason, nestling conditions are likely to be an important factor influencing the timing of the pre-basic moult. Indeed, feather renewal requires substantial amounts of energy (Dietz et al. 1992) that, in cases of limited resources, birds have to detract from other costly processes or behavioural activities (Siikamaki et al. 1994, Barta et al. 2008). Alternatively, in order to reduce overlapping their moult with other costly phases of their life cycle, low-condition individuals can delay the start of their moult and increase the number of concurrently growing feathers, although this negatively affects the feather quality (Badyaev and Duckworth 2003, Dawson 2004, Serra et al. 2010). An effect of individual conditions on moult performance has been demonstrated on barn swallows (*Hirundo rustica*), where males in prime condition showed an advanced stage of moult as compared to low condition males (Møller et al. 1995). In the same study it was showed also a negative correlation between start of the moult and

abundance of feather holes. Chewing lice are one of the possible causes that produce feather holes (Møller 1991, Vas et al. 2008, Vágási 2014), and a recent study on house sparrows (*Passer domesticus*) demonstrated that an increase of feather holes corresponds to a postponed start and increased speed of the moult (Moreno-Rueda 2014). Ectoparasites are therefore predicted to affect individual conditions as highly infested birds, i.e. those with more feather holes, delayed the moult start and accelerated the moult speed.

Considering that nest ectoparasites adversely affect the development of nestlings and that moulting is an energy-demanding activity, it can be hypothesized that the first pre-basic moult could be affected by downstream effects due to the presence of the ectoparasites in the nest. The start of the first pre-basic moult is regulated by a number of factors including age, date of birth, timing of migration, and seasonal and geographical factors (latitude, photoperiod, temperature), whereas moult duration is primarily regulated by the daylength (Jenni and Winkler 1994). Moult duration, in turn, is positively correlated to the quality of flight (Serra 2001, Dawson 2004) and ornamental feathers (Serra et al. 2007, Vágási et al. 2010). The effect of the parasite load on adult moult pattern has been demonstrated in a number of studies. In adult house sparrows (*Passer domesticus*), birds with coccidian infection produced shorter and lighter feathers as compared to those produced by birds in good condition (Pap et al. 2011). In house martins (*Delichon urbica*), individuals with a malaria infection and haemosporidian parasites moulted over a longer period than non-infected individuals; although the quality of the resulting plumage was not impaired by the infection (Marzal et al. 2013). Individual condition also influences the timing of the moult in the long living Laysan albatrosses (*Phoebastria immutabilis*), in which birds with more nematodes began moulting later and replaced a lower number of primary feathers (Langston and Hillgarth 1995). Collectively, these studies suggest that parasites usually have a negative impact on the moult pattern and plumage quality in adult birds.

To investigate the effect of nest-dwelling ectoparasites on the moult pattern of juvenile European starlings, we treated a group of nests with insecticide and used a group of unmanipulated (i.e. naturally infested) nests as the control. At fledging, birds were sexed molecularly and males from the two nest treatments were moved into indoor aviaries. We used hematocrit as an index of individual condition during the course of the moult because hematocrit is positively correlated to the body mass (Eeva et al. 2008) and is lower in birds exposed to a high parasite load (Merino and Potti 1998, Gwinner et al. 2000, Brommer et al. 2011) or stressed by a lack of food (Hoi-Leitner et al. 2001). We predicted that nestlings grown in ectoparasite-free nests would fledge in better conditions and hence show higher hematocrit levels and start the first pre-basic moult at an earlier age as compared to the birds from control nests that, in contrast, were exposed to ectoparasites

during their somatic growth. If so, birds from ectoparasite-free nests have more time to complete their moult and are expected to moult over a longer period (Dawson 2004).

Materials and Methods

Origin and housing of starlings

The starlings used in this experiment originated from a nest box breeding colony situated in Northern Italy (Ozzano Emilia, Bologna, 44°28'N, 11°29'E). Although different ectoparasites have been observed within the nest boxes of this colony, including fleas and mites, the dominant species is the fly *Carnus hemapterus* which mainly feeds on the blood of nestling starlings (Liker et al. 2001), and can thus be considered as one of the major stressors for growing nestlings. In the 2011 breeding season, 25 out of 45 nest boxes were occupied by starling pairs. A second clutch was laid in 5 nest boxes (3 ectoparasite-free and 2 control nests, see below) which were cleaned and sanitized between the two breeding attempts.

From the middle of March to the end of the breeding season (June-July), we checked the nest boxes daily for the progression of egg laying. After clutch completion, we randomly assigned each nest to the ectoparasite-free (N=15) or to the control (naturally infested) treatment (N=15). The nest-dwelling ectoparasites were removed from the nests of the ectoparasite-free treatment using an antiparasitic spray (Frontline spray, Fipronil 0.25 g, Merial – Tolose, France), whereas the control nests were sprayed with tap water. Every three days from clutch formation to fledging, we removed the eggs or the nestlings from the nest before spraying one shot of antiparasitic solution (or water for the control group) onto the nest cup. When the nest material was completely dry (usually within three minutes), eggs or nestlings were returned to the nest. When the nestlings were removed from the nest, each nestling was positioned into a cardboard box (10 x 10 x 20 cm) previously filled with cotton wool. We then counted the number of *Carnus hemapterus* on each nestling and those felt on the cotton wool in order to calculate the total amount *C. hemapterus* hosted by the brood during each treatment event. We considered the mean ectoparasite load of the nest as the mean number of *C. hemapterus* counted during the nestling phase. The antiparasitic spray removed almost all the ectoparasites from the nests (mean ectoparasite load: 0.2 ± 0.1 SE in ectoparasite-free nests and 14.0 ± 0.9 SE in control nests, Kruskal-Wallis test: $\chi^2 = 24.28$, $P < 0.001$), and the difference between treatments was consistent over the entire nestling period.

All nestlings were successfully sexed using the method originally developed by Griffiths et al. (1998), which we already used in a previous study on starlings (Serra et al. 2012). We collected a

blood sample (<70 µl) from the brachial vein of 4-day nestlings. 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). Sex analyses were completed before the brood fledged. We decided to study the moult of only males because the moult pattern is sex-dependent (Serra et al. 2010) and we had a limited number of aviaries to keep in captivity also the females.

A total of 34 males (17 from ectoparasite-free and 17 from control nests) were randomly distributed among four indoor aviaries 2-3 days before they left the nest. At this age (18 days) birds become readily capable of feeding autonomously and can be maintained in the absence of the parents. Each aviary measured 200 × 80 (base) × 200 cm (height), and birds were provided with food and water *ad libitum*. The shape of these aviaries is that recommended by a recent study (Asher et al. 2009) where starlings housed in long-shaped cages displayed low stereotypic behaviour, which is widely considered as an indicator of an inadequate environment. When moved to the aviaries, all of the starlings were treated against endoparasites (coccidia, bacteria, and fungi).

The same birds were used in a parallel study aimed at investigating the effect of the dietary carotenoid content during the moult on beak and throat feather colours, which are two sexually selected traits in starlings. To this purpose, from the start to the end of the moult, 18 birds were fed a carotenoid-rich diet while the other 16 birds were deprived of carotenoids. We did not expect any effect of the dietary treatment on the moult duration and, indeed, it was not different between birds fed with or without carotenoids (Linear Mixed Model with moult duration as response variable, nest and dietary treatments as fixed factors, brood identity as random factor, and hatching date and brood size as covariates, $F_{1,29} = 1.35$, $P = 0.26$). Similarly, we did not find any effect of dietary treatment on the hematocrit levels at the end of the moult (LMM, $F_{1,29} = 0.35$, $P = 0.56$). We therefore pooled the two dietary groups within each nest treatment for the following analyses.

Moult parameters

The timing and duration of the primary moult was assumed as a proxy of the complete moult (body and wing feathers). The moult progression of the 9 long primary feathers was checked weekly. The starting date of the moult was considered as the previous checking date to the checking date when the first primary feather (=the innermost) was found shed. The moult was considered completed at the checking date when all the primary feathers were found fully grown. We considered the variable "start of moult" as the time lag from hatching to the start of the moult.

Hematocrit assay

Hematocrit shows seasonal variations which are influenced by various factors (i.e. age, reproductive status, geographical elevation, season, parasitism, and nutritional status) that concurrently affect the hematocrit level (Fair et al. 2007). However, the contribution of any single factor to changing hematocrit can be isolated if all the other factors remain constant. This is the case for our study where we manipulated the ectoparasite load of the starlings that were i) born the same year in the same breeding colony (therefore age and geographical factors remain constant), and ii) in the non-reproductive season.

At the start and at the end of the moult, a blood sample was drawn from the brachial vein into 3-4 micro-hematocrit capillary tubes. We used a Centric 150 centrifuge (Intercontinental Srl, Anzio, Italy) to centrifuge the blood samples at 11500 rpm for 10 min (centrifuge radius 94 mm) and to separate the plasma from the red blood cells. The hematocrit (proportion of red blood cells over total blood volume) was measured on capillary tubes with a dial caliper (accuracy of 0.1 mm).

Statistical analyses

All the response variables were normally distributed and were therefore analysed with parametric tests. We used linear mixed models (LMMs) to test the effect of the nest treatment on the start and duration of the moult and on the hematocrit level at the beginning and at the end of the moult. Brood identity was entered as a random factor to control for the non independence of nestlings fledged from the same nest. Hatching date was entered as a covariate because of its effect on the timing of the moult and the hematocrit levels. Indeed, birds that fledge later in the season naturally advance the start of their moult and carry out a shorter moult with respect to the birds that fledge earlier (Jenni and Winkler 1994). Moreover, it has been demonstrated that the age of juveniles is one of the main parameters which contributes to determining the variation of hematocrit in birds (Fair et al. 2007). We also included the brood size as a covariate, because it is negatively correlated both to nestling body mass at fledging (Pearson's correlation = -0.42, P = 0.013) and to the hatching date (Pearson's correlation = -0.53, P = 0.001). In particular during the course of the breeding season, the birds that fledged later were in smaller broods and weighed less than birds that had fledged earlier. Furthermore, the nestling body mass at fledging was not different between ectoparasite-free and control nests (LMM with body mass as the response variable, nest treatment as the fixed factor, hatching date and brood size as the covariates, and brood identity as the random factor, $F_{1,17.5} = 2.27$, P = 0.15). Statistical analyses were carried out using the software R 3.0.3 (R Development Core Team). All of the tests were two-tailed and significant when $P < 0.05$.

Results

Moult parameters

The start of the moult was earlier in birds from nests without the nest-dwelling ectoparasites as compared to birds from nests with ectoparasites (effect of the antiparasitic treatment, -3.82 days ± 1.70 SE, $F_{1,17.3} = 5.04$, $P = 0.038$; Fig. 1) and was delayed as the brood size increased (brood size, 3.2 days ± 1.0 SE, $F_{1,20.1} = 10.2$, $P = 0.005$). In contrast, we did not find a significant effect of hatching date on moult start ($F_{1,19.2} = 0.06$, $P = 0.80$).

Birds whose nests were treated with the insecticide showed a significantly longer moult after statistically correcting for hatching date (antiparasitic treatment, 4.30 days ± 2.03 SE, $F_{1,30} = 4.49$, $P = 0.043$; hatching date, -0.43 days ± 0.06 SE, $F_{1,30} = 45.9$, $P < 0.001$; Fig. 1). Brood size did not significantly affect moult duration ($F_{1,30} = 0.30$, $P = 0.59$). Once statistically controlling for moult starting date, the effect of treatment on moult duration was attenuated (antiparasitic treatment, 2.45 days ± 1.75 SE, $F_{1,31} = 1.96$, $P = 0.17$; starting date of the moult, -0.46 days ± 0.05 SE, $F_{1,31} = 82.8$, $P < 0.001$), suggesting that birds that started to moult later increased their moult speed. As a result, the date at which the moult was completed was positively correlated with hatching date but did not differ between birds from ectoparasite-free and control nests (antiparasitic treatment, 2.40 days ± 3.48 SE, $F_{1,31} = 0.48$, $P = 0.49$; hatching date, 0.52 days ± 0.09 SE, $F_{1,31} = 31.19$, $P < 0.001$).

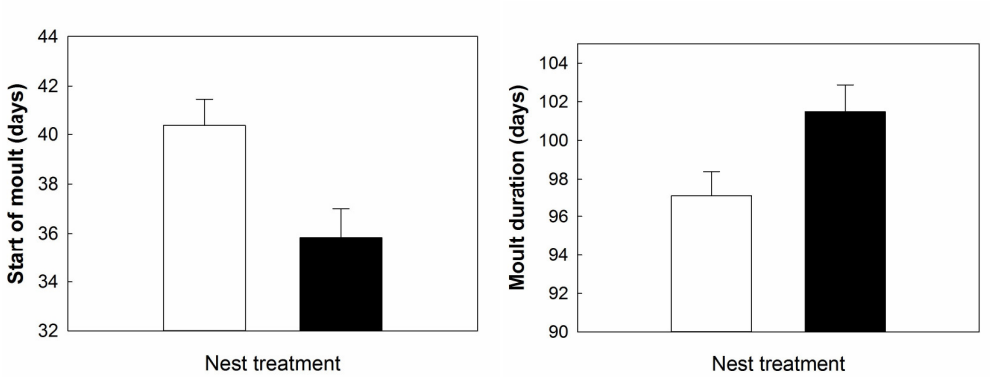


Fig. 1 - Timing of the first pre-basic moult in birds grown in ectoparasite-free (solid bars) and naturally infested nests (empty bars). Start of the moult stands for the time from the birth to the shed of the first primary feather. The values are the marginal means ± 1 standard deviation.

Hematocrit assay

Contrary to predictions, hematocrit levels at the start of the moult were unaffected by the nest treatment (antiparasitic treatment, $F_{1,17.3} = 0.07$, $P = 0.79$; hatching date, $F_{1,19} = 2.88$, $P = 0.11$; brood size, $F_{1,19.9} = 0.16$, $P = 0.69$; Fig. 2). At the end of the moult, individuals that fledged from

ectoparasite-free nests showed higher hematocrit levels than the individuals from control nests (antiparasitic treatment, 2.02 ± 0.91 SE, $F_{1,30}$ = 4.98, P = 0.033; hatching date, $F_{1,30}$ = 1.78, P = 0.19; brood size, $F_{1,30}$ = 1.40, P = 0.24; Fig. 2). Control birds significantly decreased their hematocrit from the beginning to the end of the moult (paired *t*-test: t = -2.27, d.f. = 16, P = 0.037, mean ± SE = -2.5 ± 1.1; Fig. 2); whereas individuals from ectoparasite-free nests maintained their unaltered hematocrit during the moult (paired *t*-test: t = 0.66, d.f. = 16, P = 0.52, mean ± SE = 0.6 ± 0.9; Fig. 2).

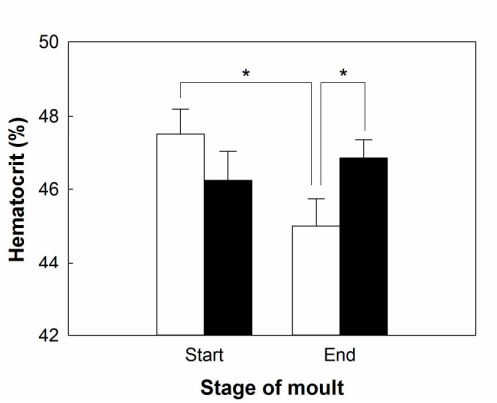


Fig. 2 - Hematocrit levels at different stages of the moult in birds grown in naturally infested (empty bars, N = 17) and in ectoparasite-free nests (solid bars, N = 17). The values are mean ± 1 standard error. Asterisk = significantly higher within stage of moult.

Discussion

Several studies have demonstrated that nestlings and parents compensate for the stress caused by the presence of nest-dwelling ectoparasites through behavioural, physiological, and immune responses which can produce adverse effects either immediately or in later stages of life (Hurtrez-Boussès and Renaud 2000, Metcalfe and Monaghan 2001, Fitze et al. 2004, Tomás et al. 2008, Martínez-de la Puente et al. 2011, Cantarero et al. 2013). While parents are dealing with a trade-off between current and subsequent reproductive attempts within the same breeding season (Trivers 1972, Møller 1993, Richner and Tripet 1999), the nestlings have to face energy-demanding events soon after fledging whose performance is likely to be influenced by their individual conditions.

In this study, we experimentally demonstrated that nest-dwelling ectoparasites influenced the temporal pattern of the first pre-basic moult in the European starling. In particular, we found that the removal of ectoparasites was associated with an earlier moult start and a slower moult progression as compared to what was observed in nestlings which were grown in naturally infested

nests. We can expect a selective advantage associated with an earlier start of moult, because foraging conditions are expected to deteriorate as the autumn progresses, temperatures decrease and the energetic cost of feather moulting is therefore likely to increase with the season (Barta et al. 2008). Furthermore, all the rest being equal, the earlier the moult is started the earlier will be completed, and this may reduce the overlapping or time constraints associated with subsequent autumn migration and/or post-natal dispersal. Our results indicate that nest ectoparasites may represent a significant fitness cost mediated by a delay in moult start possibly mediated by a reduced nestling condition. Indeed, other studies have evidenced that ectoparasites can cause a decreased growth rate of nestlings in other species (Christe et al. 1996, Tomás et al. 2008, Brommer et al. 2011). The effect of ectoparasites on our starlings was more subtle than that found in the above studies, as we did not find evident effects of the experimental treatment on body mass at fledging. Nonetheless, the conclusion that the start of the moult was delayed in birds from control nests because of the negative effect of ectoparasites on nestling conditions is indirectly supported by the observed correlation between moult start and brood size. A larger brood size is expected to be associated with a reduced amount of food delivered to each nestling and with an increased competition for the food (Wright and Cuthill 1990, Wiehn and Korpimäki 1997), which both should negatively affect nestling conditions.

In contrast, brood size did not affect moult duration, suggesting that the *ad libitum* diet received by the birds in the aviaries compensated for previous food limitation in the nest. Moult duration was also significantly influenced by our experimental treatment, with ectoparasite-free birds moulting slower as compared to their control counterparts. The faster moult of birds from naturally infested nests is probably the result of a compensation for the delay in moult start along with a time constraint to complete the moult. This is suggested by the strong correlation between moult duration and the hatching date, i.e. birds that hatched later performed shorter moults, which is in accord with previous studies (e.g. Bojarinova et al. 1999, Coppack et al. 2001). Furthermore, the effect of nest-dwelling ectoparasites on moult duration was not anymore statistically significant when the starting date of the moult was entered as a covariate in the mixed model and moult ended, on average, at the same time of the season in the two groups, indicating that birds from infested nests were able to temporally compensate for the initial delay in the moult process. However, it is likely that this compensation occurred at a cost.

First, a longer primary moult has been shown to result in the production of better quality flight feathers in terms of resistance against abrasion (Serra et al. 2010, De La Hera et al. 2010, Vágási et al. 2012). Furthermore, the quality of ornamental pigmented feathers also increases with moult duration (Serra et al. 2007, Vágási et al. 2010). Moult duration also affects plumage structural

colours. For example, in blue tits (*Cyanistes caeruleus*) males that moulted at a slower rate had more colourful ultraviolet and blue crown feathers (Griggio et al. 2009). Considering that female starlings prefer males with a higher ultraviolet reflectance on throat feathers (Bennett et al. 1997), male starlings grown in control nests should produce lower quality throat feathers and thus be less attractive to females. Another possible cost of nest-dwelling ectoparasites is that starlings that are forced to accelerate their moult will probably have a higher daily energy expenditure as compared to individuals from ectoparasite-free nests. The energy invested by birds in a faster moult will probably be subtracted to other energy-demanding daily activities, such as vigilance against predators, foraging, and self-maintenance (Redpath 1988, Cucco and Malacarne 1997). Accordingly, birds from naturally infested nests showed lower hematocrit levels, possibly an effect of the higher energetic investment associated with a faster moult. Although hematocrit levels show rapid seasonal changes (Williams et al. 2004, Dickens et al. 2009), it seems unlikely that the difference in hematocrit levels observed at the end of the moult could be explained by the seasonal effect considering that the two groups, on average, completed the moult at the same time. The lower hematocrit levels observed at the end of the moult were therefore more likely the consequence of the interaction between the initial conditions (which caused the delay in moult start) and the metabolic cost of renewing the plumage at a faster rate. This result partly accords with previous evidence of a decrease in hematocrit levels during the moult in free-living passerines (Fair et al. 2007, but see Goldstein and Zahedi 1990). In contrast, our ectoparasite-free birds did not show reduced hematocrit levels at the end of the moult, probably as a result of their good initial conditions and slower moult. In more natural conditions the costs of nest-dwelling ectoparasites are expected to be amplified by the interaction with endoparasite infestation, predation, and food limitations which could occur in the wild.

In conclusion, our analyses revealed that in the European starling, the presence of nest-dwelling ectoparasites which are known to be an early stressor that negatively affects nestling growth also have detrimental effects at later stages of life. The removal of ectoparasites from the nest allowed birds to i) start the first pre-basic moult at an earlier age, ii) extend moult duration (so they probably produced better plumages), and iii) have better conditions at the end of the moult as shown by hematocrit. The difference in the start and duration of the moult between ectoparasite-free males and their naturally infested conspecifics, although significant, was small in absolute value (approximately 4 days of delay in moult start and 4.3 days in moult duration over a total duration of about 100 days). Whether these relatively small differences in the moult temporal pattern will translate into differences in the quality of flight feathers and sexual plumage ornaments clearly needs to be tested.

MANUSCRIPT 6

A supplement of carotenoids during the post-juvenile moult increases the preening time of male European starlings in winter

Simone Pirrello[a,b], Andrea Pilastro[b], Lorenzo Serra[a]

[a]*Istituto Superiore per la Protezione e la Ricerca Ambientale, 40064, Ozzano Emilia, Bologna, Italy*
[b]*Dipartimento di Biologia, Università degli Studi di Padova, 35121, Padova, Italy*

Abstract

Birds invest considerable time and energy to maintain the good state of their plumage through preening. There are contrasting results regarding the effect of individual conditions on preening time, and no studies investigated the potential downstream effects of early stresses on these maintenance activities. In this study, we manipulated environmental conditions of male European starlings during the nestling stage and during their first moult to assess the effects of early stresses on preening time a few months after moult completion. We used starlings fledged from nests with or without ectoparasites that were fed with or without carotenoids during the first moult. Preening time was not associated with the current individual conditions. While the nest ectoparasite load did not affect preening time, birds supplemented with carotenoids preened more often than non-supplemented birds. Our results suggest that preening time reflects nutritional condition during moult, and considering that an increased investment in preening should be associated to better plumage condition, it is likely that the availability of carotenoids during moult have a positive effect on male plumage quality in the subsequent breeding season.

Introduction

Survival and reproduction are influenced by the individual investment in preserving the good state of the integuments (e.g. skin, scales, plumage, pelage). These types of maintenance behaviours are energy-demanding and time-consuming (Croll and McLaren 1993), and thereby the good state of the integument is likely to reflect individual condition.

Birds invest considerable time and energy in preening, a suite of behaviours aimed at cleaning (dirt and ectoparasites), arranging, oiling, and ordering the feathers (Cotgreave and Clayton 1994; Walther and Clayton 2005; Clayton et al. 2010; Waite et al. 2012). Such behaviours are primarily

devoted to maintain the thermoregulatory and ornamental functions of the plumage (Zampiga et al. 2004; Lenouvel et al. 2009; Clayton et al. 2010). Recently, it has been suggested that preening conveys also public information to other group members and potential mates (Danchin et al. 2004), although there is contrasting evidence that such behaviours are associated to individual condition (Yorinks and Atkinson 2000; Surmacki and Hill 2013). To date, only two studies tested whether preening is condition-dependent. A study on juvenile apapanes (*Himatione sanguinea*), a honeycreeper living in the island of Hawaii, showed that individuals infected with malaria spent significantly less time preening when compared to healthy birds (Yorinks and Atkinson 2000). In contrast, a study on the American goldfinch (*Spinus tristis*) failed to find a positive correlation between coccidian infestation and preening rate (Surmacki and Hill 2013). These studies manipulated individual conditions at the time when preening time was measured, whereas no study investigated the effect of past conditions on the following maintenance performance.

Several experiments showed that early development conditions can influence the lifetime of a bird at different time scales (e.g. (Lindström 1999; Metcalfe and Monaghan 2001; Krause et al. 2009; Tschirren et al. 2009). Birds experiencing adverse nest condition grow at a slow rate, have low body mass at fledging, and have a low recruitment in the following breeding season (Fitze et al. 2004; Brommer et al. 2011; Cantarero et al. 2013). For example, nest ectoparasites are an early stress for growing nestlings, which usually invested part of their growth energy to a suite of physiological and behavioural compensatory responses (Christe 1996; Tomás et al. 2008; Martínez-de la Puente et al. 2011). The removal of nest ectoparasites has a positive effect for nestlings not only immediately (Richner et al. 1993; Cantarero et al. 2013), but also during moult (Manuscript 5), and in the following breeding season (Manuscript 7).

Adverse early nutritional condition not only impairs current individual condition, but also affects subsequent responses to stress (Krause et al. 2009), and survival (Hochachka and Smith 1991). Nutritional deficiencies during the moult affect the quality of the new feathers, including detrimental effects on plumage ornaments (McGraw et al. 2002; Siefferman and Hill 2005). Carotenoids are a limiting factor for birds, given that they can only be assumed with diet (Goodwin 1980). A limited intake of carotenoids early in life resulted in a reduced assimilation capacity of carotenoids as adult (Blount et al. 2003), with negative consequence for the overall antioxidant capacity or the general condition of the organism (Butler and McGraw 2013). In the European starling (*Sturnus vulgaris*), an early carotenoid supplementation increased the overall song rate of males (Van Hout et al. 2011) and the yellow beak colouration in the following breeding season (see Manuscript 7). It is predictable that birds fed with or without carotenoids in a certain period of their life may present a different carotenoid assimilation capacity when returned to a diet with

carotenoids. Moreover, this difference should be more evident in winter when birds naturally increase their basal metabolic rate, and therefore their food intake, in response to seasonal acclimatization to cold (Swanson 2010). In cold climate, preening is expected to have a crucial role in preserving the thermoregulatory function of the plumage, thereby it represents an energy demanding and time-consuming activity for birds.

In this study, we investigated the effect of early individual conditions on preening time of male European starlings during the months following the moult completion. At this time starlings do not perform other time and energy consuming life-history activities, so preening time is likely to be mainly related to current individual condition. Since we used birds in captivity, the temporal trade-off between preening and other time-demanding activities that are normally performed in nature, such as seeking food and monitoring predators, should be very limited (Redpath 1988; Cucco and Malacarne 1997). To investigate the effect of nest-dwelling ectoparasites on the moult pattern of juvenile European starlings, we used male starlings fledged from nests with or without ecoparasites. These males were subsequently assigned to a carotenoid-rich and a carotenoid-free diet only during the course of their post-juvenile moult, following the same treatment that was used in previous studies (Van Hout et al. 2011; Manuscript 5). Our predictions were that birds fledged from ectoparasite-free nests and that received a carotenoid-rich diet were in better condition during the months following the end of moult and thereby should invest more time in preening.

Materials and Methods

Study area and origin of starlings

The study was carried out in Northern Italy (Ozzano Emilia, Bologna, 44°28'N, 11°29'E) between December 2012 and January 2013. We used 42 male starlings fledged in 2012 from a nest-box breeding colony that was first established in this area in 2009. In February 2012, we installed 38 nest boxes on the roof of two buildings at a height of approximately 15 m and orientated randomly with respect to cardinal points. In the course of the breeding season, 27 out of 38 nest boxes were occupied by starling pairs. During the breeding season, a second clutch occurred within 12 nest boxes that were cleaned and sanitized between the two depositions.

Nest and dietary treatment

From the middle of March to the end of the breeding season (June-July), we checked all of the nest boxes daily, and after clutch completion we randomly assigned each nest to the

ectoparasite-free (N=25) or to the control (naturally infested) group (N=27). The nest ectoparasites were removed from the ectoparasite-free nests using an antiparasitic spray (Frontline spray, Fipronil 0.25 g, Merial – Tolose, France), whereas the control nests were sprayed with tap water. Each treatment consisted of removing all the eggs or chicks from the nest and then spraying one shot of antiparasitic solution or water onto the nest cup. When the nest material was completely dry (usually within three minutes), the eggs or chicks were returned to the nest. Both treatments were repeated every three days until fledging. The nest ectoparasite load was considered as the mean number of ectoparasites that were counted on nestlings during each treatment procedure. This treatment was successfully used in a previous study (Manuscript 5) to remove the ectoparasites of the nest. We obtained a clear difference in the ectoparasite load between the nest groups (mean number of ectoparasites: 0.4±0.3 SE in ectoparasite-free nests, and 21.4±3.4 SE in control nests). All nestlings were molecularly sexed, and 42 males (20 from ectoparasite-free and 22 from control nests) were randomly distributed among four indoor aviaries 2-3 days before they left the nest. At this age (18 days) birds feed autonomously so that they can be separated from parents. Each aviary measured 200 × 80 (base) × 200 cm (height) and birds were provided with food and water *ad libitum* (see below for dietary details). Moreover, birds were provided with bath water every three days to favour the plumage cleaning. The aviaries had a shape coherent with that recommended by a recent study (Asher et al. 2009), where starlings housed in long-shaped cages displayed low stereotypic behaviour, which is indicative of an inadequate environment. Here, all the starlings were treated against endoparasites (coccidia, bacteria and fungi). At the start of moult, 21 birds received a supplement of carotenoids in diet for about three months (i.e. during the post-juvenile moult) while the other 21 birds' diet was deprived of carotenoids. The base food consisted of a mixture of 50% TH White Extra and 50% of TH White Soft (Raggio di Sole, Piacenza - Italy) that contains a concentration of lutein between 0.9 and 1.2 mg per kg. The dietary carotenoid supplementation consisted of an additional 50 g Versele-Laga Yel lux [Oropharma, Deinze - Belgium; extracted from marigolds (*Tagetes erecta*) and containing 8 g of lutein per kg] per one kg of base food which is a concentration already used on starlings (Van Hout et al. 2011, Manuscript 5).

At moult completion, all birds were fed the same diet containing carotenoids. We therefore obtained four experimental groups: 1) ectoparasites + low carotenoids, 2) ectoparasites + high carotenoid, 3) ectoparasite-free + low carotenoids, and 4) ectoparasite-free + high carotenoids.

Behavioural analyses

In December 2012 and January 2013, all birds were weighed with an electronic balance (accuracy of 0.1 g), and moved to single cages (60 × 25 × 35 h cm) 24h before recording their

behaviours to allow them to acclimatise. In the cage, all birds received a commercial food for insectivorous species and water *ad libitum*. Each month we recorded all birds in consecutive days (from 13 to 21 December, and from 15 to 23 January) for 2 hours between 9.00 and 12.00. Video recordings were carried out using a Nikon P6000 digital camera that was positioned in front of the cages at a distance of 2 m. At the end of each recording session, the birds were returned to the aviaries whereas at the end of the experiment all birds were released.

The video analyses were performed blindly by the same person (SP), and using VLC media player 2.1.3 (Free Software Foundation, Inc., Boston, MA). Considering the time required to set the digital camera, leave the experimental room, and allow birds to calm down, we did not consider the initial 14 minutes of each video recording. We then sampled the behaviour of each individual every 15 seconds for 90 minutes, thereby collecting 360 data from each video. More specifically, we counted the times in which the focal bird performed the following activities: preening (the bird takes a feather in its beak), scratching (the bird scratches a part of its plumage with a foot) and ruffling (the bird shakes the entire plumage). We then considered the variable "total preening" as the sum of events in which the focal bird preened, scratched or ruffled its plumage.

Statistical analyses

The effect of nest and dietary treatment on preening time was analysed using a mixed model assuming a Poisson distribution. Nest treatment, dietary treatment, and their interaction were included as explanatory factors in the initial model, but the interaction was removed from the final model as not significant. Nest identity and bird identity were entered as random nested factors to account for repeated measures of the same individual and of different individuals from the same nest. Mixed models were carried out using the library *lme4* within the software R 3.0.3 (R Development Core Team).

Results

On average, male starlings spent 3.9±0.3% of their time preening. Total preening was not correlated to body mass (Pearson's correlation, December: -0.23, P = 0.14; January: -0.07, P = 0.65). Birds supplemented with carotenoids preened, scratched or ruffled more often than individuals deprived of carotenoids during the previous moult (effect of carotenoid supplementation: 0.55 ± 0.17 SE, z = 3.26, P = 0.001), while there was no effect of nest ectoparasites (effect of ectoparasite removal: 0.05 ± 0.17 SE, z = 0.30, P = 0.76; Fig. 1).

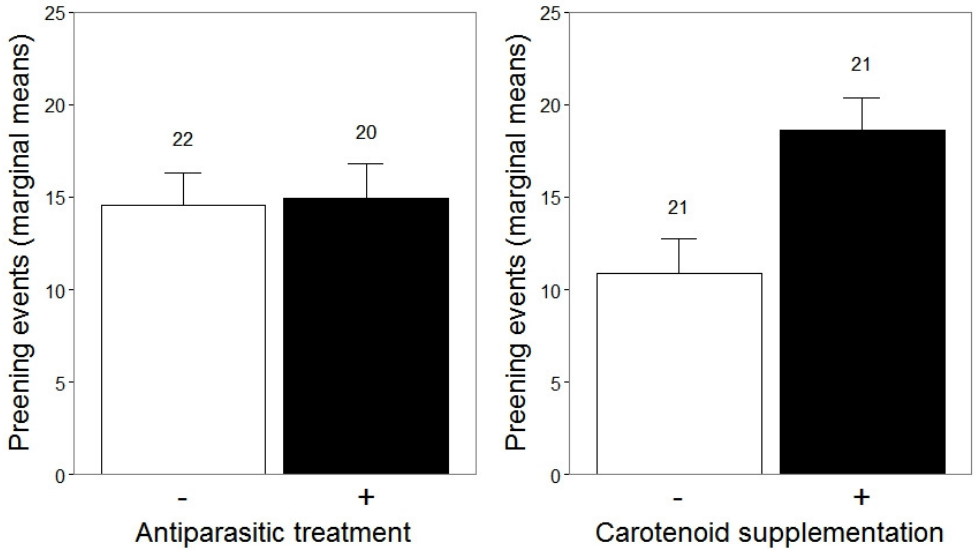


Fig. 1 - Preening events in birds fledged from nests with (+) or without (-) ectoparasites, and supplemented with (+) or deprived of (-) carotenoids during the moult. Values are marginal means + SE, and the sample size of each groups is shown above each bar.

Discussion

This study provides the experimental evidence that nutritional condition during the moult, i.e. availability of carotenoids, influences the preening time of male starlings during the following months. Contrary to predictions, individuals fledged from ectoparasite-free nests did not preen more often than their conspecifics fledged from nests with ectoparasites. Although preening time was not correlated to body mass, our findings suggest that individual condition was affected by carotenoid deprivation in a more subtle way than just causing a reduction of body mass a few months after the end of the stress.

Previous studies showed that severe nutritional conditions can produce detrimental effects not only at the time when the deficiency occurs, but also at subsequent stages of life (Lindström 1999; Metcalfe and Monaghan 2001). Poor nutritional conditions during moult are associated to the low quality of growing feathers, as widely demonstrated in birds that exhibit carotenoid-based plumages (Hill and Montgomerie 1994; McGraw et al. 2002; Senar et al. 2003). In fact, carotenoids are traded off between pigmentation (of feathers and bare parts) and immunity (Lozano 1994). A low concentration of carotenoids in diet is therefore expected to stress such trade-off, and birds may adaptively respond by disrupting the use of carotenoids for plumage ornaments (to favor reproduction) or immunity (to favor survival). While a shortage of carotenoids during moult is

directly involved in producing low quality carotenoid-based feathers and impaired immune responses, its effect on a species without carotenoid-based plumage ornaments should be primarily deleterious for immunity (Butler and McGraw 2013). In the present study, we used a species that does not exhibit a carotenoid-based plumage in a period of the year when the beak just starts to change from horny black to yellow, thus when the amount of carotenoids that starlings apply for ornamentation is limited. Preening time can therefore reflect the general heath status of the organism, thereby prime condition individuals invest more time (and energy) in preening. It has been shown that a shortage of carotenoids during development reduced the carotenoid assimilation capacity at adulthood (Blount et al. 2003). Because the roles of carotenoids as antioxidant substrates or modulators of immunity (Burton and Ingold 1984), a reduced assimilation capacity of carotenoids can be deleterious for individual condition. A reduced capacity of adult starlings to assimilate carotenoids if they experienced a shortage of carotenoids during moult has been shown in another study, where males deprived of carotenoids produced a less yellow beak in the following breeding season (Manuscript 7). We therefore showed that the availability of carotenoids during moult, that is an energy demanding event for the lifetime of a bird, increased the time spent by males to maintain the good state of their plumage, and this probably affects their attractiveness in the following breeding season.

Contrary to predictions, the removal of nest ectoparasites did not increase the preening time of males. Many studies showed that nest ectoparasites have negative consequences for nestling growth and immunity (Christe 1996; Tomás et al. 2008; Martínez-de la Puente et al. 2011; Cantarero et al. 2013), while the long-term effects of this stress are so far poorly understood. Our result suggests that preening time about 5-6 months after fledging is not affected by carry over effects due to the stress of the nest ectoparasites. This is particularly interesting because in a previous study we showed that starlings fledged from ectoparasite-free nests moulted earlier than starlings fledged from naturally infested nests (Manuscript 5). Collectively, these studies suggest that nest ectoparasites induce carry-over effects on individual life-history within a few months from fledging, but they become not significant 5-6 months after fledging.

In conclusion, the preening time of male starlings in the months following the end of their moult is directly related to nutritional condition experienced during moult, i.e. to the stress temporally closer to our observations. Since starlings are social birds that form large flocks in winter, it would be interesting to investigate whether males use preening as a social (or sexual) signal to convey information regarding their condition to other group members or females. Indeed the proportion of time that males invest in maintenance activities probably reflect their overall antioxidant capacity, or their ability to acquire adequate nutritional resources. If so, other

conspecifics should follow the males that preen more to find the feeding grounds with better nutritional resources, or the females could potentially assess the male quality by evaluating the time that each prospecting mate devotes to preening.

MANUSCRIPT 7

Male juvenile condition influences the female choice in the European starling *Sturnus vulgaris*

Simone Pirrello[a], Andrea Pilastro[b], Andrea Brunelli[c], Lorenzo Serra[a]

[a]*Istituto Superiore per la Protezione e la Ricerca Ambientale, 40064, Ozzano Emilia, Bologna, Italy*
[b]*Dipartimento di Biologia, Università degli Studi di Padova, 35121, Padova, Italy*
[c]*Dipartimento di Scienze Biologiche, Geologiche e Ambientali, Università degli Studi di Bologna, 40126, Bologna, Italy*

Abstract

Different studies on sexual selection showed that females gain direct and indirect advantages by choosing high quality males. The male quality can be signalled by the expression of condition-dependent sexually selected traits which are used by females to evaluate the prospective partners in order to maximize their fitness return. Whether male condition in the first stages of life influences the expression of their sexually selected traits and, in turn, their attractiveness in adulthood is less clear. In the present study, we experimentally manipulated nest and post-fledging moult conditions in 33 male European starlings. In their first breeding season, the colourations of the beak and the throat were measured, and their attractiveness was evaluated with a two-way choice chamber. We showed that the beak colouration, which is a plastic sexual trait that responds nearly instantaneously to changes in condition, is also affected by the nutritional condition experienced during post-fledging moult. Despite nest manipulations not affecting the expression of male sexually selected traits, females showed a clear preference in the choice experiment for both the males grown in nests without ectoparasites and those who moulted with a diet containing carotenoids. Overall, our findings demonstrated that the juvenile condition of males has the potential to influence future aspects in their lifetime including the perspective to mate in the first breeding season.

Introduction

Female choice has driven the evolution of exaggerated male traits that are conspicuous ornaments used by males to attract females (Andersson, 1994). Exaggerated sexually selected traits

(SSTs) are costly to produce and maintain (Zahavi, 1975) and often are condition-dependent, i.e. males in better condition pay a smaller unitary cost for ornamentation (e.g. Getty 1998; Getty 2006). Individual condition is ultimately determined by the resources available for maintenance and reproduction, but also by the genetic quality of an individual and its capability to withstand the environmental challenges that occurred i) during development (i.e. before SSTs are expressed) (Spencer & MacDougall-Shackleton, 2011), ii) at the time when the SSTs are produced (e.g. Hill 1991; Scheuber et al. 2004; Martín and López 2010), and iii) after their production if the SSTs need to be maintained (Griggio, Hoi, *et al.*, 2010; Hill, 2011). Therefore, condition-dependent SSTs could potentially convey various information to females about the individual condition of prospecting partners.

When SSTs are produced in different stages of a male's life cycle, it is predictable that earlier stressors mostly affect those SSTs that are developed earlier, whereas the SSTs produced at a later stage should be less affected (e.g. Scheuber et al. 2003). This simple prediction is complicated, however, by the different physiological and developmental pathways involved in producing different types of sexual ornaments (discussed by Hill 2011), and also by the different nature of the stressors that may occur during the life. Different types of environmental stress are therefore likely to differentially affect the suite of sexual ornaments that contribute to male sexual attractiveness. Consequently, females may evaluate past and current conditions of the potential partners according to the intrinsic characteristics of their SSTs, especially in those cases in which males bear multiple ornaments of whose evolutionary advantage has been debated (Møller & Pomiankowski, 1993; Johnstone, 1996; Candolin, 2003). The evolutionary advantage for males to invest in multiple traits has been explained by two alternative hypotheses, i.e. the multiple message (MMH) and the redundant signal hypotheses (RSH). The MMH states that different ornaments either convey information on various aspects of male quality or reflect male condition at different time scales (e.g. Møller and Pomiankowski 1993; Scheuber et al. 2003; van Doorn and Weissing 2004; Freeman-Gallant et al. 2010). The RSH, also called the backup hypothesis, posits that each SST provides only a partial piece of information on the overall condition of an individual; thus females should rely on multiple signals to obtain a more honest representation of male condition (e.g. Alonso-Alvarez and Galván 2011).

These issues on sexual selection have been investigated widely in birds which typically have multiple ornaments involved in mate attraction (e.g. Galván 2010; Taff et al. 2012). Colour ornaments are often produced in different and temporally distinct periods of a male's life. For example, plumage ornaments are usually formed a few months before the start of the breeding season, and once completed, they show a limited phenotypic flexibility (but see Ornborg et al. 2002;

Delhey et al. 2007; Griggio et al. 2010a). On the contrary, the colouration of bare parts, such as skin and beak, is usually expressed just before the start of the breeding season and typically shows high phenotypic flexibility (e.g. Faivre et al. 2003).

Early environmental stressors that are typically faced by nestlings and young birds include parasites and food availability. For example, it has been shown that nest conditions influenced the growth of nestlings which may later withstand carry-over effects (Hochachka & Smith, 1991; Scheuber *et al.*, 2003). Several studies demonstrated that the nestling growth is adversely affected by the abundance of ectoparasites in the nest (Christe, 1996; Tomás *et al.*, 2008; Cantarero *et al.*, 2013) either directly (Martínez-de la Puente *et al.*, 2011) or because they influence parental behaviour (Richner & Tripet, 1999). Poor environments or scarce feeding ability may produce detrimental effects both immediately and at longer time scales (Krause *et al.*, 2009). Considering that moult is one of the most costly events in the lifetime of a bird (Jenni & Winkler, 1994), the ability to acquire adequate energy resources, in terms of both macro- and micro-nutrients, during moult is a major challenge during the life of an individual (Siikamaki *et al.*, 1994; Barta *et al.*, 2008). In several cases, birds must rely on both food obtained during moult and on previously stored reserves to fulfil the energetic requirements of the moult (Fox *et al.*, 2009; Fox & King, 2011). Freshly fledged birds unable to satisfy their nutritional needs may face a decline in conditions (Metcalfe & Monaghan, 2001; McGraw *et al.*, 2002; Searcy *et al.*, 2004; Krause *et al.*, 2009; Harrison *et al.*, 2011) and therefore perform an impaired post-juvenile moult (Badyaev & Duckworth, 2003). Thus, although nutrition during moult is expected to have the strongest effect on the expression of feather signals (and hence on male attractiveness), its effect may be amplified by nestling condition either additively or multiplicatively.

Carotenoids are considered to be a key component of animal diet, as they can only be obtained from food (Goodwin, 1980), and insufficient carotenoid content in the diet has detrimental effects to the organism, in particular during moult (McGraw *et al.*, 2011). As expected, the effect of carotenoid limitation is particularly pronounced in those species that exhibit yellow/red carotenoid-based SSTs (e.g. Blount et al. 2003b; Biard et al. 2006). Indeed, carotenoid-pigmented feathers honestly signal the nutritional status of the bird during moult (e.g. Hill and Montgomerie 1994; Hill 2000; Navara and Hill 2003). However, little is known on the effect of carotenoids on the structural coloration of plumages, and the only study conducted so far suggests that, in blue tits, carotenoid supplementation does not affect the UV/blue feathers (Peters *et al.*, 2011). Carotenoids are also found in the beak of several birds which is a trait used in female choice that honestly signals short-term changes in individual condition (Faivre *et al.*, 2003; Navarro *et al.*, 2010; Rosenthal *et al.*, 2012).

We investigated the influence of early conditions on the expression of SSTs in the European starling where reproductive males show iridescent and elongated throat feathers and yellow nuptial beaks. In particular, our general aim was to investigate whether multiple ornaments convey information about different stress types experienced by the birds at different developmental stages. Indeed, plumage colour ornaments are usually completed in autumn about five months before the beginning of the breeding season. In contrast, the beak gets its nuptial colouration immediately before the breeding season. We evaluated the effect of individual conditions during two crucial developmental stages in altricial birds, namely the nestling stage and the moult stage, on male sexual attractiveness. We experimentally manipulated individual conditions by reducing the ectoparasite load at the nest and the availability of carotenoids during the post-juvenile moult, and then obtained the spectrometric measures of the beak and the throat feathers of reproductive males to understand which, and how, SSTs were affected by our experimental treatments. Nestling males were obtained from ectoparasite-free and control clutches from a starling population with a high rate of ectoparasite infestation. After fledging, these males were randomly subdivided into two further groups which were fed with either a carotenoid-rich or a carotenoid-free diet during the post-juvenile moult. We predicted that males experiencing more favourable juvenile conditions, i.e. without nest ectoparasites and with a carotenoid-rich diet during the moult, should be able to produce better quality SSTs and be more attractive to females. Considering that we performed two manipulations of different nature, we cannot predict *a priori* which manipulation mostly affected the SSTs. On one hand, if the different manipulations were equally effective the SSTs would be more influenced by the stress that was temporally closer to the time when the signal is produced. In other words, carotenoid restriction would have a greater effect on sexual attractiveness than ectoparasites, and feather colours would be more affected than beak colour as all males received an enriched-carotenoid diet after moult conclusion during about five months before beak nuptial colouration was expressed. On the other hand, it has been suggested that early stresses are more likely to have persistent effects on individual condition as they interfere with development (Lindström, 1999; Tschirren *et al.*, 2009), and may trigger compensatory responses which affect the life-history trajectory of an organism (Metcalfe & Monaghan, 2001). Under this scenario, birds from naturally infested nests should produce poorer SSTs than birds from treated nests or, alternatively, they can produce SSTs of similar quality but pay later in life the costs of the relative compensatory response (Metcalfe & Monaghan, 2001). Moreover, if plumage quality reflects individual conditions during moult, we predicted that males supplemented with limited carotenoids in the diet produce lower quality throat feathers and were therefore less preferred by females (Bennett *et al.*, 1997). Conversely, since the beak colouration should reflect current individual

conditions (e.g. Faivre et al. 2003), we did not expect to observe any effect of diet on beak colour as all males, from the end of the moult to the breeding period, were fed with the same amount of carotenoids in their diet. Hence, any difference in beak colours could be ascribed to carry-over effects of early conditions.

Materials and Methods

In February 2011, we installed 45 nest boxes in Northern Italy (Ozzano Emilia, Bologna, 44°28'N, 11°29'E) on the roofs of two buildings at a height of approximately 15 m and orientated randomly with respect to cardinal points. The nest boxes were made of softwood panels (2 cm thick) with inside dimensions of 15 × 15 cm (base) × 45 cm (height) and an entrance hole size diameter of 4.5 cm (the distance of the hole from the base was 31 cm). Overall, 25 out of 45 nest boxes were occupied by starling pairs. Nest boxes were cleaned and treated with an insecticide (Frontline spray, Fipronil 0.25 g, Merial – Tolose, France) after a breeding attempt was concluded, so that new breeding attempts were not affected by the ectoparasite load of previous broods.

Nest and dietary treatments

From the middle of March to the end of the breeding season (June-July), we checked all of the nest boxes daily to control for egg deposition progress. After clutch completion, we randomly assigned each clutch to the insecticide-treated (I, N = 15) or control group from which ectoparasites were not removed (parasitized group, P, N = 16). The dominant ectoparasite of the starling broods is *Carnus hemapterus*, a small (ca. 2 mm) haematophagous fly which feeds on the blood of nestlings and adults (Liker *et al.*, 2001). In the insecticide-treated clutches, the ectoparasites were removed using an antiparasitic spray (see above for details), whereas the control clutches were sprayed with tap water. Each treatment consisted of removing all of the eggs or chicks from the nest and then spraying one shot of antiparasitic solution or water onto the nest cup. When the nest material was completely dry (usually within three minutes), the eggs or chicks were returned to the nest. Both treatments were repeated every three days until fledging, and the total amount of ectoparasites on nestlings were counted in order to assess the effectiveness of our treatment. After the first treatment, ectoparasites disappeared almost completely from insecticide-treated clutches (mean number of ectoparasites = 0.2 ± 0.1, N = 15), whereas control clutches constantly showed a very large ectoparasite load (mean number of ectoparasites = 14 ± 3, N = 16). All of the nestlings were individually marked and molecularly sexed using the protocol already used in a previous study

(Serra *et al.*, 2012). Overall, 39 males (17 from 11 insecticide-treated clutches and 22 from 14 control clutches) were caged in four indoor aviaries 2-3 days before they left the nest. In some cases, two or three males originated from the same clutch. When there were two males from the same clutch, they were caged in different aviaries, whereas in cases of three males of the same clutch, two of them were randomly housed in the same aviary. Each aviary measured 200 × 80 (base) × 200 cm (height) and birds were provided with food and water *ad libitum* (see below for dietary details). The shape of these aviaries is that recommended by a recent study (Asher *et al.*, 2009) where starlings housed in long-shaped cages displayed low stereotypic behaviour which is widely considered as an indicator of an inadequate environment. Here, all birds were treated against endoparasites (coccidia, bacteria, and fungi) and fed without carotenoids until the start of the moult. When the moult started, 20 birds received a carotenoid-rich diet (C) for about three months (i.e. during the post-juvenile moult), while the other 19 birds' diet was deprived of carotenoids (NC). At moult completion, all birds were fed the same diet containing carotenoids. The food consisted of a mixture of 50% TH White Extra and 50% of TH White Soft (Raggio di Sole, Piacenza - Italy) which contains a concentration of lutein between 0.9 and 1.2 mg per kg. The dietary carotenoid supplementation consisted of an additional 50 g Versele-Laga Yel lux [Oropharma, Deinze - Belgium; extracted from marigolds (*Tagetes erecta*) and containing 8 g of lutein per kg] per one kg of base food which is a concentration already used on starlings (Van Hout *et al.*, 2011).

We therefore obtained four experimental groups: 1) I/C; 2) P/C; 3) I/NC; and 4) P/NC. During winter, five P individuals (one C and four NC birds) died and one escaped, so in the subsequent breeding season we could use 9 I/C, 9 P/C, 8 I/NC, and 7 P/NC males. At the beginning of January 2012, all starlings were moved to single cages (60 × 25 × 35 h cm), where they were kept until SSTs and sexual attractiveness was estimated (see below).

Spectral analyses of SSTs

In the middle of March 2012, we measured the spectral reflectance of the beak and throat of each male. We took two measurements of the beak colour, one at the yellow distal part and one at the blue proximal part and both on the right side of the upper mandible. The feathers reflectance spectrum was obtained by averaging the spectra measured on five iridescent green feathers randomly plucked from the right side of the lower throat where purple and green meet (Svensson, 1992). We measured each feather separately following established protocols (Meadows *et al.*, 2011).

The spectral reflectance (range 300-700 nm) was measured with an Ocean Optics S2000 spectrometer and measurements were relative and referred to a WS-2 white standard and to

darkness. This calibration was repeated before starting each measurement session. The spectral reflectance of feathers was measured in laboratory whereas the beak spectra were measured on daily natural light near to the aviary to limit the stress for birds. The ambient light was excluded with a black PVC tube mounted on the ferrule tip of the probe which was held at 90 degrees from the feather or the beak. Each measurement (two for the beak and five for the throat) was the average of five consecutive measures taken automatically by the spectrometer.

The mean reflectance corresponded to the average value of reflectance in the 300-700 nm range and was regarded as an index of feather and beak brightness (Hill & McGraw, 2006). Given that in the starling female mate preference is influenced by the ultraviolet range of spectra (Bennett *et al.*, 1997), we also calculated the UV chroma of throat feathers and beak as the proportion of reflectance between 300 and 400 nm with respect to total reflectance (UV chroma = $R_{300\text{-}400}$ / $R_{300\text{-}700}$, Griggio et al. 2010b). As the spectrum of the yellow part of the beak is characterised by two peaks respectively in the UV (300-400 nm) and yellow (450-570 nm) ranges, we separately considered their contribution to total brightness by calculating UV and yellow brightness (indices already used in other studies, e.g. Freeman-Gallant et al. 2010; Pickett et al. 2013; Midamegbe et al. 2013). Similarly, the spectrum of throat feathers has two peaks respectively in the UV (300-400 nm) and green (500-600 nm) ranges, so we considered the UV and green brightness separately. For both colours of the beak, we calculated the carotenoid-chroma [($R_{450 - R700}$) / R_{700}] as it represents the most appropriate objective colorimetric parameter for quantifying spectral purity (Hill & McGraw, 2006).

Female preference

We estimated the attractiveness of males in a choice chamber in which a female could choose between two males from different experimental groups. We used 14 females fledged in 2010 from the same nest box population which were kept in large indoor aviaries. Males and females were kept in different aviaries and did not have any interaction before the choice test in order to exclude any effect of previous knowledge on female preference (Senar *et al.*, 2013). The choice chamber had two lateral rooms (choice rooms, 50 × 200 × 80 h cm) and one central room (neutral room, 100 × 200 × 80 h cm) which were separated by dividers that had an opening (35 cm wide) that allowed the female to freely move through the rooms but prevented the stimulus males on the two side ends of the choice chambers to see each other (see below). We positioned a cage with a male on the side of each lateral room. In each lateral room, the female could see the male positioned at that side but not the one positioned to the opposite side. The choice tests were performed indoors, in the aviaries, and in a room (18 x 9 x 10 h m) where all the experimental birds were housed during the study. The

two males used in a trial were visibly but not acoustically isolated from the other birds in the room (Swaddle *et al.*, 2005). The central room contained food and water, and the feeders were positioned in a part of the room from which the female could not see males. This arrangement of the choice chamber allowed the unambiguous estimation of the time spent by the female observing males from the time spent in feeding or resting. Each female was moved into the choice chamber 24h before starting the choice trials and to allow to acclimatisation. Six different pairs of males were presented to each female in six consecutive days at the same time between 10.00-13.00 h.. Before each trial, the two males were introduced in the lateral cages, and after 5 min of acclimation, the female position was recorded for one hour using a Nikon P6000 digital camera. Video analyses were performed by the same person (AB) using the software LongoMatch (ver. 0.16.9 - http://www.longomatch.ylatuya.es). We regarded the female preference for each male as the proportion of time spent by the female in each lateral room of the choice chamber.

Statistical analyses

The investigated range of wavelengths (300-700 nm) was first divided into 40 classes of 10 nm, and then the mean brightness of the whole spectrum was subtracted from the mean brightness of each class. This standardisation eliminates the strong correlation between the first PCA score and brightness (Cuthill *et al.*, 1999). We therefore performed three PCAs to analyze the spectrum of each SST (blue and yellow parts of the beak and green throat feathers). We only considered the first and second PCA scores, since combined they explained more than 80% of the total spectral variance. Pearson's correlations were used to investigate the co-variation between PCA scores and colour parameters. Spectral data was normally distributed, so we used parametric tests.

Three ANOVAs were used to test if PC1 and PC2 (predictors) of the SSTs differed between nest (fixed factor, 2 levels: Parasitized, Insecticide) or dietary groups (fixed factor, 2 levels: Carotenoid supplementary, No-Carotenoid supplementary). The interaction "nest group" x "dietary group" was always excluded from the model as never significant (yellow part of the beak: $P > 0.488$; blue part of the beak: $P > 0.437$; throat: $P > 0.140$).

The time spent by the female in association with each of the males was square-root transformed and fitted as a dependent variable in a Linear Mixed Model (LMM), whereas nest treatment (Parasitized, Insecticide) and dietary treatment (Carotenoid, No-Carotenoid) were considered as fixed effects. The model was first tested using the interaction term "nest treatment x dietary treatment" as a fixed effect, but it was removed from the final model as not significant ($P > 0.188$). We also considered the identity of the female and the male as random factors in order to take into account repeated measures on the same individuals.

Statistical analyses were performed with the software IBM SPSS Statistics (ver. 20). All of the tests were significant when $P < 0.05$.

Results

Expression of male SSTs

In their first breeding season, males that received a diet with carotenoids during moult developed brighter beaks, both in the UV and yellow range, along with higher UV chroma than males that received a diet without carotenoids. Considering the main colour parameters that describe the spectral shape of carotenoid-based ornaments, the UV saturation and the UV and yellow brightness of the yellow part of the beak was higher in C than NC birds (Tab. 1, Fig. 1). These colour parameters were positively correlated to the second explanatory factor of the PCA, which was different between dietary treatments. In contrast, the first PC score of the yellow part of the beak was positively correlated to the yellow chroma and, in turn, negatively correlated to the UV chroma. Indeed, the first PC score described the spectral shape of the beak colouration which is characterised by two peaks in the UV (300-400 nm) and yellow (450-570 nm) range of spectra, respectively. Our results therefore indicate that the spectral shape of the yellow part of the beak was not different between treatments.

		yellow part of beak		blue part of beak		throat feathers	
		PC1	PC2	PC1	PC2	PC1	PC2
Pearson's correlation	UV chroma	**-0.59**	**0.67**	**-0.95**	-0.06	0.14	**-0.36**
	UV brightness	-0.21	**0.60**	**-0.46**	**-0.43**	**-0.22**	**-0.86**
	Yellow (or Green) chroma	**0.73**	0.14	**0.45**	**-0.80**	**-0.79**	-0.03
	Yellow (or Green) brightness	0.21	**0.49**	0.04	**-0.44**	**-0.45**	**-0.78**
	% Variance	63	22	77	14	43	37
ANOVA	Nest treatment (P, I)	0.275	0.711	0.528	0.295	0.918	0.954
	Dietary treatment (C, NC)	0.124	**0.011**	0.779	0.560	0.196	0.453

Tab. 1 - Effects of nest and dietary treatments on colouration of male SSTs. Resulting scores from the PCAs on male sexually selected traits and their correlation with the main colour parameters. For the breast colouration, we considered the Green chroma and Green brightness. Significant correlations are highlighted in bold.

Conversely, the blue part of the beak and the throat colouration did not differ significantly between treatments (Tab. 1). The first PC score of the blue part of the beak was positively correlated to the UV component of the spectra, whereas the second PC score was positively correlated to the yellow component of the spectra (Tab. 1, Fig. 1). The first PC score of the throat feathers was negatively correlated to the green part of the spectra, indicating that lower PC1 are representative of more green feathers. The second PC score is, indeed, negatively correlated to the UV and green brightness, so that higher PC2 values indicate less bright throat (Tab. 1, Fig. 1). Our analyses therefore suggest that the experimental manipulations did not influence individual conditions and, thus, the expression of these SSTs in a measurable way. Alternatively, the colouration of throat feathers is not influenced by individual conditions, although its structural colouration is used by females to choose their partner (Bennett *et al.*, 1997).

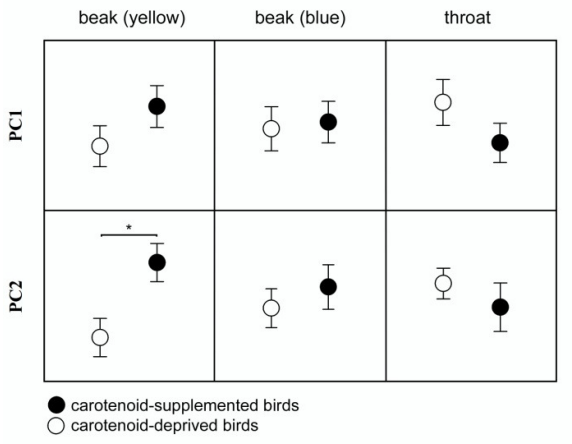


Fig. 1 - Mean value ± SE of the first and second explanatory factors of the PCA for the colourations of the beak (yellow and blue) and the breast in birds fed with carotenoid-rich (C) and carotenoid-free (NC) diets. The only significant difference between dietary groups is in PC2 of the yellow part of the beak.

Female preference

Females showed a significant preference for males grown in ectoparasite-free broods and fed with a diet containing carotenoids during moult (Tab. 2, Fig. 2). The preference for C males could readily be explained by the difference in beak colouration observed between dietary treatments, whereas the female preference for I males did not match with our measure of SSTs expression. Female mate choice was not influenced by the male size (tarsus length) which did not differ between experimental groups ($F_{3,29} = 0.382$, $p = 0.77$) and neither between I and P birds ($F_{1,31} = 0.636$, $p = 0.43$). Interestingly, our experimental treatments did not affect the adult body condition

of males at the time when the choice tests were carried out as the body mass index (BMI, calculated as tarsus/weight and considered as a measure of individual condition) was not different between experimental groups ($F_{3,29} = 0.496$, $p = 0.69$).

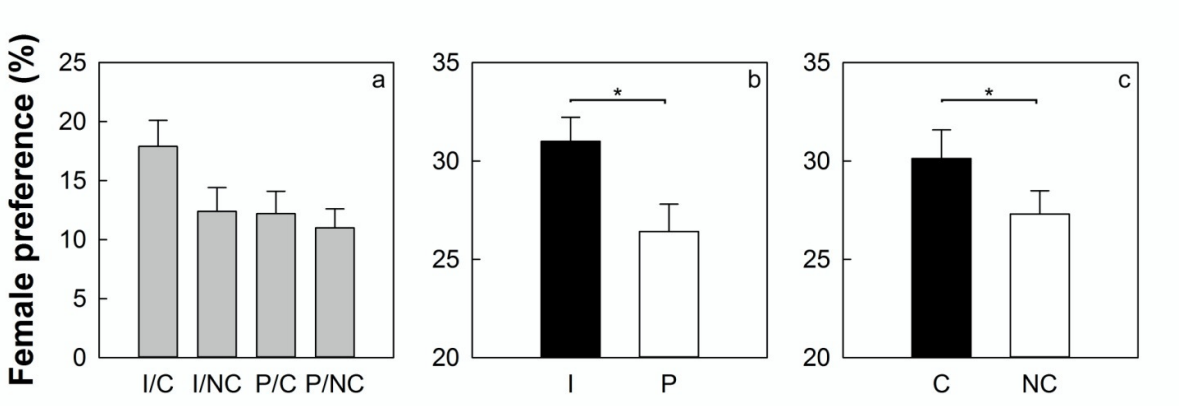


Fig. 2 - Female preferences among the males of different experimental groups (% mean ± SE). The female preference is the percentage of time that females spent on average in front of the males of the 4 experimental groups (a), for males from ectoparasite-free (I) and control (P) nests (b), and for males supplemented with carotenoids (C) or deprived of carotenoids (NC) in diet during moult (c).

Discussion

In order to maximise their fitness returns, females are driven to choose males that honestly signal their quality as mate through highly expressed condition-dependent SSTs (e.g. Hill 1991). Often, males bear multiple SSTs allowing females to better evaluate their quality as prospective partners (Scheuber *et al.*, 2004).

In the present study, we considered a wild passerine species where the males bear multiple ornaments in order to investigate whether the environmental conditions experienced by males in their first year of life can influence their likelihood to mate the following breeding season. Our findings show that favourable juvenile conditions, i.e. ectoparasite-free nests and the availability of carotenoids during their first moult, produce downstream benefits for males in the following breeding season as they are preferred by females. We also demonstrate that beak colouration is a SST whose expression, although mostly influenced by current individual condition, is also affected by the nutritional condition of individuals during moult.

Expression of male SSTs

A number of studies showed that adverse juvenile conditions produce carry-over effects to the organism, such as by decreasing survival during migration (Mitchell *et al.*, 2011), worsening the physiological response to a stress occurring in adulthood (Krause *et al.*, 2009), or affecting song learning (Buchanan *et al.*, 2003). However, carry-over effects on the expression of SSTs have been less studied (Harrison *et al.*, 2011; Doutrelant *et al.*, 2012).

For the first time, we experimentally demonstrated that the beak colour, which is a carotenoid-based sexual trait, is related to the availability of carotenoids during moult, i.e. a physiologically demanding activity that occurs early after fledging in the European starling. Whilst there is evidence that beak colour is a plastic trait that responds rapidly to fluctuations in individual condition (Navarro *et al.*, 2010; Rosenthal *et al.*, 2012), no study has demonstrated until now that past nutritional condition could influence the expression of beak colour. The strength of our findings lie in the fact that males were provided with the same dietary carotenoid content from the end of the moult (October-November) to the choice-chamber experiment (March-April); thus it is unlikely that the different expression of yellow beak colouration between treatments was due to different nutritional conditions of males after the end of the moult. However, the physiological process under the lowly expressed yellow beak colouration in males that did not receive carotenoids during moult deserves further investigation. Indeed, a study on zebra finches (Blount, Metcalfe, Arnold, *et al.*, 2003) has demonstrated that the antioxidant assimilation capacity of adults that received a poor quality diet as chicks, even in terms of carotenoid content, was lower when compared to adults that received a standard quality rearing diet. These findings may suggest that during the breeding season, the antioxidant assimilation capacity of male starlings is regulated by the amount of carotenoids assimilated during moult. As a consequence, males that experienced a shortage of carotenoids during moult will be unable to assimilate and apply these pigments to carotenoid-based ornaments in the following breeding season. Additionally, a compensatory response of birds deprived of carotenoids during moult could contribute to explaining the less bright beak colour of these birds during the breeding season (Metcalfe & Monaghan, 2001). Indeed, it could be suggested that starlings can tolerate the nutritional deficiency experienced during moult by using other elements (i.e. vitamins C and E, and antioxidant enzymes) than carotenoids as antioxidants and immune substrates (Hartley & Kennedy, 2004) or, alternatively, by down-regulating the antioxidant and immune response during moult (Kostelanetz *et al.*, 2009). In the first case, birds underwent a progressive reduction of these elements during moult and dealt with such depletion later in life. In the second case, birds may face the subsequent life-history events by carrying the burden of the detrimental effects due to the down-regulation of the antioxidant and

immune responses earlier in life. The final result is that birds deprived of carotenoids during moult produced less attractive SSTs (beak colours) in their first breeding season (Metcalfe & Monaghan, 2001).

Considering the hypotheses for multiple SSTs, our results support the MMH as the beak and throat colours did not respond equally to different stressors. More precisely, while we found that the colouration of the yellow part of the beak is influenced by the availability of carotenoids during moult, we failed to find an effect of a shortage of carotenoids in the diet on the colouration of growing throat feathers. This suggests either that the quality of feathers is not related to individual conditions or that our experimental manipulations did not deplete individual conditions significantly enough to adversely affect the quality of growing feathers. Additionally, it is likely that throat colouration did not differ between dietary groups because moult duration, which is the main factor regulating the plumage quality (Jenni & Winkler, 1994), was not different between treatments. In support to these findings, earlier studies on the European starling failed to find a clear effect of a daily 4-hour food deprivation on both the chronic stress of moulting birds (Bauer *et al.*, 2011) and the quality of feather regrowth in birds undergoing an induced moult (Strochlic & Romero, 2008). Moreover, another study showed that moulting starlings can rapidly compensate an increased allostatic load by down-regulating the response to stressors in order to maintain a constant moult rate (Kostelanetz *et al.*, 2009). Our study therefore provides further supports for the prediction that an appropriate and complete moult takes precedence over other concurrent physiological challenges.

Female preference

Despite our experimental manipulations only having a significant effect on the yellow part of the beak, as predicted females showed a clear preference for males that experienced the most favourable juvenile conditions, i.e. those grown in nests without ectoparasites that received carotenoids during moult. Our findings can be explained by two alternative but not mutually exclusive hypotheses: i) females can perceive different aspects of male quality that we have been unable to measure or ii) female choice is influenced by male sexual traits that we did not investigate.

The European starling has a black iridescent plumage produced by a combination of melanin-based and structural colourations (Cuthill *et al.*, 1999). While the plumage brightness is associated with the amount of melanin deposited in feathers, with brightness being higher in feathers with lower melanin concentrations, the ultraviolet colouration is indicative of the micro-structural organization of the feathers. Considering the moult duration as the ultimate factor

influencing the micro-structural organization of the feathers, it may be assumed that a longer moult will produce more well-organized feathers and consequently highly UV saturated plumages than those produced by a shorter moult. In a previous study we shown that birds grown in ectoparasite-free clutches started to moult at an earlier age and moult over longer periods than birds from naturally infested clutches (Pirrello et al. submitted). Nonetheless, the colouration of throat feathers did not differ between nest and dietary treatments. Studies aimed at investigating the condition dependence of structural colours have recently provided confounding results. For instance, it has been demonstrated that the UV colour of wing coverts and breast feathers in wild turkeys (*Meleagris gallopavo*) is negatively affected by coccidial infection (Hill *et al.*, 2005), the UV-blue coloration of the tips of the primary feathers in male Eastern Bluebirds (*Sialia sialis*) is partially linked to nutritional condition (Doyle & Siefferman, 2014), whereas the UV/blue (crown) and white (cheeks) colourations of blue tits are not influenced by the diet quality during moult (Peters *et al.*, 2011). Our study therefore suggests that the structural colouration of throat feathers in starlings is not linked to the nutritional condition or to the presence of ectoparasites in the nest. However, females showed a clear preference for males grown in ectoparasite-free nests. It must be considered that despite measuring the spectral reflectance of the throat feathers at a 90 degree angle, plumage iridescence is an extremely variable colouration which continuously changes when observed at different angles of viewing, and that probably females perceive the pattern of variation to better assess the male quality (Dakin & Montgomerie, 2013). Hence, it cannot be excluded that individual conditions may affect the iridescence of throat feathers at angle of viewing different from 90 degrees, and that males from ectoparasite-free nests showed more iridescent throat feathers than males from naturally infested clutches. Finally, females are likely to perceive and use this information in order to choose the best partner.

Another aspect that we did not consider advisedly in this study is the song quality of male starlings, although it is a SST that reflects the developmental conditions of individuals (Buchanan *et al.*, 2003). Indeed, we performed several attempts to recording the male song after the choice experiment, by positioning a male apart from the other males in front of the aviary containing the females. However, we observed that because of such movement the male was inhibited to sing for the entire recording period (2 hours). As a consequence, considering our experimental design where the males were introduced in the lateral cages only 5 min before starting the observations, it can be assumed that in this study the song of males did not represent a SST that could influence the female choice.

Finally, male behaviour as a factor determining female choice is another aspect which could deserve further investigations. For example, if birds in good condition are more active than those in

poor condition, in a multiple message context females could also use the male's activity as a cue to select mates.

Conclusions

Our study experimentally demonstrates that poor environmental conditions during the early stages of life can produce carry-over effects in adult male starlings which will decrease the likelihood of mating during their first breeding season. Since female preference was influenced by the rearing conditions of males, we predict that nest ectoparasites represent an early stressor in wild starlings and in turn adversely affect the reproductive performance of males in their first breeding season even though the nest conditions did not influence the expression of male SSTs. The availability of carotenoids during moult has a carry-over effect on the beak colouration which is a SST mainly related to current individual condition.

Conclusively, the ability of females to evaluate past conditions experienced by the prospective partners and adaptively prefer high quality individuals is a result which deserves further investigations in future studies on mate choice.

Riassunto

Sulla base delle teorie di Darwin, i biologi evoluzionisti hanno formulato diverse ipotesi per spiegare l'origine dei tratti sessuali secondari esibiti dai maschi, anche detti ornamenti. Una di queste ipotesi suggerisce che le femmine valutino gli ornamenti maschili per massimizzare il proprio successo riproduttivo nella scelta del partner e che la preferenza femminile sia una delle forze selettive principali alla base dell'evoluzione dei tratti sessuali secondari. Lo scenario evolutivo si complica se consideriamo che i maschi di molte specie esibiscono ornamenti multipli. E' stato ipotizzato che diversi ornamenti possano segnalare differenti aspetti della qualità maschile. In questo caso la loro espressione potrebbe riflettere la condizione del maschio in fasi diverse della *life-history*, oppure differenti ornamenti potrebbero essere influenzati in modo diverso da uno stesso stress. Molti studi sui tratti sessuali secondari maschili hanno mostrato che i costi associati all'espressione di alcuni ornamenti sono correlati alla condizione dell'individuo, cioè sono condizione-dipendenti. Tuttavia, sono pochi gli studi sull'effetto di eventi di stress in fasi diverse del ciclo vitale sull'espressione di ornamenti multipli. Nel mio progetto di Dottorato ho studiato i meccanismi che regolano l'espressione dei tratti sessuali secondari di Storno (*Sturnus vulgaris*), un passeriforme di medie dimensioni in cui i maschi presentano ornamenti multipli. In questa specie, vi sono evidenze sperimentali che le femmine preferiscono penne della gola più lunghe e con un'alta brillantezza nella radiazione ultravioletta, canti complessi e (probabilmente) colori del becco più intensi. Nel corso del mio Dottorato ho effettuato tre manipolazioni sperimentalmente per modificare la condizione dei maschi in due fasi costose della *life-history*, durante lo sviluppo giovanile e nel corso della prima muta, per valutare l'effetto di stress precoci sull'espressione degli ornamenti nel corso della prima stagione riproduttiva. L'attrattività dei maschi manipolati è stata testata utilizzando un esperimento di scelta femminile. La prima manipolazione sperimentale aveva l'obiettivo di studiare l'effetto di uno stress immunitario sulla condizione dei pulcini in nidi naturali (manoscritto 1), oltre che sui tempi di muta del primo e del secondo anno di vita (manoscritto 2). Con la seconda manipolazione sperimentale ho testato l'effetto della rimozione dei parassiti del nido sull'investimento parentale durante il periodo d'incubazione (manoscritto 3)

e sull'interazione genitori-prole (manoscritto 4). La terza manipolazione sperimentale è stata effettuata sulla dieta dei maschi nel periodo della muta giovanile; ad un gruppo di individui è stata somministrata una dieta ricca di carotenoidi, mentre un altro gruppo ha ricevuto una dieta priva di carotenoidi. Quindi ho esaminato i tempi della muta giovanile dei maschi involatisi da nidi con e senza parassiti che hanno ricevuto una dieta ricca o priva di carotenoidi (manoscritto 5). Ho analizzato l'effetto delle differenze di carico parassitario e di dieta i) sul tempo investito dai maschi nel mantenere in ordine il piumaggio (manoscritto 6), ii) sull'espressione degli ornamenti maschili e iii) sulla preferenza femminile (manoscritto 7). I risultati ottenuti suggeriscono che uno stress precoce, come un'infezione batterica o la presenza degli ectoparassiti nel nido, non abbia un effetto rilevante sulla crescita dei pulcini. Il potere antiossidante (Capacità Antiossidante Totale, TAC) e la concentrazione di metaboliti reattivi dell'ossigeno (ROM) non sono risultati significativamente differenti tra i pulcini trattati con lipopolisaccaridi (LPS) e i pulcini di controllo trattati con una soluzione salina. D'altra parte, l'ematocrito è risultato più alto nei pulcini trattati con LPS ma solo in quelli nati tardivamente. Nei nidi in cui sono stati rimossi gli ectoparassiti, i pulcini hanno eseguito un *begging* posturale mediamente più intenso, mentre le strategie di allocazione degli adulti non sono state influenzate dalla presenza dei parassiti nel nido, anche se durante la fase di incubazione delle uova gli adulti hanno trascorso un tempo significativamente maggiore nei nidi privi di parassiti. L'inizio della muta post-giovanile è stato anticipato dagli individui trattati con LPS e da quelli involatisi da nidi senza ectoparassiti, probabilmente perché in condizioni migliori rispetto agli individui di controllo (trattati con una soluzione salina, o involatisi da nidi infestati da parassiti). La muta è stata completata in tempi più lunghi dai maschi involatisi dai nidi senza parassiti, mentre il trattamento con LPS non ha prodotto differenze significative rispetto ai controlli nella durata della muta. La presenza dei carotenoidi nella dieta durante la muta non ha influenzato la colorazione delle penne della gola. Tuttavia i risultati suggeriscono che il trattamento abbia avuto un effetto positivo sulla condizione nei mesi successivi alla muta, poiché i maschi che hanno assunto carotenoidi nel corso della muta hanno investito più tempo nelle attività di mantenimento del piumaggio e hanno prodotto un becco più brillante nel corso

della successiva stagione riproduttiva. Nell'esperimento di scelta, le femmine hanno mostrato una preferenza significativa per i maschi involatisi dai nidi senza parassiti e che hanno ricevuto una dieta ricca di carotenoidi durante la muta.

In conclusione, i risultati del mio Dottorato forniscono prove sperimentali di effetti *carry-over* nello Storno. Infatti, due differenti stress giovanili hanno ridotto l'attrattività dei maschi nella prima stagione riproduttiva, anche se i dati ottenuti dalle misure strumentali hanno evidenziato differenze significative soltanto per la colorazione del becco e non per la lunghezza o la colorazione delle penne della gola. E' noto che l'attrattività dei maschi è legata alle caratteristiche del canto, ma nel corso del mio Dottorato non è stato possibile stimare l'effetto delle manipolazioni sperimentali su tale ornamento. Ad ogni modo il risultato ottenuto dall'esperimento di scelta femminile suggerisce che le femmine considerano in modo complessivo gli ornamenti multipli del maschio, la cui espressione è influenzata da effetti *carry-over*. Questi risultati sembrano in accordo con l'ipotesi del segnale ridondante (*Redundant Signal Hypothesys*), secondo cui le femmine integrano le informazioni ottenute dagli ornamenti maschili per valutare la qualità dei potenziali partner.

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