UNIVERSITÀ DEGLI STUDI DI MILANO

PhD course in Environmental Sciences

XXX Cycle

Colouration as an intraspecific signal in birds

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Academic Year: 2016-2017

SSD: BIO/07

Thesis performed at Department of Environmental Science and Policy, University of Milan, Italy.

Contents

Abstract

Colour variation seems to be a pivotal trait in avian communication dynamics, where information is provided to recipients by 'signallers' through colouration. Plumage colours can be structural or due to pigments incorporated into the keratin of feathers, and melanin-based colouration, produced by the melanocortin system, is one of the most common in birds. Melanin-based colouration is under genetic control and the degree of melanism is often associated with variation in physiological, behavioural and life-history traits, due to pleiotropic effects of the compounds regulating the melanin synthesis.

However, variability in colouration is observed not only among plumage traits but also in fleshy ornaments, legs or eggshells. Variation in eggshell pigmentation, determined by biliverdin and protoporphyrin pigments, may accomplish several different functions, the most obvious of which are camouflage and background matching. It has been also proposed that intra-specific variation in eggshell pigmentation patterns could reflect egg, maternal or paternal traits and hence provides reliable cues to conspecifics about egg, maternal or paternal quality.

The first part of the studies presented in this thesis is aimed at investigating the role of melanin-based colouration from different points of view. I started by investigating the role of melanin-based colouration in barn swallow nestling (*Hirundo rustica*) as a signal of individual quality and its covariation with behavioural stress response. Moreover, I investigated the covariation of genes regulating melanin-based plumage colouration and plumage polymorphism in lesser kestrel males (*Falco naumanni*).

Secondly, since the relationship between protoporphyrin-based eggshell pigmentation and egg or maternal/paternal traits appears to be highly variable among species, I investigated the possible signalling role of protoporphyrin-based eggshell pigmentation in both study species.

Using both experimental and correlative approaches, I found that even if pleiotropic effects of melanocortin system may still drive the associations between melanin-based colouration and individual quality, the melanocortin pleiotropic hypothesis may not apply to plumage colouration deriving from mixing of eu- and pheomelanins. Moreover, results on the potential signalling role of protoporphyrin-based eggshell pigmentation in two distantly related bird species support the idea that intraspecific signalling via eggshell pigmentation is species-specific feature rather than a general pattern among avian taxa.

Chapter 1

General introduction

Many vertebrates exhibit a lot of conspicuous ornamental traits, including horns, long tails or bright colouration, which are mainly used for intraspecific communication and have little bearing for survival (Andersson, 1994). Ever since Darwin (1859, 1871), behavioural ecologists and evolutionary biologists have been interested in intra- and interspecific variation in animal colouration and its adaptive function, as well as in factors affecting colour expression and in the role of colours in communication dynamics.

With regard to intraspecific colour variation, there are species whose individuals show small differences, while in others each individual looks unique. This variability seems to play a crucial role in several communication dynamics in which 'signallers' use colouration to provide information to 'receivers' (Dale, 2005). Several studies highlighted a role of colouration in camouflage, warning, thermoregulation and especially in social interactions (Dale, 2005). In this respect, in many species colouration acts as a signal of phenotypic and genetic attributes for potential mates, thus enhancing possibilities of mating success and being under sexual selection (Andersson, 1994). Indeed, since production and maintenance of brightest colourful ornamental traits have a cost only sustainable by high quality individuals, colouration may convey honest information about the quality or the condition of the bearer/signaller (Zahavi, 1975; Andersson, 1994). Furthermore, according to models of runaway selection proposed by Fisher (1930), genes for the expression of colourful ornamental traits (generally expressed in males) may evolve under the effect of preferences for those traits (generally expressed in females) that reliably signal genotypic or phenotypic quality and may also predict sexual attractiveness of future offspring (Andersson, 1994). However, not all colour traits are deliberately used by the bearer/signaller to indicate its quality, but may still be used as a cue by the receiver to decide on its future actions, as in the case of a predator or a brood parasite (Hasson, 1994; Brulez et al., 2015).

In addition, intraspecific colour variation seems to be also linked to alternative strategies to cope with various environmental factors, and in a majority of species it is indeed associated with reproductive parameters and behavioural, physiological and life-history traits (Roulin, 2004).

Birds are the most colourful group of vertebrates thanks to the marvellous shapes, forms and shades of their feathers and the spectral sensitivity of their vision. This great colour variation exists among and within species, providing one of the best expressions of variability in animal colouration. In birds, plumage colour polymorphism involves 61% of the 23 orders

and 37% of the 143 families and can be found in all age classes and in both sexes (Roulin, 2004). The huge variability in colouration exhibited by birds occurs not only in plumage traits but also in hard and soft body parts, such as bills, crests, legs and eggshells. The latter in particular show a great colour variability as well, that still remains a little studied aspect of avian reproductive investment (Kilner et al., 2006; Cassey et al., 2010). Among birds, most species display a similar (white) background eggshell colouration whose variation is determined by the different presence and concentration of the two key pigments responsible for avian eggshell colouration across diverse avian lineages (Kennedy & Vevers, 1976; Cassey et al., 2010).

Melanin-based plumage colouration

Bird plumage colours can be ascribed to two different categories: they can be due to structural features of feathers or they may be due to pigments (Fox, 1976; Brush, 1978). Structural colours are produced by the interaction between highly ordered keratin layers of feather bars and light, generating non-iridescent UV, violet and blue colours (Fox, 1976; Brush, 1978; Doucet et al., 2006). Pigmentary colours are determined by pigments such as porphyrins, responsible for pink, brown, red and green colouration (Toral et al., 2008), carotenoids, responsible for bright red, orange and yellow colouration (Fox & Vevers, 1960; Fox, 1976; Brush, 1978) and melanins, responsible for brown, reddish, black and grey colouration.

Porphyrins are all derived from the same precursors, succinyl Coenzyme A and glycine, and are produced in many widespread orders of birds, as in owls and bustards. However, there are very few studies regarding the biochemical role of porphyrins or their function in bird feathers (McGraw, 2006a).

Carotenoids are synthesized only by photosynthetic organisms, and animals can acquire them only from their food. Although birds cannot synthesize them *ex novo*, they can modify their structures and incorporate them into several tissues, among which feathers (Fox & Vevers ,1960; Fox, 1976, 1979; Brush, 1978). Carotenoids are present in birds with around 30 different molecules, that are also necessary for synthesis of vitamins and are involved in the enhancement of the immune system due to their anti-oxidant and immunostimulating proprieties (McGraw, 2006b). For these reasons, a more expressed carotenoid-based colouration might directly signal higher individual ability to forage and nutritional health and thus a greater quality of the bearer as a potential mate (McGraw, 2006b).

Melanin is the most common endogenous pigment involved in bird plumage colouration (Majerus, 1998). In tissues, melanin is present in two different forms: eumelanin, responsible for grey to black colouration, and pheomelanin, which determines yellow to reddish one (Prota, 1992; Hearing, 1998; Simon et al., 2009). Melanins are synthetized into dedicated organelles called melanosomes, located in melanocytes (Schraermeyer, 1996). Both melanin forms are produced by the same biosynthetic pathway (Figure 1), and the final perceived colouration is determined by the relative and absolute concentration of the two forms in pigmented tissues (Haase et al., 1992; Graw, 2006c; Ito et al., 2011).

Understanding the melanogenesis pathway is useful to acquire knowledge on the evolution, maintenance and adaptive function of melanin-based colour traits (Roulin & Ducrest, 2011).

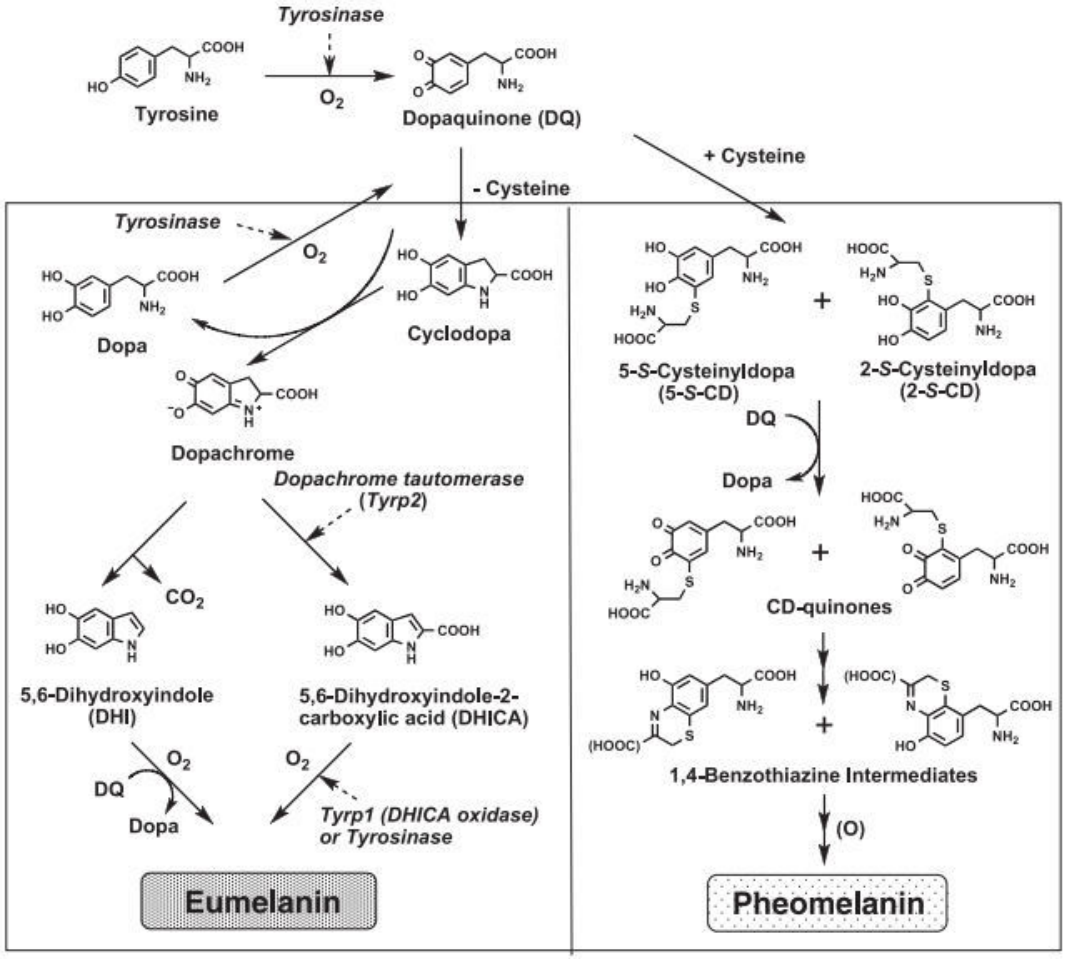

Figure 1. Melanogenesis pathway (Wakamatsu & Ito, 2002).

The relative amount of eu- and pheomelanin pigments is mainly regulated by the *melanocortin-1 receptor* (*MC1R*), belonging to a family of transmembrane G protein coupled receptors (*MC1-5Rs*). When in feather follicles *MC1R* binds its agonist, the melanocortins (i.e. melanin-stimulating hormones α- β- and γ-MSH and the adenocorticotropin hormone,

ACTH), the production of intracellular cAMP (cyclic adenosine monophosphate) is activated. This leads to the transcription of Mitf factor (microphthalmia-associated transcription factor) and increases the activities of the eumelanin-related enzymes *TYR* (*tyrosinase*) and *TYRP1* (*tyrosinase-related protein 1*), favouring the eumelanin synthesis (Walker & Gunn, 2010). On the other hand, when *MC1R* binds its antagonist, the *Aguti Signalling Protein* (*ASIP*), the pheomelanin synthesis is favoured (Walker & Gunn, 2010). Melanocortins are post-translation products of the *proopiomelanocortin* (*POMC*) gene, which just like *MC1R* is a highly conserved gene among vertebrates (Ducrest et al., 2008). Moreover, melanocortins can bind not only *MC1R*, but also four other melanocortin receptors (*MC2-5Rs*) regulating several physiological and behavioural functions, thus resulting in a covariation between melanin-based colouration and other phenotypic traits due to pleiotropic effects of the melanocortin system (Ducrest et al., 2008; Figure 2).

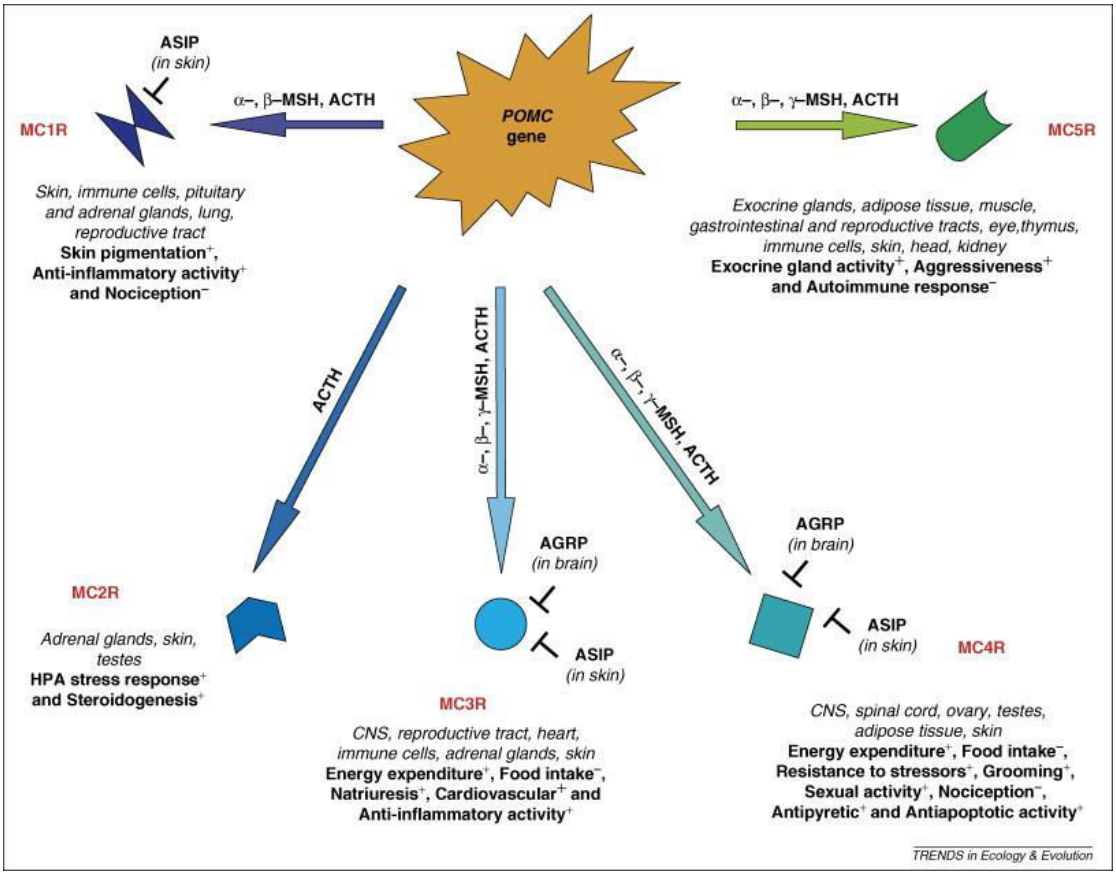

Figure 2. Melanocortin system. The products of POMC gene bind the five different melanocortin receptors (MC1-5Rs) expressed in different tissues. For each functions, positive (+) or negative (-) effects on a specific physiological function are reported (Ducrest et al., 2008).

Assuming that activities of melanocortins and their antagonists are correlated among tissues where melanins are produced (i.e. epidermal tissue) and where physiological and

behavioural responses are regulated (i.e. brain tissue), the melanocortin system may explain the observed covariations between melanin-based plumage colouration and other physiological and behavioural traits. Indeed, it has been shown in many vertebrates that dark and pale conspecifics display differences in behaviour and physiology (Ducrest et al., 2008). In particular, in the majority of cases darker individuals are (1) sexually more active, (2) more aggressive, (3) more resistant to stressful factors (higher activation of hypothalamic-pituitary-adrenal (HPA) stress response and (4) to oxidative stress, or (5) show an enhanced exocrine gland activity, (6) a better regulation of the energy balance between food intake and energy expenditure and (7) a better antibody response (for details see Ducrest et al., 2008 and Roulin & Ducrest, 2011). All these associations between melanin-based colouration and behavioural and physiological traits raise the idea that variation in melanin-based colouration may advertise about the genetic and/or phenotypic quality of the bearer. The covariation between melanin-based colouration and these traits has been particularly studied in birds thanks to polymorphism in plumage colouration. Knowledge of the pleiotropic effects of the melanocortin system may be useful to understand the role of colouration in social interactions (Roulin & Ducrest, 2011).

Protoporphyrin-based eggshell pigmentation

Another fascinating example of bird colouration is offered by the multiple colour shades that eggshells can show. Eggshell colouration seems to be a key component of the avian reproductive system for two main reasons: firstly, despite the huge variation in ecology and life-history traits of avian species, birds are very conservative in their reproductive mode, characterized by a period of external egg incubation. Secondly, birds are unique among amniotes to have evolved a pigmented eggshell showing huge variation among species (Cassey et al., 2010).

Eggshell pigmentation is determined by two different pigments: biliverdin, an antioxidant pigment producing blue-green colouration, and protoporphyrin IX, a pro-oxidant pigment resulting in brownish colouration of eggshell maculation and spots (Kennedy & Vevers, 1976). Both pigments are tetrapyrrole molecules that are involved in the synthesis and catabolism of the haem group (Milgrom, 1997). Several non-mutually exclusive hypotheses have been proposed in order to explain the evolution of the large variability observed in eggshell pigmentation (Kilner et al., 2006; Cassey et al., 2010, 2012). At the inter-specific level, variation in eggshell pigmentation may accomplish a variety of adaptive functions, such

as camouflage and crypsis (Sanchez et al., 2004; Stoddard et al., 2011; Lovell et al., 2013), resistance to bacterial penetration (Fargallo et al., 2014), structural compensation for eggshell thinning caused by calcium deficit (Solomon, 1997; Gosler et al., 2005), egg recognition (Davies & Brooke, 1989; Soler et al., 2000), and/or increasing detectability to parents in cavity-nester species (von Haartman, 1957; Solomon, 1997; Cherry & Gosler, 2010). Besides, at the intraspecific level, eggshell colouration shows remarkable among-female variation. This has lead evolutionary biologists to investigate whether such intra-pecific variation is related to intraspecific communication. Since pigment production and release into eggshells during egg formation may represent a cost for breeding females, Moreno and Osorno (2003) proposed the so-called 'Sexually Selected Eggshell Colouration Hypothesis' (SSECH), arguing that in species where bi-parental care occurs eggshell pigmentation might reliably signal female quality and/or the quality of eggs and of the future progeny. If eggshell pigmentation reliably reflects individual quality, the social partner could be able to judge the quality of his mate and optimally modulate parental investment according to the information conveyed by eggshell pigmentation and the expected fitness returns of the reproductive event (Moreno & Osorno, 2003). The SSECH was originally formulated for biliverdin-based eggshell pigmentation: since biliverdin is an antioxidant, females could showcase their antioxidant capacity by depositing a larger amount of blue-green pigment on their eggs, which is expected in greater share of parental duties by their partners (Moreno & Osorno, 2003). The SSECH has been subsequently hypothesized to apply to species laying protoporphyrin-pigmented eggs, but the results have been conflicting (Martínez-de la Puente et al., 2007; Sanz & García Navas, 2009; Holveck et al., 2012; Krištofík et al., 2013; Giordano et al., 2015; Hargitai et al., 2016a,b; Poláček et al., 2017). This is due to the fact that, contrary to biliverdin, protoporphyrin is a pro-oxidant whose accumulation in the liver induces oxidative stress in females (Afonso et al., 1999). A more intense eggshell pigmentation, caused by a larger amount of protoporphyrin released on the eggshell, may be related to two opposite individual states: 1) higher female quality and higher maternal investment into the eggs due to her higher ability to sustain high levels of circulating pro-oxidants thanks to a higher capacity of the antioxidant system; or 2) lower female quality and lower maternal investment, as high eggshell protoporphyrin may indicate female inability to remove this harmful molecule (Moreno & Osorno, 2003).

Protoporphyrin-based eggshell pigmentation may also reflect egg traits and the potential reproductive value of the clutch (Hargitai et al., 2016b): maternal effects can occur through variation in egg mass, macro-constituents (e.g. albumen content) and quantitatively

minor components that may affect post-natal performance, such as antioxidants (e.g. carotenoids), immune factors (e.g. immunoglobulins) or hormones (e.g. androgens, corticosterone), influencing offspring development and future progeny quality (Groothuis et al., 2005). However, empirical evidence for an association between protoporphyrin-based eggshell pigmentation and egg quality are once again conflicting (Martínez-de la Puente et al., 2007; Sanz & García Navas, 2009; Holveck et al., 2012; Krištofík et al., 2013; Giordano et al., 2015; Hargitai et al., 2016a,b; Poláček et al., 2017).

Mate quality may play a role in the regulation of female deposition of protoporphyrin on eggshell as well. Females may indeed modulate their eggshell pigment deposition according to the condition and/or the ornament expression of their mates (Morales et al., 2012). Life-history theory predicts that individuals of iteroparous species could spend different efforts in parental care according to the reproductive value of current progeny because there may be a trade-off between current and future reproduction (Stearns, 1992). According to the differential allocation hypothesis (Burley, 1988; Harris & Uller, 2009), females could invest more in their offspring when paired with relatively more attractive mates because their offspring will inherit desirable genetic qualities and they will be preferred as mates in the future (Andersson, 1994). However, species that use courtship feeding were not considered when examining the idea of eggshell pigmentation as a post-mating sexual signal. Martínez-Padilla et al. (2010) thus proposed that for species in which males play a key role in providing food to females during pre-laying and laying periods, influencing their body condition (Newton, 1989; Village, 1990), male quality may be reflected by variation in eggshell pigmentation. To the best of my knowledge, only few studies have investigated the relationship between protoporphyrin-based eggshell pigmentation and male quality (Martínez-Padilla et al., 2010; Holveck et al., 2012; Poláček et al., 2017).

Outline of the study

The present thesis follows along a still active line of studies investigating whether bird plumage and eggshell colouration could play a role as intraspecific signal in several communication dynamics. Identifying the causes and functions of intraspecific colour variation is pivotal in understanding the evolution of different traits in modern evolutionary ecology (Hubbard et al., 2010).

The present thesis is divided in two parts. In the first part (**Chapters 2 to 4**), I investigated the possible signalling role of melanin-based plumage colouration from a variety of perspectives. Identifying the association between melanisation and physiological, behavioural and life-history traits in populations under a sexual and natural selection is pivotal to any study focused on the evolution of melanism. For this reason, firstly I investigated whether the pleiotropic effects of the melanocortin system accounted for covariation between melanin-based breast plumage colouration and individual quality in terms of expected future reproductive success (**Chapter 2**) or behavioural stress response (**Chapter 3**) in European barn swallow (*Hirundo r. rustica*) nestlings. Moreover, I investigated (**Chapter 4**) whether sequences variation at well-known genes (*MC1R* and *TYRP1*) implicated in the regulation of melanin-based plumage colouration in bird species is related to inter-individual plumage variation displayed by lesser kestrel males (*Falco naumanni*).

The role of melanin-based ventral plumage colouration as a honest signal of individual quality was investigated in **Chapter 2**. Despite parents are equally related to all of their progeny, they may differentially invest in individual offspring according to their expected reproductive value in order to maximize their fitness. Sons and daughters can differ in reproductive value, mainly in species where fitness is predicted by the expression of sexually selected traits. For instance, in the barn swallow individuals displaying darker ventral plumage colouration obtain larger reproductive success compared to paler ones (Safran & McGraw, 2004; Safran et al., 2005; Vortman et al., 2011, 2013). This variation in reproductive success according to ventral plumage colouration is larger in males than in females (Safran et al., 2005; Vortman et al., 2011; Romano et al., 2016). Considering that barn swallow nestlings may honestly display their quality by means of acoustic or visual signals, we created dyads of same sex siblings with similar body mass, whereby ventral plumage colour was experimentally darkened in one of the two siblings, while the other served as a control. We therefore tested whether parents allocate different amounts of food depending on plumage colour of their male and female offspring. We expected that parents

preferentially allocate more resources to experimentally darkened males because in adult males of this species darker ventral colouration is associated with greater reproductive success.

Based on previous observations on the covariation between personality and colouration in other species (review in Roulin et al., 2004), in **Chapter 3** I investigated the relationship between melanin-based breast plumage colouration and behavioural stress response in barn swallow nestlings, to assess whether melanisation could also be also related to individual personality of a sexually dimorphic species in a sexual communication context. Previous studies of barn swallows reported contrasting results concerning the association between plumage colouration and stress response (Jenkins et al., 2013; Saino et al., 2013a). This is due to the fact that, compared to the other melanocortin pleiotropic effects, differential regulation of HPA stress response according to mixed melanin plumage is more complex. In the range of colouration of barn swallow ventral feathers, darker colouration was associated with a higher concentration of eu- and pheomelanins and a higher relative concentration of pheo- on eumelanin (Saino et al., 2013b). I used three behavioural tests as proxies of behavioural stress response: tonic immobility (Jones and Faure, 1981; Jones, 1986), breath rate (Carere & Van Oers, 2004; Fucickova et al., 2009) and agitation (Brommer & Kluen, 2012; van den Brink et al., 2012; Kluen et al., 2014). These three proxies should be inter-correlated, representing strategies that the nestlings may use to cope with demands from their environment. As a consequence of the pleiotropic effects of the melanocortin system, I expected darker individuals to show a stronger behavioural stress response as a consequence of higher activation of HPA axis. They were therefore expected to show a longer tonic immobility duration, higher breath rate and lower agitation.

In **Chapter 4** I tackled the issue of the whether genetic variation at *MC1R* and *TYRP1* explained intraspecific variation in complex melanin-based plumage colouration in the lesser kestrel. Variations in melanin-based colouration at intra- and interspecific levels are not necessarily associated with mutations in *MC1R* gene, indicating the possible presence of a mixture of modifications in the structure and/or in the regulation of other genes involved in the melanogenesis pathway (Bourgeois et al., 2016). This is especially true for species with a complex pattern of eu- and pheomelanin deposition, such as the lesser kestrel. Lesser kestrel adult males present marked inter-individual variations in melanin-based traits, including reddish ventral plumage colouration and the presence and the extension of black spots on breast, belly and underwing coverts. Specifically, I investigated whether single nucleotide polymorphisms (SNPs) of *MC1R* and *TYRP1* were related to inter-individual variation in

breast and underwing spottiness and belly reddish plumage colouration of lesser kestrel males.

In the second part (**Chapters 5** and **6**), I examined the possible signalling role of protoporphyrin-based eggshell pigmentation in both the barn swallow and the lesser kestrel (see **General Introduction**), two distantly related bird species, with the aim of disentangling the results provided by previous studies.

Specifically, in **Chapter 5** I investigated the patterns of intraspecific variation of protoporphyrin-based eggshell pigmentation in barn swallow, analysing its association with egg and clutch characteristics, maternal (health status, intrinsic quality, reproductive success and survival) and paternal traits (sexual ornaments) and with parental feeding effort. Eggshells were classified using a qualitative visual criterion (Gosler et al., 2000), categorizing eggshell pigmentation according to four different components: pigment distribution (pigment distribution along the surface of the egg), pigment intensity (colour of pigmented area of the eggshell), pigment coverage (amount of eggshell pigmented surface) and spot size. Predictions about the relationships between protoporphyrin eggshell pigmentation and female/egg traits/quality were ancipital. We did not have *a priori* expectation because of the ambiguous results indicated by previous study.

Differently from barn swallow, in lesser kestrel female food provisioning during pre-laying and laying periods depends mainly on male efforts (Newton, 1979; Village, 1990; Donázar et al., 1992). Hence, females can use quality and quantity of food provisioning in mate choice decisions (Mougeot et al., 2002). Consequently, if male quality affects female body condition, both parent condition and/or 'intrinsic' quality can be reflected on eggshell pigmentation. As previously said, species that use courtship feeding were not considered when examining the idea of eggshell pigmentation as a post-mating sexual signal. For this reason, in **Chapter 6**, by means of a food supplementation experiment where we increased the food abundance for a group of individuals in order to manipulate perceived male quality and parental condition, we investigated the possible associations of protoporphyrin-based eggshell pigmentation with male and female condition and potential quality and with clutch and egg traits.

Based on previous evidence from the closely-related Eurasian kestrel (*Falco tinnunculus*, Martinez Padilla et al., 2010), we expected a positive association between protoporphyrin-based eggshell pigmentation and parental quality. Since pigment production may represent a cost for breeding females (Moreno & Osorno, 2003), we expected that eggs with greater protoporphyrin-based eggshell pigmentation were laid by food-supplemented

females, both because of the improvement on their body condition (reducing the costs of protoporphyrin eggshell deposition consequently) and also because of the higher foraging efforts by their mates that food supplementation should simulate, potentially inducing a greater egg investment by the female. Moreover, we expected a positive association between protoporphyrin-based eggshell pigmentation and parental traits denoting high 'intrinsic' quality. If protoporphyrin-based eggshell pigmentation operated as a signal of parental quality, we also expected a positive association between intensity of protoporphyrin-based eggshell pigmentation and egg/clutch traits associated with high fitness.

To conclude, in **Chapter 7** I present an overview of the main findings emerging from the studies discussed in detail in the previous chapters.

Part I

Melanin-based plumage colouration

Chapter 2

Nestling sex and plumage color predict food allocation by barn swallow parents

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Behavioral Ecology 27 (2016): 1198-1205.

Behavioral Ecology

The official journal of the
ISBE
International Society for Behavioral Ecology

Behavioral Ecology (2016), 27(4), 1198–1205. doi:10.1093/beheco/arw040

Original Article

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Received 9 November 2015; revised 5 January 2016; accepted 25 February 2016; Advance Access publication 18 March 2016.

Despite parents are equally related to all of their progeny, they may differentially invest in offspring that provide the highest fitness return. Sons and daughters can differ in reproductive value, especially in species where fitness is predicted by the expression of sexually selected traits. In many birds, offspring plumage coloration functions as a honest signal of individual quality, thus allowing parents to differentially invest in offspring of either sex accordingly. Here, we tested whether parents allocate different amounts of food depending on plumage color of their male and female offspring. As a model, we used the barn swallow (*Hirundo rustica*), a species where large among- and within-brood variation in ventral plumage color exists and male reproductive success varies according to ventral plumage coloration. We recorded the proportion of feedings obtained and body mass variation by dyads of same-sex and similar-sized nestlings subjected to either experimental darkening of their ventral plumage color or to a sham treatment. Plumage darkening enhanced food provisioning and body mass gain of males but not of females. Because darker ventral coloration is associated with larger reproductive success in male barn swallows, these results suggest that parents tune their effort toward more valuable male offspring that are likely to provide the greatest fitness returns. Our study thus suggests that parents are selected to differentially invest in offspring of either sex according to a trait expressed in early life, which is relevant to intrasexual competition for access to mates at sexual maturity.

Key words: **food allocation, parental investment, parent–offspring conflict, plumage color, sexual selection, sibling competition.**

INTRODUCTION

In multiparous species with altricial offspring, parents have to decide how to partition limiting resources among the progeny in order to maximize their fitness. Because biological parents are equally related to all of their offspring, an even parental investment in individual members of the progeny should theoretically be expected (Hamilton 1964; Trivers 1974; Royle et al. 2012). However, whenever individual offspring differ in quality and viability, parents will benefit from providing greater care to the more valuable descendants (Haig 1990; Mock and Forbes 1995; Stenning 1996; Mock and Parker 1997, 1998; Lessells 2002; Glassey and Forbes 2002; Royle et al. 2012).

One crucial source of variation in offspring reproductive value is sex: Owing to fundamental genetic differences, male and female offspring may differ in several characters, such as developmental trajectories (Seller and Perkins-Cole 1987; Cook and Monaghan 2004), susceptibility to parasites or rearing conditions (Nager et al. 2000; Tschirren et al. 2003; Bize et al. 2005; Siefferman et al. 2008; Romano et al. 2011), absorption of food or energy expenditure (Martins 2004; Vedder et al. 2005), begging for food or competitive ability (Fargallo et al. 2003; Boncoraglio et al. 2008), and hence fitness return per unit parental investment. Parents are therefore expected to tune their parental investment accordingly (Trivers and Willard 1973; Charnov 1982; Lessells 2002; Uller 2006). In addition, sex-related variation in reproductive value is expected to be larger in species where male breeding success depends on the expression of sexually dimorphic traits under female preference (Komdeur 2012). This is the case because of the unequal breeding opportunities for males and females of different phenotypic qualities, leading to a larger variance in reproductive success of males (Trivers and Willard 1973). Indeed, high-quality males are expected to produce more offspring than females of the same quality, whereas the opposite holds true for poor-quality individuals.

Because decisions by parents about resource allocation in a brood according to offspring reproductive value and sex can have profound consequences on their lifetime fitness, natural selection should have favored the evolution of offspring phenotypic traits that convey reliable information about their quality (Kilner and Johnstone 1997; Wright and Leonard 2002; Royle et al. 2012), allowing parents to adaptively modulate their investment (e.g., Kilner 1997; Saino et al. 2000; Fargallo et al. 2003; de Ayala et al. 2007; Dugas 2009; Avilés et al. 2011). In birds, nestlings

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may honestly display their quality, in terms of current need of care or condition, by means of multiple visual and acoustic signals (Johnstone 1995, 1996; Kilner and Johnstone 1997; Wright and Leonard 2002), and parents may also exploit various other types of reliable cues (e.g., body mass) potentially revealing the reproductive value of their progeny (see Kilner and Johnstone 1997; Wright and Leonard 2002; Royle et al. 2012). Although it is rare for immature birds to have conspicuous feather coloration (but see Krebs and Putland 2004), increasing evidence exists that plumage chromatic traits also serve as signals of offspring quality to parents (Galván et al. 2008; Tanner and Richner 2008). Indeed, many studies have demonstrated that nestling plumage coloration can be a condition-dependent trait, which may, for example, mirror parasites infection, nutritional state, or rearing conditions (Hõrak et al. 2000; Tschirren et al. 2003; Fitze et al. 2003; Jacot and Kempenaers 2007; Siefferman and Hill 2007; Siefferman et al. 2008; Parejo and Silva 2009). In addition, it has been shown that the expression of ornamental plumage traits in juveniles may also influence the level of parental care (Lyon et al. 1994; Griggio et al. 2009; Ligon and Hill 2010; Barrios-Miller and Siefferman 2013; but see Tschirren et al. 2005).

Because plumage coloration honestly signals individual quality to competitors and potential mates in sociosexual contexts (see Roulin 2004; Hill and McGraw 2006; McGraw 2008 for reviews), in species where nestlings plumage color shows large interindividual variability, parents may differentially invest in male and female offspring according to their feather coloration. In particular, when variation in heritable plumage color traits is related to reproductive success in one sex, parents should favor the offspring of the sex that will benefit the most by displaying such traits (i.e., generally males). To date this hypothesis has received little attention: To our knowledge, only 2 studies on the same species, the eastern bluebird (*Sialia sialis*), provided evidence of sex-related parental care according to offspring plumage color traits that are associated with fitness traits in adults (Ligon and Hill 2010; Barrios-Miller and Siefferman 2013).

Here, we first provide a description of natural among-brood and between-sex variation of barn swallow (*Hirundo rustica*) nestling white to brownish coloration of ventral body feathers. Like in several other Palearctic birds, barn swallow juveniles fledge with a plumage that is a paler representation of that of the adults (Moreno and Soler 2011; Hubbard et al. 2015), suggesting that adult-like plumage in immature individuals could be mainly due to selection pressures on adults (Hawkins et al. 2012). We thus investigated whether parents provide a different amount of food to their offspring according to experimental alteration of nestling plumage. We tested this hypothesis by recording parental food allocation (e.g., proportion of feedings and body mass variation) to dyads of same-sex nestlings subjected to a different manipulation of their ventral contour feathers coloration: One nestling was experimentally darkened within the range of natural variation, while a sham-colored sibling served as control.

In adult barn swallows, ventral plumage shows moderate sexual dichromatism, males having darker pheomelanin- and eumelanin-based ventral coloration than females, but also large within-sex variability (Saino, Romano, Rubolini, Ambrosini, et al. 2013; Saino, Romano, Rubolini, Teplitsky, et al. 2013). The contour feather coloration is heritable (Saino, Romano, Rubolini, Ambrosini, et al. 2013; Hubbard et al. 2015; Vortman et al. 2015), and in different populations, paler individuals of either sex pay sexual selection costs compared with darker ones (Safran and McGraw 2004; Safran et al. 2005; Vortman et al. 2011, 2013), with variation in reproductive success according to plumage color being larger in males than in females (Safran et al. 2005; Vortman et al. 2011). Moreover, within individuals, coloration at the nestling stage predicts coloration in adulthood (Hubbard et al. 2015). Because of the potential reproductive advantage for males showing brownish ventral feathers, parents were expected to favor their male offspring displaying experimentally darkened plumage by providing them with more food compared with the control sibling. This parental favoritism was not expected among females.

MATERIALS AND METHODS

Study species

The barn swallow is a small (ca. 20 g) semicolonial migratory passerine bird, breeding synanthropically in rural buildings. Females lay 1–3 clutches of 1–7 eggs (modal size: 5 eggs) per breeding season and incubate them for circa 14 days (Møller 1994). Nestlings are fed by both parents and display sex-related begging features, thus allowing parents to recognize offspring of either sex (Saino et al. 2003; Saino, de Ayala, et al. 2008). Nestlings fledge when they are circa 20 days old, and parents usually provide food to a single nestling per visit to the nest (Romano et al. 2011, 2012).

General field procedures and sex determination

The present study was carried out between April and July 2014 in 4 barn swallow colonies (= farms) located in the countryside southeast of Milan (Northern Italy). All nests were visited every second day to record breeding events. Considering the day of hatching of the first egg(s) in a nest as the day 0, at day 7 all nestlings in broods with 3 or more nestlings were banded with an aluminum ring. Nestlings were sexed molecularly (Griffiths et al. 1998; Saino, Martinelli, et al. 2008) using a small blood sample (ca. 40 µL) collected by puncturing their brachial vein. We thus identified the sex of all nestlings before the day of the manipulation of plumage color and the assessment of its potential effect on parental food allocation.

Plumage color manipulation

At 15 days of age, we manipulated the ventral plumage color of half of the male and half of the female nestlings (hereafter, "darkened" nestlings) within each brood by using a nontoxic marker (Letraset Promarker, satin, item code Y129). A similar procedure was used to manipulate color of adult barn swallows in previous studies (Safran et al. 2005, 2008; Vitousek et al. 2013). At this age, the ventral part of the body is almost entirely covered by contour feathers and nestlings are already soliciting parental care by protruding part of their body from the edge of the nest cup (i.e., showing to parents not only the gape as younger nestlings do but also the breast plumage; Romano A, personal observation) to achieve an advantage in scrambling for food. A small number (3–5) of feathers were plucked both before and after the experimental treatment for spectrometric color analyses (see below). To control for the effect of marker application, a sham-control treatment was performed on the remaining nestlings of the same broods (hereafter "sham-colored" nestlings) by painting their ventral plumage with a transparent marker (Letraset Promarker, clear blender, item code BL; see also Safran et al. 2005).

Nestlings were randomly assigned to the darkened or control treatment. Whenever the number of nestlings of either sex was

odd, the odd nestling was assigned randomly to either treatment. Overall, we darkened the ventral coloration of 58 nestlings (29 males and 29 females) from 28 broods, whereas 63 siblings served as controls (32 males and 31 females). However, not all nestlings were included in feeding trials (see below).

Feeding trials

To test for a difference in the food allocation between darkened and sham-colored nestlings, we compared both body mass variation and the proportion of feedings received during feeding trials, including pairs of same-sex and opposite-treatment siblings (i.e., darkened male vs. sham-colored male; darkened female vs. sham-colored female; hereafter "dyads"). We decided to focus on dyads of nestlings, rather than on the entire brood, in order to adhere to theoretical models of parental food allocation, which were developed for pairs of offspring (e.g., Godfray 1995; Johnstone 2004), and to simplify the behavioral observations. Because in the barn swallow the sex of nestlings and that of their siblings are known to affect parental food allocation (Bonisoli-Alquati et al. 2011), we tested only same-sex dyads in order to experimentally account for this confounding effect. In addition, because in the barn swallow the allocation of food varies in relation to nestling size (Bonisoli-Alquati et al. 2011), dyads were composed by selecting the 2 nestlings showing the least difference in body mass within the brood.

Feeding trials were performed 10–15 min after the color manipulation in order to allow the plumage to dry after the application of colored and sham marker, and started at 8.00–9.00 AM. Feeding trials were carried out as following: First, we measured body mass of the 2 focal nestlings with an electronic scale (accuracy 0.1 g) and their tarsus with a digital caliper (accuracy of 0.01), randomly marked them on their forehead with 2 white spots (aligned longitudinally or transversally) to make them individually recognizable, and left them in their nest for 90 min while removing all their siblings. Concomitantly, the nestlings not included in the feeding trial were kept in a safe place at ambient temperature. All parental feeding visits were videotaped with a Sony DCR-SR72E camera placed in front of the nest so as to identify which nestling received each food item provided by parents. At the end of the feeding trial, the focal nestlings were weighed again to record body mass variation, indicating individual food intake and also accounting for the balance between cost and benefit of scrambling and for the defecation rate (see also Boncoraglio et al. 2008; Romano et al. 2011, 2012).

When 2 other nestlings of the same-sex and opposite treatment existed in the same brood, the above-described procedures were repeated to obtain information for an additional dyad. However, no nestling was used in more than 1 feeding trial. Although nestlings in different dyads from the same brood had different levels of hunger (i.e., the second dyad was always tested after a period of food deprivation during the feeding trial of the first dyad), we emphasize that nestlings within each dyad were always tested under the same hunger level. Indeed, despite the fact that the feeding rate was greater in second tested dyads compared with first tested ones (Poisson mixed model including dyad order as fixed effect and nest identity as random effect: $F_{1,14} = 20.77$; $P = 0.0004$), dyad order did not affect the quality of results (see Supplementary Materials).

The number of feedings provided by parents to each nestling was counted on video recordings by using VLC Media Player 2.2.1 (Free Software Foundation, Inc., Boston, MA). The proportion of feedings received was computed as the ratio between the number of feedings obtained by each nestling and the total number of feedings delivered by parents to the nest. The proportion of feedings obtained by each nestling was used to assess whether food allocation differed between experimental and control nestlings. However, because feeding rates do not account for variation in quality and size of individual food item, body mass variation during trial was also used to account for the whole food allocation in different nestlings by parents.

In the barn swallow, parental food allocation to individual nestlings is positively related to the intensity of their begging behavior (Saino et al. 2000; Boncoraglio et al. 2008; Bonisoli-Alquati et al. 2011; Romano et al. 2012). Thus, we also compared the intensity of postural begging between darkened and sham-colored nestlings. To this aim, the intensity of postural begging (hereafter "begging intensity") during each feeding visit by parents was scored on a 4-level scale ranging from 0 (nestling did neither open the mouth nor move toward the attending parent) to 3 (nestling begged intensely by shaking head and fully stretching neck toward the attending parent) (see Romano et al. 2012 for details).

The whole protocol was accomplished for 36 dyads (18 male and 18 female dyads). For practical reasons (e.g., impossibility of placing the camera in front of the nest), for other 10 dyads (6 male and 4 female dyads), only the data on body mass variation during the feeding trial, not food provisioning data, were available. Before the experimental manipulation of plumage color, no significant difference in body mass and tarsus length was observed between darkened and sham-colored nestlings, both considering all dyads (paired $t_{45} = -0.30$; $P = 0.77$) and when analyses were separated for males (paired $t_{23} = -1.30$; $P = 0.21$) and females (paired $t_{21} = 0.73$; $P = 0.48$).

At the end of the experiment, all nestlings were returned to their nest. We did not observe any nest desertion by parents nor mortality of nestlings during the study.

Control broods

To test for the possibility that a larger investment in the darkened nestlings was rather caused by a disadvantage paid by their sham-colored siblings because of the sham treatment on plumage (i.e., sham-colored nestlings may have been made less valuable for parents by the experimental manipulation), feeding trials were also established on a sample of 11 control broods, including sham-colored and un-manipulated nestlings. Un-manipulated nestlings were handled for the same amount of time as their sham-colored siblings, while on their plumage, we did not apply any colored or sham marker. For practical reasons, only data concerning the body mass variation during the feeding trial were available for these broods. On the whole, feeding trials were carried out for 10 male and 8 female dyads in control nests. No difference in body mass and tarsus length was observed between sham-colored and un-manipulated nestlings before the feeding trials (in both sexes: paired $t < 1.19$; $P > 0.26$).

Color measures

Reflectance spectra of a single ventral feather per nestling were measured in a dark chamber, using an Avantes DH-2000 spectrometer. Every measure was taken twice. Importantly, as previously demonstrated (see Romano et al. 2015), color measurements taken on a single feather, on 3 feathers or directly on ventral plumage of the individuals from which the feathers were plucked provide very consistent estimates (correlation coefficients between the color variable values obtained by the 3 methods were $r > 0.88$ for θ and $r > 0.91$ for both φ and *rA*). Color quantification was performed

relatively to white and black standards by using the tetrachromatic color space model with the TetraColorSpace program version 1a (Stoddard and Prum 2008). This program also includes information about bird cone sensitivity and therefore provides biologically realistic color metrics (Antonov et al. 2011; Saino, Romano, Rubolini, Ambrosini, et al. 2013). Here, we assumed a ultraviolet-sensitive cone-type retina and used spectral sensitivity of the blue tit (*Cyanistes caeruleus*), the most closely phylogenetically related species to the barn swallow for which information on cone spectral sensitivity is available in TetraColorSpace. For each measurement, the program provided 3 color components: θ, φ, and *rA* (see Stoddard and Prum 2008; Antonov et al. 2011). φ and θ, respectively, reflect the ultraviolet and the red–green–blue components of hue, whereas *rA* is a proxy of color saturation. For barn swallow ventral feathers, decreasing θ indicates darker, brownish coloration and a higher concentration of both eumelanin and pheomelanin (see Saino, Romano, Rubolini, Ambrosini, et al. 2013). Further details on color measures are provided in Supplementary Materials.

The experimental manipulation enhanced ventral plumage coloration within 1 or 2 standard deviation (SD) of the mean value of un-manipulated nestlings depending on the color variable considered (see Supplementary Materials). The sham treatment slightly altered *rA* of nestling plumage coloration. However, we stress that the alteration of the plumage color of the sham-colored nestlings was in the same direction with respect to that observed in darkened nestlings, indicating that our results were conservative (see also Results for analysis of "control broods"). Importantly, after the experimental manipulation (i.e., during the feeding trial), darkened nestlings were significantly darker than their sham-colored siblings (see Supplementary Materials).

Statistical analyses

Natural among-brood variation in ventral plumage coloration was investigated by estimating the contribution of brood identity to the variance of the 3 color variables (θ, φ, and *rA*). This was done by means of variance components analyses, including nest identity as a random factor, while accounting also for the effect of nestling sex included as a fixed factor. The same analyses were also performed on male and female nestlings separately. Broods containing a single nestling of either sex were removed from both analyses. In addition, sex-related variation of color variables was analyzed in linear mixed models (LMMs) including the sex of individual nestling as a fixed effect and brood identity as a random effect. These analyses were performed using ventral feathers plucked before the application of the color marker or the sham marker.

All the analyses on parental food allocation were performed for males and females separately because dyads were composed by same-sex nestlings, thus preventing us to test the effect of the statistical interaction between treatment and sex on the whole dataset (see also Romano et al. 2011).

We investigated whether the proportion of feedings received by the darkened nestling in each dyad deviated from 0.5 (i.e., the value expected in case of 2 same-sex and similar-size nestlings in the absence of any parental favoritism) by means of intercept-only binomial mixed model where the dependent variable was the proportion of feedings obtained by the darker nestlings over the total number of feeding events (i.e., events/trials syntax), and brood identity was included as a random intercept effect. This analysis is mathematically equivalent to a mixed model of the absolute number of feeding received by each nestlings in each dyad assuming a Poisson error distribution, including experimental manipulation (sham colored vs. darkened) as a 2-level fixed effect, with brood and dyad identity as random intercept effects (details not shown for brevity).

The difference in body mass between the end and the start of the trial (hereafter "body mass variation") was analyzed by means of LMMs with experimental manipulation (sham colored vs. darkened) as a 2-level fixed effect, with brood and dyad identity as random intercept effects. The analysis of body mass variation in the sample of control dyads was carried out by LMMs with the same random structure as above and with treatment (un-manipulated vs. sham colored) as a fixed effect.

Variation in begging intensity between control and darkened nestlings was analyzed in multinomial mixed models for ordered outcomes by using the cumulative logit link function (Schabenberger 2006; see also Romano et al. 2012) with experimental manipulation as a fixed effect, and brood and dyad identity as random intercept effects.

All analyses were carried out using SAS 9.1.3 (SAS Institute 2006). The analyses of variability of color variables among nests were carried out with the VARCOMP procedure, whereas mixed models analyses using the MIXED and GLIMMIX procedures. In LMMs, degrees of freedom were computed with the Kenward–Roger approximation, and in multinomial generalized linear mixed models, the degrees of freedom were conservatively set equal to the number of dyads included in each model.

RESULTS

Natural variation in nestling plumage color

All color variables significantly varied among broods (analysis of variance tests: θ: $F_{27,91} = 2.10$, $P = 0.005$; φ: $F_{27,91} = 2.33$, $P = 0.002$; *rA*: $F_{27,91} = 3.24$, $P < 0.001$), indicating that ventral plumage color was more similar between nestlings reared in the same nest than between nestlings of different broods. However, the variance explained by the nest of rearing accounted for less than half of the total observed variance (variance components analyses: θ: 0.20; φ: 0.24; *rA*: 0.34), as previously demonstrated in nestlings of a different subspecies of the barn swallow (*Hirundo rustica erythrogaster*; Hubbard et al. 2015). This result was confirmed when the analyses were carried out on males (variance components analyses: θ: 0.26; φ: 0.25; *rA*: 0.52) or females (variance components analyses: θ: 0.33; φ: 0.33; *rA*: 0.29) separately. These findings thus imply that large differences existed in ventral coloration among same-sex siblings. A large within-brood variability in color is a fundamental prerequisite to enable parents to differentially allocate parental care among individual progeny members based on their ventral plumage coloration. The within-brood variation was not explained by nestling sex, as no statistically significant difference in any color variable was observed between males and females (mixed models with brood identity as a random factor; effect of sex: θ: $F_{1,90} = 1.11$, $P = 0.29$; φ: $F_{1,90} = 0.28$, $P = 0.60$; *rA*: $F_{1,90} = 0.07$, $P = 0.79$; see Supplementary Figure S1).

Parental food allocation according to experimental manipulation of nestling ventral coloration

The mean number of feedings delivered by parents during feeding trials was 20.69 ± 12.35 SD, ranging from 4 to 64 feeding events per trial. Analyses on male dyads showed that darkened nestlings obtained a larger proportion (and number) of feedings than

sham-colored siblings, whereas this was not the case among female dyads (Table 1; Figure 1). Indeed, in 14 out of 18 male dyads, darkened males obtained more food items than the sham-colored siblings, indicating a nonrandom food allocation by parents (binomial test: $P = 0.031$). On the other hand, darkened females obtained more food than sham-colored siblings in 9 out of 18 cases (binomial test: $P = 1.00$), as expected in case of random food allocation. As a consequence, color darkening had also a positive effect on male, but not female, body mass gain (Table 1).

Begging intensity did not differ between darkened and sham-colored nestlings of both sexes (males: $F_{1,18} = 1.13$; $P = 0.30$; females: $F_{1,18} = 0.11$; $P = 0.74$). Therefore, begging intensity did not confound our results. Finally, no significant difference in body mass variation during the feeding trials was observed between sham-colored and un-manipulated nestlings in the control broods in dyads of either sex (males: $F_{1,9} = 1.22$; $P = 0.23$; females: $F_{1,7} = 0.18$; $P = 0.69$), indicating that the sham treatment did not affect food allocation to the nestlings.

DISCUSSION

We showed that barn swallow parents modulated their investment in male, but not female, offspring according to plumage coloration. In fact, experimentally darkened males obtained more food and gained more mass compared with their sham-colored siblings. As expected, this pattern of differential food allocation between offspring displaying different plumage colors was not observed in females. The mean standardized effect size $\mathcal{Z}$ associated with the relationships between experimental manipulation and measures of parental investment (i.e., considering the proportion of feedings obtained and the body mass variation) was 0.50 and 0.21 in male and female dyads, respectively, indicating that the effect of treatment in males was more than twice as large as in females.

Between- and within-sex variation in plumage melanin-based coloration is common in birds (Hill and McGraw 2006). Melanogenesis, and thus the expression of melanin-based pigmentation, is also known to covary with several physiological and behavioral traits, including immune or hormonal functions, response to stressors, as well as social aggressiveness and sexual behavior (Roulin 2004; Ducrest et al. 2008; Roulin et al. 2008; Galván and Alonso-Alvarez 2009). Variation in melanin-based colorations can therefore reliably indicate individual genetic/phenotypic quality, which can be used in intrasexual and intersexual interactions, as showed by a large variability in breeding performance according to melanization, with darker individuals being generally more sexually active and obtaining larger benefits in terms of reproduction (Roulin 2004; Meunier et al. 2011). This is also the case for the species we studied here, in which darker individuals, especially males, accrue breeding advantages over paler ones (Safran and McGraw 2004; Safran et al. 2005; Vortman et al. 2011, 2013). Therefore, by allocating more resources to darker sons, which will be recruited as darker adult breeders in the next breeding season (Hubbard et al. 2015), parents were likely favoring the more valuable offspring, which should provide them the largest fitness return. This is consistent with our expectation because there is greater variance in reproductive success associated with brownish coloration in males than in females. While brownish females only produce slightly larger clutches than paler ones (Safran and McGraw 2004), darker males have shorter prelaying period, enjoy larger fidelity by social mate, prevail in sperm competition, and extrapair copulations, thus resulting in a larger production of biological offspring in each breeding season (Safran and McGraw 2004; Safran et al. 2005; Vortman et al. 2011, 2013). Larger parental investment in darkened males may thus function to enhance parental fitness.

Our results are also compatible with the non-mutually exclusive interpretation that parents invested more in darkened nestlings because their darkness reliably signals better body condition or quality compared with siblings, as shown in other species (Tschirren et al. 2003; Fitze et al. 2003; Parejo and Silva 2009). However, a brood size manipulation experiment carried out on 12 pairs of broods that were either increased or decreased in size showed that an experimental increase in brood size (see Bonisoli-Alquati et al. 2008 for details of the experimental protocol) negatively affected body mass (LMM with brood of origin and of rearing, and dyad as a random effects; effect of treatment: $F_{1,64} = 4.68$; $P = 0.034$), but did not alter nestling ventral coloration (LMMs as above; effect of treatment on θ, φ, and rA: all $P \geq 0.24$; Costanzo A et al., unpublished data). The lack of effect of brood size manipulation on plumage coloration does not support the hypothesis that coloration is a condition-dependent trait. However, other sources of nongenetic variation, such as maternal and paternal effects, external environment (e.g., rearing temperature and air pollution), and social environment (e.g., sex ratio of siblings), may contribute to variation in plumage coloration of nestlings. In fact, in a recent study on the same species, Hubbard et al. (2015) showed that only half of the variance in nestling plumage color is explained by the combination of genes and brood of rearing, suggesting that other sources of environmental influences affect plumage coloration.

In addition, plumage color may mirror other aspects of the nestling quality. In the same population where this study was performed, we showed that darker nestlings of both sexes harbor longer telomeres (Costanzo A et al., unpublished data). Individuals with longer telomeres and/or smaller rates of their shortening are generally more viable (Barrett et al. 2013; Boonekamp et al. 2014) and have better reproductive and physiological performance (Le Vaillant et al. 2015). It is therefore possible that barn swallow

Table 1

Mixed models of the effects of experimental manipulation (darkened or sham colored) on proportion of feedings received by darkened nestlings and on body mass variation by dyads of same-sex nestlings

	Males				Females			
	Coefficient (SE)	$F/\mathcal{Z}$	df	P	Coefficient (SE)	$F/\mathcal{Z}$	df	P
Proportion of feedings	0.243 (0.106)	2.30[a]	—	0.021	0.142 (0.103)	1.38[a]	—	0.17
Body mass variation	0.175 (0.082)	4.54	1,23	0.044	0.045 (0.084)	0.29	1,21	0.59

Analyses were performed for nestlings of either sex separately. $\mathcal{Z}$ values were computed by dividing the intercept estimate by the associated standard error in intercept-only logistic regression models. See Methods for details and sample sizes. df, degrees of freedom; SE, standard error.
[a] $\mathcal{Z}$ value.

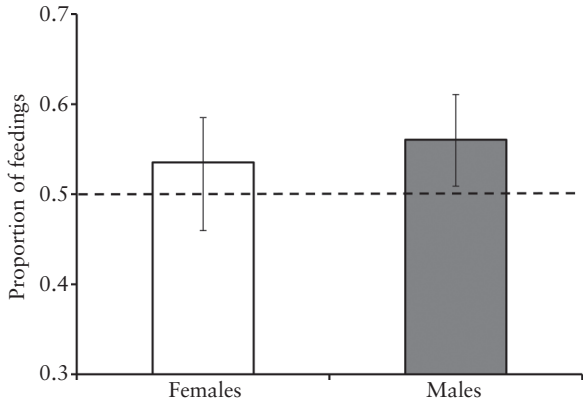

Figure 1
Proportion of feedings received during feeding trials by darkened nestlings in 18 male and 18 female dyads. Values are estimates from mixed models shown in Table 1, together with the 95% confidence intervals reported on the original scale. The broken line indicates the expected proportion of feedings received in case of 2 same-sex and similar-size nestlings in the absence of any parental favoritism. The *y* axis is bound between 0.3 and 0.7 for ease of representation.

parents invested more resources in darker nestlings because they were honestly advertising their quality in terms of telomere length. Although darkened females also obtained more (though statistically nonsignificantly so; Figure 1) food than their control female siblings, the effect of darkening on parental investment was markedly larger among male offspring, possibly because they benefit more than females from carrying this sexually selected trait at sexual maturity. We emphasize however that, irrespective of the mechanism that generated preferential food allocation toward darker sons, our results are consistent with the idea that barn swallow parents adaptively modulate their parental investment based on offspring coloration.

Proximately, although larger investment in darkened nestlings should not increase their fledging success, because the mortality in the last few days of rearing is almost negligible (Møller 1994), an increased food intake could favor their survival in the very crucial postfledging period, when mortality is very high (Grüebler et al. 2014).

Favoritism toward darkened males emerged after controlling for the effect of sex of their direct competitors (i.e., always males), and irrespective of difference in body size and begging behavior, which are known to affect competitive interactions among siblings and food allocation in the barn swallow (Saino et al. 2000; Boncoraglio et al. 2008; Bonisoli-Alquati et al. 2011; Romano et al. 2011). Thus, our results should reliably reflect parental decisions on food allocation according to offspring reproductive value, as clearly indicated by the observation that in a very large proportion of male dyads the darkened nestlings obtained more food than their sham-colored siblings. In addition, the differential investment toward darkened males or females was observed despite a lack of sex-related difference in plumage coloration in this sample of immature barn swallows (but see Costanzo A et al., unpublished data), as in most of bird species (Moreno and Soler 2011; Hawkins et al. 2012). This is not surprising because the parental ability to distinguish offspring of either sex, and therefore to differently invest in them, is mediated by other traits, such as vocalizations and gape coloration, which are known to differ between male and female nestlings (Saino et al. 2003; Saino, de Ayala, et al. 2008). The lack of sex-dependent coloration in nestlings used in the present study is partly consistent with the evidence from a recent study on the North-American subspecies (*H. r. erythrogaster*), where a slight difference between male and female nestlings was observed in plumage saturation (*rA*), but not in the other color variables considered here (Hubbard et al. 2015).

In our study population, darker males are less viable than paler ones once they reach sexual maturity (i.e., between the first and the second breeding season; Saino, Romano, Rubolini, Teplitsky, et al. 2013). However, viability according to ventral plumage color has not been investigated in other populations, and it is still unknown if survival in the first year of age varies according to coloration, thus preventing to generalize to all age classes the pattern observed between the first and the second breeding seasons (Saino, Romano, Rubolini, Teplitsky, et al. 2013). Indeed, there is no a priori reason to discard the possibility that an opposite pattern of color-related mortality may occur between fledging and sexual maturation, approximately 1 year later. In addition, the barn swallow is a short-lived species with a life expectancy at sexual maturation of 1–2 years; indeed, most adults only enjoy a single breeding season in their life (Møller 1994; Saino et al. 2012). Furthermore, although the variance in the number of clutches, of eggs, and of fledglings is very small (Møller 1994; Saino N, unpublished data), sperm competition is intense, leading to a high level of extrapair paternity (in our population, the biological sire of up to 30% of nestlings is not the social father; Saino et al. 1997). This suggests that the cost paid by darker adult males in terms of survival may be overcompensated by the larger success in sperm competition, thus justifying an investment toward brownish male nestlings.

The above speculation rests on the assumption of a role of plumage color in affecting male lifetime breeding success in our focal population, which is matter of current investigation. However, in a recent study (Romano et al. 2015), we demonstrated that male-like females with darker ventral plumage produce male-biased broods. Because plumage color is a heritable trait (Saino, Romano, Rubolini, Ambrosini, et al. 2013; Hubbard et al. 2015; Vortman et al. 2015), this finding suggested an overproduction of "sexy sons" implying a possible role of ventral ornamentation in increasing male attractiveness. Taken together, the present findings and those by Romano et al. (2015) therefore indicate the possibility that the white to brownish plumage color in males may be under sexual selection also in European barn swallows.

Male and female parents may differently contribute to bias food allocation according to the phenotype of their offspring (Tschirren et al. 2005; Barrios-Miller and Siefferman 2013). Unfortunately, in the present study, we had no information about the feeding behavior of individual parents, and we therefore have no clue as to whether differential food allocation toward darker sons was driven more by mothers or fathers, or both parents. Further investigations by identifying the sex of attending parents during feeding trials would disclose this interesting issue.

Previous studies of other bird species demonstrated that plumage color can convey reliable information about nestling quality, allowing parents to adjust food allocation accordingly (see above and Introduction for details). This is also the case for the expression of colored plumage patches that can confer direct fitness benefits in intrasexual competition at sexual maturity, as suggested by studies on great tits (*Parus major*; Galván et al. 2008; Tanner and Richner 2008), eastern bluebirds (Ligon and Hill 2010; Barrios-Miller and Siefferman 2013), and rock sparrows (*Petronia petronia*; Griggio et al. 2009). However, to the best of our knowledge, only a single study investigated whether parental favoritism according to nestling plumage color varied between sons and daughters: Male, but not

female, eastern bluebird parents showed a preferential postfledging defense behavior toward the most ornamented sons (Barrios-Miller and Siefferman 2013). In addition, preferential food allocation toward brighter male nestlings has been observed in the same species, but differential food provisioning according to offspring coloration was not tested in females (Ligon and Hill 2010).

Our novel results are therefore consistent and complementary with those obtained in the eastern bluebird. They in fact provide further evidence on the parental ability to differentially invest in male and female offspring according to a trait expressed during early development, which will be involved in intrasexual competition for access to mates at sexual maturation by adding new knowledge about the mechanisms of parental favoritism of more valuable offspring.

A.R. conceived of the study, designed the study, collected data in the field, performed data analyses, drafted the manuscript; G.B. collected data in the field; M.Ca. carried out the molecular lab work; M.Co. collected data in the field; A.C. carried out the color measures; D.R. conceived of the study, participated in data analysis, helped draft the manuscript; N.S. conceived of the study, designed the study, helped draft the manuscript. All authors gave final approval for publication.

Handling editor: Nick Royle

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Nestling sex and plumage color predict food allocation by barn swallow parents

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Received 9 November 2015; revised 5 January 2016; accepted 25 February 2016; Advance Access publication 18 March 2016.

Behavioral Ecology (2016), 27(4), 1198–1205. doi:10.1093/beheco/arw040

Supplementary Data

Materials and methods

Colour measures

Reflectance spectra of one ventral feather per nestling were measured in a dark chamber, using an Avantes DH-2000 spectrometer equipped with deuterium-tungsten halogen light source (see Saino et al. 2013a for details). Every measure was taken twice. Importantly, as previously demonstrated (see Romano et al. 2015) colour measurements taken on a single feather, on three feathers of the same individual or directly on ventral plumage of individuals provide very consistent estimates (correlation coefficients between the colour variable values obtained by the three methods were $r > 0.88$ for θ and $r > 0.91$ for both φ and rA). Colour quantification was performed relatively to white and black standards by using the tetrachromatic colour space model with the TetraColorSpace program version 1a (Stoddard and Prum 2008). This program includes information about bird cone sensitivity and therefore provides biologically realistic colour metrics (see also Antonov et al. 2010; Saino et al. 2013a). Here, we assumed a UVS cone type retina and used spectral sensitivity of the blue tit (*Cyanistes caeruleus*), the most closely phylogenetically related species to the barn swallow for which information on cone spectral sensitivity is available in TetraColorSpace.

Idealized stimulation of the four cone types of passerines were normalized to a sum of 1, so that tetrahedral coloration was described by a vector of {uv, s, m, l} values, which represents stimulation of the cones sensitive to ultraviolet, short, medium and long wavelengths, respectively. Tetrahedral colour space vectors were then transformed into the spherical coordinates θ, φ, and r (see Antonov et al. 2010; Stoddard and Prum 2008). φ and θ respectively reflect the ultraviolet and the red-green-blue components of hue, while r is a proxy of colour saturation. For barn swallow ventral feathers, decreasing θ indicates darker, brownish coloration and a higher concentration of both eu- and pheo-melanin (see Saino et al. 2013a). Because the tetrahedral colour space is not a sphere and different hues vary in

maximum potential saturation (r_{max}), we therefore computed a 'achieved saturation' as the ratio between the obtained and the maximum potential value of saturation ($rA = r/r_{max}$).

Effects of experimental manipulation on plumage colour

We tested whether the experimental manipulation increased the ventral plumage colour in mixed models including sex, experimental manipulation (sham-coloured vs. darkened), and sequence (before or after manipulation) as fixed effects, and brood and nestling identity as random intercept effects. The experimental manipulation by experimental phase interaction term was also included in the model. The same model was applied to all colour variables (θ, φ, and rA). Colour measurements of three individuals were removed from the analyses because outliers.

The results of experimental manipulation on plumage colour of the two experimental groups are shown in Table S1. No differences in plumage colouration between male and female nestlings were observed for all colour variables. Plumage colouration significantly varied both according to experimental manipulation and sequence (Table S1). Their interaction was statistically significant for θ and rA, but not for φ. Fig. S1 shows the spectral curves for ventral plumage colouration of male and female nestlings before and after experimental manipulation for control and treatment groups.

Table S1. Mixed models of the effects of offspring sex, experimental manipulation (sham-coloured vs. darkened), sequence (before or after manipulation) and their interaction on θ, φ, and *rA*. Brood and nestling identity are included as random effects in the models.

	Coefficient (SE)	F	DF	P
θ				
Sex	-0.016 (0.012)	1.72	92	0.19
Experimental manipulation	-0.081 (0.016)	13.04	92	0.0005
Sequence	-0.093 (0.013)	27.76	92	<0.0001
Experimental manipulation × sequence	0.074 (0.021)	12.44	92	0.0007
φ				
Sex	-0.013 (0.022)	0.34	92	0.36
Experimental manipulation	-0.088 (0.031)	3.96	92	0.11
Sequence	-0.142 (0.027)	18.42	92	<0.0001
Experimental manipulation × sequence	0.103 (0.042)	2.36	92	0.016
rA				
Sex	0.009 (0.009)	1.27	92	0.26
Experimental manipulation	0.070 (0.012)	20.95	92	<0.0001
Sequence	0.089 (0.011)	51.18	92	<0.0001
Experimental manipulation × sequence	-0.060 (0.017)	12.85	92	0.0002

We thus explored the differences between darkened and sham-coloured nestlings before and after the experimental manipulation by means of post-hoc tests (Figure S2). Before the marker application no difference in any colour variable was observed between nestlings included in either sham-coloured or darkened group (θ: $t_{92} = -0.40$, P = 0.69; φ: $t_{92} = 0.51$, P = 0.61; *rA*: $t_{92} = 0.84$, P = 0.40). In addition, the experimental manipulation enhanced the colour of darkened nestlings, as indicated by the significant difference between all colour variables before and after experimental manipulation (θ: $t_{92} = -6.92$, P < 0.0001; φ: $t_{92} = -5.30$, P <0.0001; *rA*: $t_{92} = 8.44$, P < 0.0001). Moreover, the sham-coloured manipulation also altered *rA* of nestlings (*rA*: $t_{92} = 2.31$, P = 0.023). However, we stress that the alteration of the plumage colour of the sham-coloured nestlings was in the same direction with respect to that observed in darkened nestlings, indicating that our results were therefore conservative. Importantly, the sham-colour treatment did not affect the θ and φ of ventral plumage colour of sham-coloured nestlings (θ: $t_{92} = 1.13$, P = 0.26; φ: $t_{92} = -1.19$, P = 0.24). This means that the red-green-blue component of hue, which consists in the spectral location representing the position of a spectrum in the colour wheel, did not change.

Importantly, after the markers application (i.e. at the moment of feeding trial), all colour variables of darkened nestlings were significantly different from those of their sham-coloured siblings (θ: $t_{92} = -5.04$, P < 0.0001; φ: $t_{92} = -2.87$, P = 0.005; *rA*: $t_{92} = 5.79$, P < 0.0001), indicating that the experimental manipulation significantly darkened ventral plumage colour (Figure S2).

Finally, the mean values of θ and φ of experimentally darkened nestlings were shifted by approximately 1 SD of the mean values of the natural colour of the nestling plumage (mean $\theta \pm$ SD of all nestlings before experimental manipulation: 0.207 ± 0.076, range 0.015 - 0.438, N = 119; mean $\theta \pm$ SD of darkened nestlings after experimental manipulation: 0.120 ± 0.076, range 0.013 - 0.341, N = 56; mean $\varphi \pm$ SD of all nestlings before experimental manipulation: -0.580 ± 0.177, range -0.777 - -0.055, N = 119; mean $\varphi \pm$ SD of darkened nestlings after experimental manipulation: -0.706 ± 0.069, range : -0.802 - -0.376, N = 56). The mean values of *rA* of experimentally darkened nestlings were shifted by approximately 2 SD from the mean value of the natural colour of the nestling plumage (mean *rA* ± SD of all nestlings before experimental manipulation: 0.106 ± 0.056, range 0.041 - 0.280, N = 119; mean *rA* ± SD of darkened nestlings after experimental manipulation: 0.191 ± 0.068, range 0.057 - 0.306, N = 56).

Figure S1. Average reflectance curves (± SD) for ventral plumage colouration of male (solid line) and female (dashed line) bar swallow nestlings both before (a: nestlings of sham-coloured and darkened groups pooled) and after experimental manipulation of plumage colour (b; sham-coloured nestlings; c: darkened nestlings). Sample size is 44 female (16 sham-coloured and 28 darkened) and 50 male (22 sham-coloured and 28 darkened) nestlings.

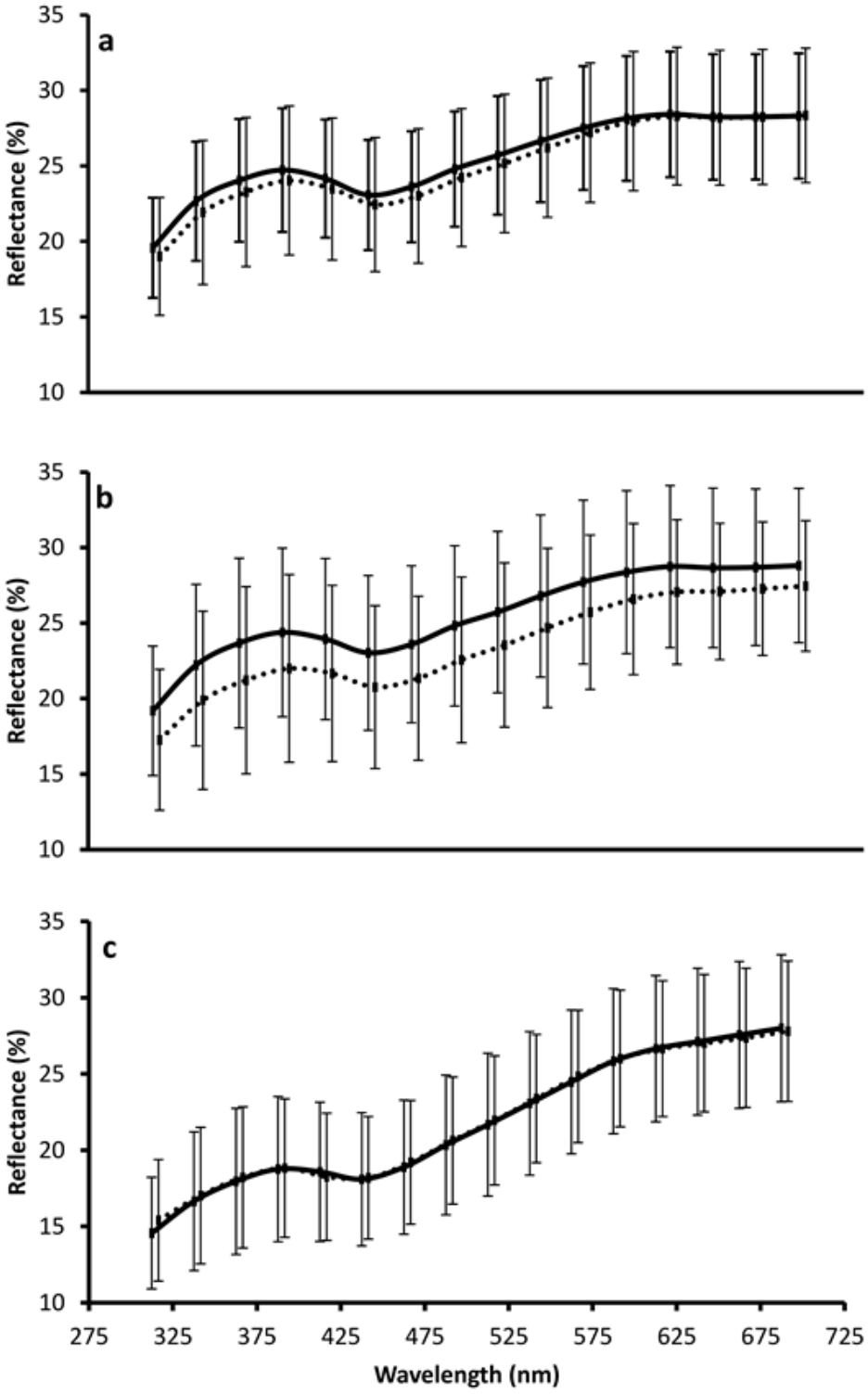
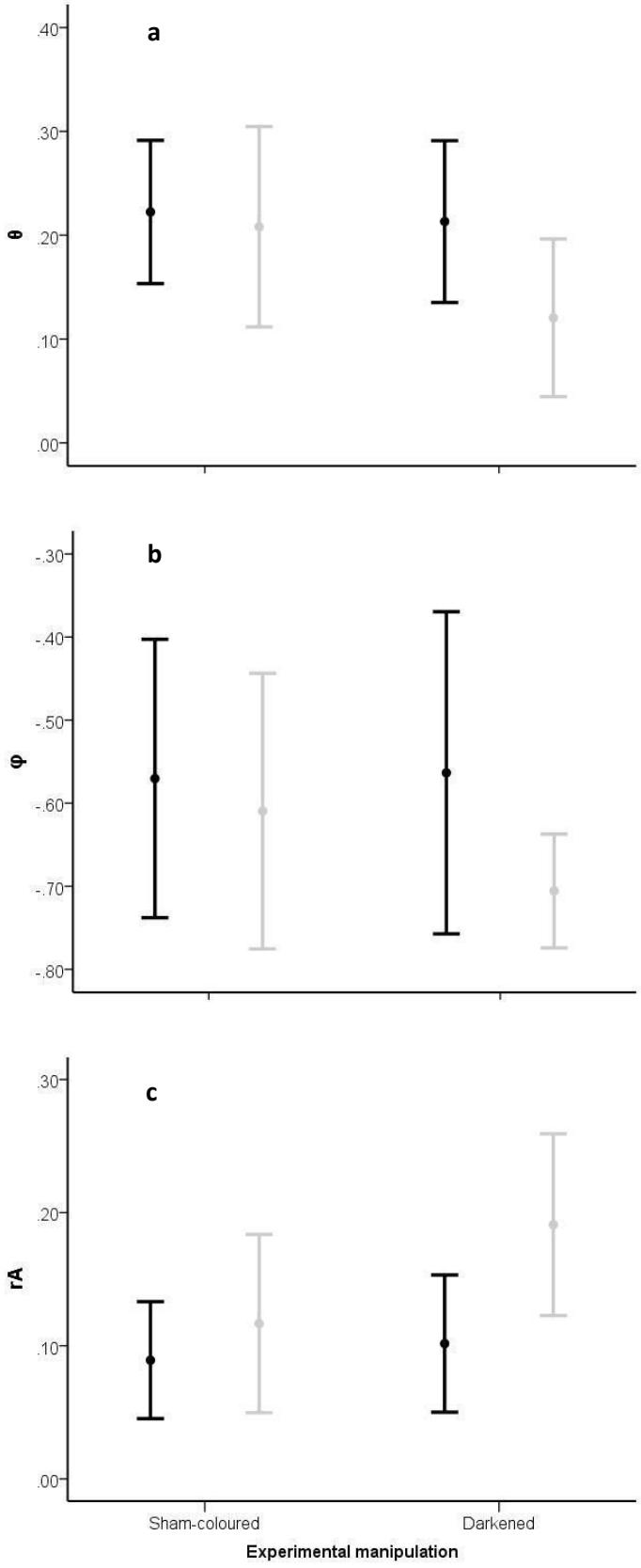


Figure S2. Mean (± SD) values of θ (a), φ (b) and *rA* (c) of sham-coloured (N = 38) and darkened (N = 56) nestlings before (dark bars) and after (light bars) the experimental manipulation of ventral plumage colour.



Results

Parental food allocation according to nestlings plumage colour while accounting for the 'dyad order'

For several broods (body mass variation: N = 21; number of feedings: N = 15) we performed feeding trials for two dyads differing in hunger level. This was the case because the second dyad was always tested after a period of food deprivation during the feeding trial of the first one. In the barn swallow, food deprivation is well known to increase hunger and begging intensity, and consequently parental feeding rate (see Saino et al. 2000; Boncoraglio et al. 2008; Bonisoli-Alquati et al. 2011; Romano et al. 2011; 2012). This is also the case for the present study as feeding rate to the second-tested dyads was larger than that of the first-tested ones (Poisson mixed model including dyad order as a fixed effect and brood identity as random effect: $F_{1,14} = 20.77$; $P = 0.0004$). To account for the different feeding rate between first- and second-tested dyads for each brood, the analysis on body mass variation was therefore repeated while also including a two-level fixed effect indicating the *dyad order*. We did not repeat the analysis on the proportion of feeding received by darkened nestlings including the dyad order because this dependent variable was not affected by dyad order ($F_{1,13} = 1.86$; $P = 0.20$). This was expected because the proportion of feedings received by each nestling is not dependent on parental feeding rate.

As shown in Table S2, the dyad order significantly predicted body mass variation of nestlings, with nestlings belonging to the second-tested dyad gaining more body mass than siblings tested in the first one. However, and importantly, the effect of the experimental manipulation was qualitatively similar to that presented in Table 1. Indeed, experimental manipulation significantly predicted the body mass variation in male, but not in female, dyads.

Table S2. Mixed models of the effects of experimental manipulation (darkened or sham-coloured) and dyad order on body mass variation by dyads of same-sex nestlings. The analyses were performed for nestlings of either sex separately. See the main text for details and sample sizes.

	Coefficient (SE)	F	DF	P
Males				
Experimental manipulation	0.175 (0.082)	4.54	1,23	**0.044**
Dyad order	0.434 (0.108)	16.27	1,20.1	**<0.001**
Females				
Experimental manipulation	0.045 (0.084)	0.29	1,21	0.59
Dyad order	0.388 (0.190)	4.19	1,6.76	0.08

Chapter 3

Behavioural stress response and melanin-based plumage colouration in barn swallow nestlings

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Behaviour 154 (2017): 853-874.

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Behaviour 154 (2017) 853–874

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Behavioural stress response and melanin-based plumage colouration in barn swallow nestlings

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Received 21 April 2017; initial decision 13 May 2017; revised 23 June 2017; accepted 10 July 2017; published online 18 August 2017

Abstract

Consistent and correlated inter-individual differences in behaviours, the so-called 'personalities', have been identified in many vertebrates. The ability to respond to stressful events is part of personalities and can have important fitness consequences, as it determines how individuals cope with environmental challenges. As a consequence of pleiotropic effects of genes involved in several functions, inter-individual differences in behavioural responses can be associated with phenotypic traits, like melanin-based plumage colouration in birds. We examined the association between three proxies of the behavioural stress response and breast plumage colouration in barn swallow (*Hirundo rustica*) nestlings. We found that nestling behavioural responses were consistent within individuals and similar among siblings, thus suggesting that these behaviours may contribute to define individual 'personalities'. However, nestling behavioural stress response was not significantly predicted by variation in breast plumage colouration, indicating that in juveniles of this species melanin-based colouration does not convey to conspecifics reliable information on individual ability to cope with stressful events.

Keywords

behavioural consistency, coping styles, *Hirundo rustica*, melanin, melanocortin, personality, stress response.

1. Introduction

In vertebrates, individuals display consistent behavioural differences in particular contexts, for example, when coping with environmental challenges, exploring a new environment or interacting with competitors or potential

DOI 10.1163/1568539X-00003447

mates (Koolhaas et al., 1999; Cockrem, 2007; Réale et al., 2007; Peleg et al., 2014). Inter-individual differences in behaviour can be highly structured and correlated across different contexts: under such circumstances, inter-individual behavioural differences are termed 'personality traits' (Sih et al., 2004; Wolf & Weissing, 2012). Personality traits are often heritable (Dingemanse et al., 2002; van Oers et al., 2005; Réale et al., 2007) and can be related to individual fitness (Dingemanse et al., 2004; Dingemanse & Réale, 2005). When different personality traits are significantly reciprocally interconnected, they are defined as a behavioural syndrome (Sih et al., 2004; Bell & Sih, 2007; Peleg et al., 2014). Individuals of the same species or population can exhibit different behavioural syndromes (e.g., individuals can be more or less aggressive; Sih et al., 2004). At the same time, a behavioural syndrome can characterize different populations or species (e.g., populations/species can be more or less aggressive; Sih et al., 2004). A well-known case of behavioural syndrome is the so-called 'proactive-reactive' axis, which describes the suite of individual behavioural and physiological responses when facing stressful events (Koolhaas et al., 1999, 2010; Coppens et al., 2010). 'Proactive' individuals respond to stressful events with an increased noradrenergic stimulation and an intense sympathetic activation, which result in lower basal glucocorticoid levels and a fight-or-fly response (Koolhaas et al., 1999, 2010; Cockrem, 2007; Carere et al., 2010; Coppens et al., 2010). In contrast, 'reactive' individuals respond to stressful events with an intense hypothalamic-pituitary-adrenocortical reactivity, resulting in a larger increase in circulating glucocorticoids and a freezing response to the stimulus (Koolhaas et al., 1999, 2010; Cockrem, 2007; Carere et al., 2010; Coppens et al., 2010). These two different 'coping styles' should represent alternative and coherent strategies which can be adaptive in different environmental contexts: the first should prevail in constant or predictable environments, while the latter should be favoured in unpredictable and changing environments (Koolhaas et al., 1999, 2010; Cockrem, 2007; Carere et al., 2010).

Behavioural syndromes can be associated with colour patterns that might serve as reliable signals of individual own personality to conspecifics in socio-sexual contexts (Réale et al., 2007; Peleg et al., 2014). For instance, several studies have reported statistically significant associations between melanin-based colouration and many physiological and behavioural traits in different phylogenetically distantly related vertebrate species (e.g., West &

Packer, 2002; Torda et al., 2004; review in Roulin et al., 2004). Melanin is the most common endogenous pigment involved in determining vertebrate colouration (Majerus, 1998). Melanin-based colouration is partly under genetic control and shows large inter-individual variability, which is determined by the deposition of different melanin pigments (Ducrest et al., 2008). In fact, melanin occurs in two different forms: eumelanin, responsible for the grey to black colouration, and pheomelanin, which determines the yellow to reddish one (Prota, 1992; Hearing, 1998; Simon et al., 2009). The same biosynthetic pathway produces both melanin forms, and the final perceived colouration is determined by the different relative and absolute concentration of the two forms in pigmented tissues (Haase et al., 1992; Ito et al., 2000; McGraw et al., 2004).

Increasing evidence indicates that associations between behavioural/physiological traits and melanin-based colouration derive from the pleiotropic effects of the melanocortins, the key regulators of melanogenesis (Ducrest et al., 2008). Indeed, melanocortins not only bind Melanocortin 1 Receptor (MC1R), their main receptor, being expressed in the skin and implicated in melanogenesis, but also other melanocortin receptors (MC2-5R) expressed in different tissues, thus influencing several other organismal traits or functions, like aggressiveness, sexual behaviour, exocrine gland activity, immune function, energy homeostasis and hypothalamic-pituitary-adrenal (HPA) stress response (for details, see Ducrest et al., 2008).

The covariation between melanin-based colouration and personality has been particularly well studied in birds, where polymorphism in plumage colouration has been related to behavioural syndromes, including aggressiveness and dominance status (Jawor & Breitwisch, 2003; Quesada & Senar, 2007). Several studies support the link between eumelanin- and pheomelanin-based colouration and stress response (Almasi et al., 2010, 2012; Saino et al., 2013b). However, compared to the other melanocortin pleiotropic effects, differential regulation of HPA stress response according to the content of the two forms of melanin is complex (Almasi et al., 2008, 2010, 2012; Jenkins et al., 2013). Previous avian studies have investigated melanin-based colouration by classifying colouration as either ‘eumelanic’ or ‘pheomelanic’, but in most cases plumage colouration is determined by both of these pigments (mixed melanin plumage; McGraw et al., 2004; Saino et al., 2013a). The only two studies investigating the relationship between mixed melanin plumage and HPA stress response reported contrasting results

(Jenkins et al., 2013; Saino et al., 2013b). Hence, the role of melanin-based colouration as a cue for stress response has yet to be fully elucidated.

In the present study, we focused on the association between behavioral stress response and melanin-based breast plumage colouration in barn swallow (*Hirundo rustica*) nestlings. In the study population, darker individuals have higher concentrations of eumelanin and pheomelanin in their breast feathers compared to paler ones (Saino et al., 2013a). Darker breast plumage colouration is also positively predicted by a higher relative concentration of pheomelanin on eumelanin, and this relationship is stronger in males than in females (Saino et al., 2013a). In addition, breast plumage colour covaries with a suite of life-history traits, such as telomere length (Costanzo et al., 2016), immune response (Saino et al., 2013b), viability (Saino et al., 2013c), and natal dispersal (Saino et al., 2014). Moreover, darker, more pheomelanic males had a higher baseline and stress induced corticosterone levels when brood size was experimentally reduced (Saino et al., 2013b). However, variation in adult and nestling male breast plumage colouration did not significantly predict circulating corticosterone levels in a different barn swallow subspecies (Jenkins et al., 2013), where the plumage coloration is on average darker, strictly related to the pheomelanin content and affects male reproductive success (Safran et al., 2005; Romano et al., 2017). Conversely, in the European subspecies the role of this plumage ornament in sexual selection has yet to be fully understood.

In order to investigate the behavioural stress response of nestlings to restraint, we used three behavioural tests, which have been extensively adopted in many studies of birds stressful environmental circumstances (reviewed in Cockrem, 2007): tonic immobility (Jones & Faure, 1981; Jones, 1986), breath rate (Carere & Van Oers, 2004; Fucickova et al., 2009) and agitation (Brommer & Kluen, 2012; van den Brink et al., 2012; Kluen et al., 2014). These three proxies of behavioural stress response should be intercorrelated: a higher breath rate is an indicator of a higher heart rate and is associated with a fearful state, resulting in a freezing behaviour (less agitation and higher tonic immobility duration; Carere & Van Oers, 2004; Fucickova et al., 2009; Brommer & Kluen, 2012; van den Brink et al., 2012; Kluen et al., 2014). Under the assumption that melanin-based coloration covaries with stress response, we expected darker individuals (i.e., those having higher concentrations of both eumelanin and pheomelanin and a larger pheomelanin/eumelanin concentration ratio) to be 'reactive' copers, showing

a stronger behavioural stress response as a consequence of higher activation of HPA axis and a larger level of circulating corticosteroid hormones. They are, therefore, expected to show longer tonic immobility duration, higher breath rate and lower agitation (see also van den Brink et al., 2012). In the analyses, we took into account the potential effects of nestling sex, rank within brood (i.e., body size reflecting hatching asynchrony), and parental colouration on behavioural stress response. Moreover, we investigated the occurrence of sex differences in the covariation between behavioural stress response and nestling colouration, as the effect of colouration on life-history traits may be sex-specific (Romano et al., 2016).

2. Materials and methods

2.1. Study species and general field procedures

The barn swallow is a small (ca. 20 g) migratory passerine. Most individual breed in rural buildings (Møller, 1994; Ambrosini et al., 2002); females lay 1–3 clutches of 1–7 eggs per breeding season (modal size: 5 eggs) and incubate eggs for 14 days. Both parents feed the offspring until fledging at ca. 18–21 days old (Møller, 1994).

The present study was carried out between April and July 2014 in five barn swallow colonies (farms) located near Milan (Northern Italy). Breeding adults were captured using mist-nets placed at doors and windows of rural buildings before dawn. All captured adults were sexed and marked with colour plastic rings to identify breeding pairs by direct observations at the nest (see Saino et al., 1999 for details). We plucked 2–3 feathers from the left paramedial breast plumage region; this position is approximately the same for all individuals. The collected feathers were stored in a plastic bag and kept in a dark box until colour analyses (see below). Every second day all nests in the farms were inspected to record breeding events. We collected information on both first and second broods.

2.2. Nestling behavioural measurements

Nestling behavior was recorded at day 13 $\pm$ 2 days post-hatching (considering the day of hatching of the first egg(s) in a nest as the age 0). All nestlings in a brood were simultaneously removed from the nest and placed individually in a cotton bag to prevent any visual contact among them. Nestlings were tested one by one, in a random sequence, and all measures were taken

by the same observer always in the same order. We quantified three standard behaviors used in previous bird studies as proxies of behavioral stress response (Carere & van Oers, 2004; Fucikova et al., 2009; Brommer & Kluen, 2012; van den Brink et al., 2012; Kluen et al., 2014; Peleg et al., 2014).

First, we measured tonic immobility response to restraint ('TI' hereafter), an innate behavioral state characterized by a catatonic condition of reduced responsiveness to external stimulation (Jones, 1986). In many vertebrates, TI is commonly observed in response to external stimuli. It is regarded as an adaptive defense mechanism against predators and the TI duration is considered to be positively realated to fearfulness (Jones, 1986; Boissy, 1995). Using the method described by Jones & Faure (1981) and Jones (1986), we obtained a measure of nestling motivation not to rest on their back. We put each nestling in a shallow bowl (diameter 15 cm, maximum depth 3 cm) on an horizontal base. We positioned each nestling on its back, restraining it with hands symmetrically placed on top of the nestling and completely covering its body until it stopped struggling to escape in order to avoid that the nestling turned its back, thus possibly confounding the test outcome. When hands were removed, we measured the time until the nestling moved to turn itself, until a maximum duration record of 90 s. Reverting from supine position to prone position in the wild seems to be a rare event: hence, nestlings were naïve to this condition before the test. We thus decided to perform the TI test only once for each individual.

Secondly, we measured the number of times an individual breathed in a fixed timespan ('breath rate' hereafter), which is a proxy of the response to emotional state and stress (Carere & van Oers, 2004; Fucikova et al., 2009). Using the method described by Brommer & Kluen (2012), each nestling was placed with its back on the palm of the experimenter's hand, ca. 40 cm from the experimenter face. Breath rate was measured by counting the number of breast movements over 20 s, using a stopwatch. The measurement was recorded twice in sequence, and it turned out to be highly repeatable (repeatability, $R = 0.88$, $p < 0.01$; see Section 2.4 for details); in subsequent statistical analyses we used the average number of breaths across the two trials (Lessells & Boag, 1987).

Finally, during the 40 s of the breath rate measurements, while the nestling was on the palm of the hand of the experimenter, another experimenter counted the number of nestling leg movements. This number was considered as a measure of nestling aggressiveness and agitation ('agitation' hereafter).

A similar variable has been previously used in behavioural studies of birds (e.g., Brommer & Kluen, 2012; van den Brink et al., 2012; Kluen et al., 2014). The measurement was performed a second time (in 10 s). Since the measure was weakly but significantly repeatable ($R = 0.22$, $p = 0.02$; see Section 2.4 for details), in statistical analyses we used the sum of the number of struggling over the two trials (Lessells & Boag, 1987).

After recording behavior, we collected a blood sample (ca. 40 μl) with capillary tubes by puncturing nestling brachial vein. This sample was used for nestling molecular sex determination after polymerase chain reaction amplification of the sex-specific avian CHD-1 gene (Griffiths et al., 1998; Saino et al., 2008). A few breast feathers (2–3) were plucked from the same ventral region of the adults for the spectrometric colour analyses (see below). We did not collect feathers from the throat region, where the colour is also determined by the concentration of both eumelanin and pheomelanin in adults (Saino et al., 2013a), because at the age when the behavioural tests were performed feathers are too small to allow obtaining a reliable spectrometric estimate of their colour (see below).

2.3. Colour measures

We measured the reflectance of a single breast feather for both nestlings and adults using an Avantes DH-2000 spectrometer in a dark chamber, illuminating it with a combined deuterium-tungsten halogen light source (for details see Saino et al., 2013a). The use of a single feather per individual is justified by the observation that the reflectance of a single feather, of three feathers or of breast plumage of the entire individual provides very consistent estimates (for details, see Romano et al., 2015). Moreover, the measure of reflectance of the same feather and of two feathers taken from different breast regions are highly repeatable (Saino et al., 2013a). Reflectance spectra were processed to quantify colouration using tetrachromatic colour space model (Goldsmith, 1990) with TetraColorSpace program (Version 1a; Stoddard & Prum, 2008) run in MATLAB 7 (MathWorks, Natick, MA). In TetraColorSpace, we assumed UVS cone type-retina and adopted the spectral sensitivity of the blue tit (*Cyanistes caeruleus*) because this is the most closely related species to barn swallow for which spectral sensitivity information is implemented in the program. Each colour vector in the tetrahedral colour space was converted into the spherical coordinates θ, φ and *rA* (Stoddard & Prum, 2008). θ represents the red-green-blue hue component, φ the ultraviolet one, while

rA is a measure of colour saturation. In the range of colouration on barn swallow breast feathers, decreasing θ values and increasing *rA* values indicate a darker colouration associated with a higher concentration of eu- and pheomelanins, and a larger ratio between pheo- on eumelanin both in males and in females (Saino et al., 2013a). θ and *rA* colour components significantly vary among broods, indicating that differences exist in breast colouration between nestlings reared in different nests (Hubbard et al., 2015; Romano et al., 2016). Variability among nestlings from the same brood also occurs in this species (Hubbard et al., 2015; Costanzo et al., 2016; Romano et al., 2016). This variability is a prerequisite to investigate whether it reflects variability in stress response. Because it is possible to formulate an a priori hypothesis about the association between behaviour and colouration only for θ and *rA*, but these variables are highly negatively correlated both in adults (Romano et al., 2015) and in nestlings (correlation between θ and *rA* in the present study was $r = -0.85$), our analyses were focused on θ only. However, the same analyses carried out on *rA* provided qualitatively very similar results (details not shown).

We do not have information about the melanin content of nestlings' plumage. However, a positive correlation ($r = 0.61$) between θ values of the same breast plumage region at nestling stage and adult (1 year old) was shown on a small sample of individuals ($N = 12$) for which a feather sample was available at both phases of the lifecycle. Since in the adults θ of the breast feathers is positively associated with the content of both melanin forms, we can expect that this relationship should be similar also among nestlings.

2.4. Statistical analyses

The covariation between the three proxies of the behavioural stress response has been tested both at the within-individual and at the within-brood level. In the former case, we simply tested the correlations between the different proxies within each individual. In the latter case, we tested the correlations between the brood mean of each proxy of behavioural stress response.

The association between nestling behavioural responses to stress tests and θ of nestlings/parents was tested in separate statistical models for each behavioural trait.

Tonic immobility response was coded as a dichotomous variable, based on the largely bimodal distribution of time of reaction (response: 0–45 s, coded

as 1, $N = 145$ nestlings; no response: >45 s, coded as 0; $N = 43$ nestlings; see Figure A1 in the Appendix). The effect of θ on tonic immobility response was tested in generalized linear mixed models (GLMMs) assuming a binomial error distribution and logit link function. The effect of θ on breath rate was tested in linear mixed models (LMMs), while the effect of θ on agitation in Poisson GLMMs. Where necessary, Poisson models were corrected for data overdispersion. Nest identity was included as a random intercept effect in all mixed models.

Because mixed models do not account for non-independence of measurements of independent variables within a given level of aggregation of data (e.g., brood), and a moderate among-brood variability in plumage colour exists (Hubbard et al., 2015; Costanzo et al., 2016; Romano et al., 2016), we aimed at separating the between- from the within-broods effects of nestling θ on behaviour. Using the approach suggested by van de Pol & Wright (2009), the original θ colour component was replaced by two new variables: the mean brood value of θ, computed separately for each brood (θ_{MEAN}), and the difference between the θ value of each single nestling and the mean of its brood (θ_{DEV}). Positive θ_{DEV} values therefore indicated nestlings with θ value larger than the mean of its brood (i.e. nestlings whose plumage is paler than their siblings).

Since the behavioural tests were performed at the age when nestlings experience pre-fledging body mass recession (Ferrari et al., 2006), we could not use nestling body mass as a reliable proxy of nestling rank within brood. Nestling rank within brood was thus evaluated considering the length of the third primary feather ('wing length' hereafter), which at this stage of development should better reflect the individual level of development, and should reflect hatch order (Jenni & Winkler, 1989; Searcy et al., 2004). Wing length was replaced by two new variables: the mean of brood value of the wing length (wing $\mathrm{length}_{\mathrm{MEAN}}$), and the difference between the wing length of each single nestling and the mean of its brood (wing $\mathrm{length}_{\mathrm{DEV}}$).

In all mixed models, we included θ_{DEV}, θ_{MEAN}, nestling age (days from hatching; same value for all nestlings in a brood), nestling sex, nestling rank (wing $\mathrm{length}_{\mathrm{DEV}}$ and wing $\mathrm{length}_{\mathrm{MEAN}}$), and brood order (first or second brood) as predictors. The two-ways interactions between nestling θ_{DEV} with brood order, nestling sex and wing $\mathrm{length}_{\mathrm{DEV}}$ and the two-way interaction between wing $\mathrm{length}_{\mathrm{DEV}}$ and nestling sex were also included in the models. Qualitatively similar results were obtained including hatching date rather

than brood order (details not shown). Multicollinearity among independent variables did not occur (Variance Inflection Factor $\leqslant 4.8$).

The effects of mother θ and father θ on nestling stress response were tested in mixed models as described above, but including mother θ value and father θ instead of nestling θ.

In all analyses, interaction terms not attaining statistical significance were simultaneously dropped from the final model (Whittingham et al., 2006). All models were run using PROC MIXED and GLIMMIX in SAS 9.1.3 software (SAS Institute, 2006). Repeatability of traits within broods (or within individuals; see Section 2.2) was calculated using the rptR package (Schielzeth et al., 2017) on R software, which returned repeatability estimates also for non-Gaussian variables (i.e., Poisson and binomial variables), reporting the link-scale approximation for the estimate for non-Gaussian variables.

The behavioural stress response was recorded in 188 nestlings from 44 broods, but only for 166 individuals we could collect blood samples and, for practical reasons (e.g., impossibility to capture both parents), we collected the complete information for 165 nestlings only, including plumage colouration of both parents.

3. Results

3.1. Covariation between behavioural stress responses

Siblings showed significantly similar behavioral stress response, despite significant but relatively low within-brood repeatability of all behavioral traits that was detected (agitation $r = 0.20$, $p < 0.01$; breath rate $r = 0.13$, $p < 0.01$; TI response: $r = 0.13$, $p = 0.02$).

We examined whether nestlings responded consistently to the handling stress by analysing the correlation between the three different proxies of the behavioural stress response, both at the individual and at the brood level (mean value within-brood). Agitation was significantly positively correlated both with TI response (individual level: $r = 0.25$, $p < 0.01$; brood level: $r = 0.31$, $p = 0.04$) and negatively with breath rate (individual level: $r = -0.26$, $p < 0.01$; brood level: $r = -0.33$, $p = 0.03$), indicating that less agitated nestlings showed significantly longer latency in TI response and a higher breath rate (Figure 1). TI response was negatively correlated with breath rate (individual level: $r = -0.39$, $p < 0.01$; brood level: $r = -0.28$, $p = 0.07$), indicating that nestlings with a larger latency in TI response have a higher

breath rate (Figure 1). Hence, there was evidence of covariation between behavioural stress responses, suggesting that differences among nestlings may represent behavioral personalities, reactive copers being characterised by longer TI duration, higher breath rate and lower agitation, whereas the opposite is the case for proactive copers.

3.2. Variation in colouration

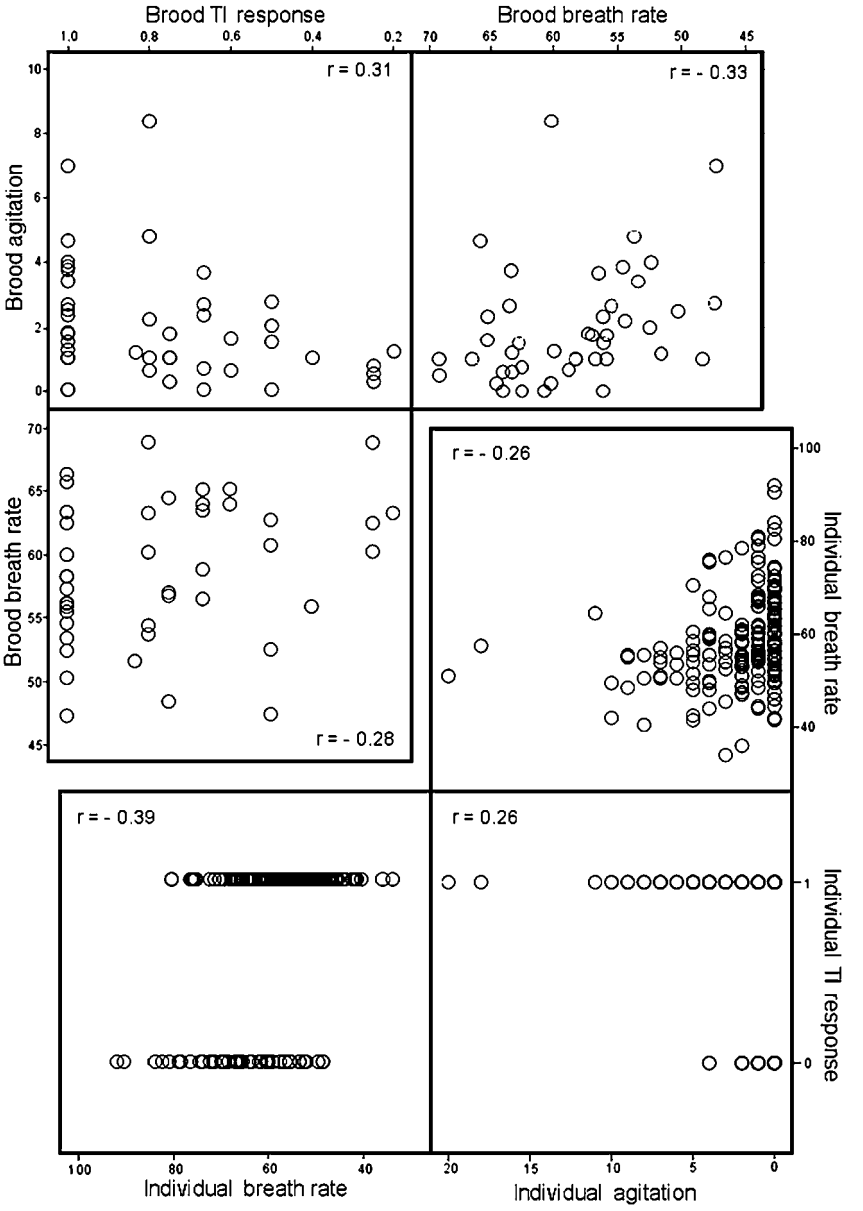
Nestling θ was significantly repeatable within broods ($r = 0.30$, $p < 0.01$), indicating that siblings share a similar breast plumage colour. However, the variance in θ explained by brood identity accounted for less than half of the total observed variance (variance components analysis: 0.35), implying large variability in θ among siblings. Furthemore, male nestlings were significantly darker than female nestlings (mixed model with brood identity as random factor, effect of sex: θ: $F_{1,148} = 10.15$, $p < 0.01$).

3.3. Behavioral stress response and colouration

Variation in nestling behavioral traits was not significantly predicted by θ of their own breast feathers (Table 1, Figure 2). Nestling breath rate and TI response were significantly predicted by wing length variation within broods, larger (i.e., early hatched) nestlings within broods having a shorter TI duration and breathing more frequently. Furthermore, agitation of second brood nestlings was larger compared to that of first brood ones (Table 1). Nestling behavioral traits were also not affected by the interaction between their own colour and the other predictors (Table 1). Adding parental colouration (both father and mother θ, included simultaneously) instead of nestling θ to the models reported in Table 1 revealed that parental colouration did not significantly affect nestling behavioral traits (details not shown for brevity). Qualitatively similar results were obtained for TI when the response was coded as a different type of dichotomous category (response: 0–90 s and no response: >90; details not shown).

4. Discussion

According to the hypothesis that genes determining melanin-based colouration have pleiotropic effects in avian species, we have examined the association between three proxies of the behavioural stress response and breast plumage colouration in barn swallow nestlings. We expected darker, more


Brood TI response
Brood breath rate
Brood agitation
Brood breath rate
Individual breath rate
Individual TI response
Individual breath rate
Individual agitation
r = 0.31
r = - 0.33
r = - 0.28
r = - 0.26
r = - 0.39
r = 0.26

pheomelanic individuals to be more reactive, i.e., to show a stronger behavioural stress response to handling restraint. First, we found that the behavioural response to stress of nestlings was moderately coherent, suggesting that these traits may collectively contribute to define individual 'personalities'. Secondly, we could not observe any statistically significant association between breast plumage colouration and the measured proxies of the behavioural stress response. Below we discuss the main findings of the study.

4.1. Covariation between behavioural stress responses

The three standard behaviours that we used as proxies of stress behavioural response were moderately and significantly inter-correlated at the individual level ($|r|$ values ranging between 0.25 and 0.39, values representing 'moderate' effect sizes according to Cohen, 1988). Similar correlations between behavioural stress proxies existed also at the brood level. These correlations indicate that siblings are weakly but significantly consistent in their behavioural response to stressful conditions. Despite correlation values were not large, we emphasize that this is typical for studies focusing on behavioural traits in wild populations (e.g., Réale et al., 2000; van den Brink et al., 2012; Markó et al., 2013), where several confounding effects may affect the contingent individual behavioural response and/or measurements. However, the covariation between these behaviours both at the individual and at the brood level suggests that the behavioural response to stress can help defining individual 'personalities' along the proactive-reactive axis (Koolhaas et al., 1999) in barn swallow nestlings, similarly to other species (Bell, 2007; Sih et al., 2012). On one end of the axis there are more 'proactive' copers that actively react to the handling stress, and are characterized by a shorter TI response, a lower breath rate and a higher agitation. On the opposite end of the axis, 'reactive' copers showed a freezing response (i.e., no response within 45 s during the TI test and lower agitation) and higher breath rate. Despite the TI response was mainly dichotomic, we note that the other behavioural responses showed a large variety of different shades between the extreme values, representing proactive-reactive copers.

Figure 1. Scatterplots of individual-level (below diagonal) and brood-level (mean value within-brood, above diagonal) correlations of the three behaviors used as proxies of the behavioral stress response: tonic immobility (0, no response within 45 s; 1, response within 45 s), breath rate and agitation (number of leg movements). Pearson's r values are reported within each scatterplot.

Table 1.
Effects of nestling θ colour components on nestling behavioural response to handling restraint (tonic immobility, breathing rate and agitation; $N = 166$ nestlings in 44 broods).

	Estimate (SE)		z	p
Tonic immobility				
θ_{DEV}	2.04	(3.41)	0.60	0.55
θ_{MEAN}	0.42	(4.67)	0.09	0.93
Nestling sex	0.30	(0.44)	0.67	0.50
Brood order	0.20	(0.63)	0.32	0.75
Nestling age	−0.17	(0.55)	−0.31	0.75
Wing length$_{DEV}$	−0.02	(0.01)	−2.45	0.02
Wing length$_{MEAN}$	0.00	(0.01)	0.04	0.97
* Wing length$_{DEV}$ × nestling sex	−0.01	(0.02)	−0.65	0.52
* θ_{DEV} × Wing length$_{DEV}$	0.23	(0.17)	1.33	0.19
* θ_{DEV} × nestling sex	4.29	(7.74)	0.55	0.58
* θ_{DEV} × brood order	1.39	(7.93)	0.18	0.86
Breath rate				
θ_{DEV}	−9.40	(11.48)	−0.82	0.42
θ_{MEAN}	24.72	(14.83)	1.67	0.10
Nestling sex	1.16	(1.48)	0.79	0.43
Brood order	−3.54	(2.01)	−1.76	0.08
Nestling age	−0.42	(1.78)	−0.24	0.81
Wing length$_{DEV}$	0.06	(0.02)	2.61	0.01
Wing length$_{MEAN}$	0.04	(0.03)	1.70	0.09
* Wing length$_{DEV}$ × nestling sex	−0.05	(0.05)	−1.15	0.25
* θ_{DEV} × Wing length$_{DEV}$	0.07	(0.47)	0.14	0.89
* θ_{DEV} × nestling sex	−21.09	(24.72)	−0.85	0.40
* θ_{DEV} × brood order	−43.31	(26.77)	−1.62	0.11
Agitation				
θ_{DEV}	−2.38	(1.59)	−1.50	0.13
θ_{MEAN}	−1.55	(2.63)	−0.59	0.56
Nestling sex	0.23	(0.21)	1.10	0.27
Brood order	0.81	(0.37)	2.19	0.03
Nestling age	0.36	(0.32)	1.11	0.27
Wing length$_{DEV}$	−0.00	(0.00)	−1.26	0.21
Wing length$_{MEAN}$	−0.01	(0.01)	−1.65	0.10
* Wing length$_{DEV}$ × nestling sex	−0.001	(0.01)	−0.11	0.91
* θ_{DEV} × Wing length$_{DEV}$	−0.06	(0.06)	−0.88	0.38
* θ_{DEV} × nestling sex	−1.81	(3.61)	−0.50	0.62
* θ_{DEV} × brood order	5.77	(4.60)	1.26	0.21

Two-way interactions between nestling sex, wing length$_{DEV}$ and brood order with nestling θ_{DEV} were removed from the final models because did not attain statistical significance (removed terms are marked with an asterisk). Estimates for main effects referred to models without interactions, whose statistics at removal are reported for the sake of completeness.

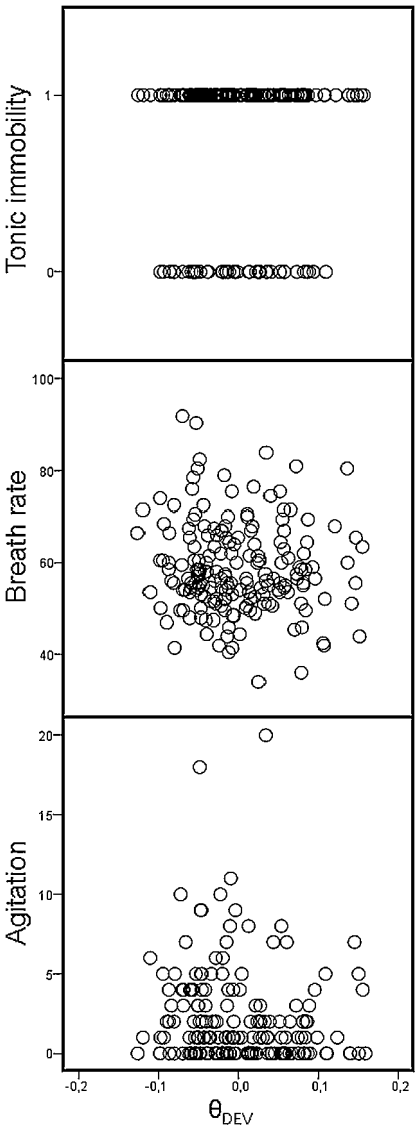


Figure 2. Variation in nestling tonic immobility (0, no response within 45 s; 1, response within 45 s), breath rate and agitation (number of leg movements) according to θ. θ values are centered within brood; positive θ_{DEV} values indicate nestlings that have larger θ compared to the brood mean.

We also assessed the within-brood repeatability of behavioural stress proxies. We found a low but statistically significant similarity among siblings in behavioural traits, indicating that common environment and/or genetic effects are important determinants of the behavioural stress response (see Bell et al., 2009 for similar results concerning within-individual variation in personality traits among different lifecycle stages).

4.2. Melanin-based colouration and stress response

Barn swallows display sexual dimorphism in melanin-based plumage colouration of breast feathers both among adults and among nestlings, males being on average darker than females (Saino et al., 2013a, c; Hubbard et al., 2015; Costanzo et al., 2016; this study; but see Romano et al., 2016). However, none of the three proxies of the behavioural stress response was significantly predicted by θ colour component of nestling breast feathers. Similarly, the behavioural response of nestlings to stress was not significantly predicted by parental colouration. These findings are inconsistent with a previous study on adults of our study subspecies, showing that darker breast plumage colouration of males attending experimentally reduced (but not enlarged) brood size was associated with a larger corticosterone level, indicating that darker males might have been more stressed (Saino et al., 2013b). The discrepancy between our findings and those obtained by Saino et al. (2013b) might be due to the fact that in the previous study hormone levels were quantified months after plumage moult, thus possibly not providing reliable information about individual hormonal state when corticosterone was measured. However, we note that our results are coherent with those obtained in nestlings of the Nearctic subspecies of the barn swallow, where breast plumage colour does not significantly covary with circulating corticosteroids (Jenkins et al., 2013).

A possible mechanism explaining the lack of covariation between melanization and stress behavior envisages the pleiotropic mechanisms that regulate eu- and pheomelanogenesis, which may have reciprocally antagonistic effects. Previous studies suggested a role of both modulators of eu- and pheomelanogenesis in the HPA axis activity (Xiao et al., 2003; Charmandari et al., 2005; Racca et al., 2005; Ducrest et al., 2008), indicating that the physiological interaction might be complex and sometimes acting in opposing ways. However, the relationship between HPA stress response and melanin-based colouration has been mostly linked to plumage traits determined by either eumelanin (Ducrest et al., 2008; Almasi et al., 2010) or

pheomelanin (Almasi et al., 2008; Roulin et al., 2008). This is unfortunate because melanin-based plumage traits of most bird species, including the breast plumage of the barn swallow, is determined by both of these pigments, and especially by their ratio (McGraw et al., 2005; McGraw, 2006; Saino et al., 2013a, c). As suggested by Jenkins et al. (2013), if the link between melanin-based colouration and HPA stress response relies only on compounds implicated in eumelanogenesis, the pleiotropic hypothesis proposed by Ducrest et al. (2008) might not apply to plumage colouration deriving from both melanin forms or where pheomelanins are the dominant driver of phenotype variation. Althought pleiotropic effects of melanocortin system may still drive the associations of melanin-based colouration and other physiological and behavioral traits, our results indicate that the behavioral stress response and mixed melanin plumage colouration are unrelated in nestlings of the European *rustica* subspecies of the barn swallow, in accordance with the lack of significant association between plumage colouration and circulating corticosteroids observed in the Nearctic *erythrogaster* subspecies, in spite of the different signalling functions of plumage colouration between subspecies (see Scordato & Safran, 2014; Romano et al., 2017).

In conclusion, our results show that barn swallow nestlings show a moderately coherent behavioural stress response at the individual and brood level, but suggest that mixed melanin-based colouration does not convey reliable information to conspecifics about individual ability to behaviorally cope with stressful conditions. Hence, our findings are coherent with the idea, proposed by Jenkins et al. (2013) for the American subspecies of the barn swallow, that the melanocortin pleiotropic hypothesis is unlikely to apply in the case of plumage traits that are the result of both eumelanin and pheomelanin pigments, because of the previously unappreciated complexity of the molecular interactions between pheomelanin products and HPA stress response.

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Appendix

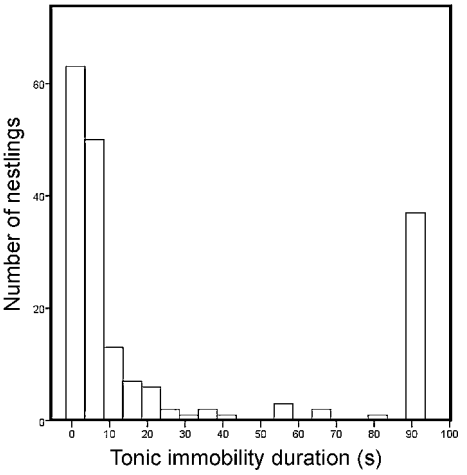

Figure A1. Histogram showing the distribution of nestling time of reaction during tonic immobility test ($N = 188$).

Chapter 4

Sequence variation in *MC1R* and *TYRP1* genes and their relationship with melanin-based plumage trait expression in lesser kestrel (*Falco naumanni*) males

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Accepted in Journal of Ornithology

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Word count (main text): 1306

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Summary

Lesser kestrel males display inter-individual variation in melanin-based plumage traits, like ventral plumage colouration and breast/underwing spottiness. We explored whether such plumage variation was associated with sequence variation at *MC1R* and *TYRP1*, two genes involved in the melanogenesis pathway. No statistically significant associations between single-nucleotide mutations and male plumage traits emerged, though in some cases very rare (<2 %) homozygous mutated individuals displayed extreme plumage phenotypes. Hence, large inter-individual male lesser kestrel plumage variation, which is consistent between years and partly age-related, was only marginally related to untranslated region and coding sequence variation at *MC1R* and *TYRP1*.

Keywords

Falconidae, genotype-phenotype associations, melanogenesis pathway, plumage melanisation, SNP, rare variants

Avian species show a remarkable diversity of plumage colouration patterns, which is maximally expressed whenever discrete colour morphs coexist within the same populations (i.e. plumage colour polymorphism; Galeotti et al. 2003). Such variability is mostly determined by different deposition of two different melanin forms, eumelanin (responsible for grey-black colouration) and pheomelanin (determining reddish-brown colouration). Variation in melanin-based traits may partly result from sexual selection, as such traits are often involved in intra– and/or inter-sexual signalling (Senar and Camerino 1998; Hoi and Griggio 2008). The final perceived colouration is determined by the different relative and absolute concentration of eu- and pheomelanin in feathers, which is known to be triggered by the *melanocortin-1-receptor* (*MC1R;* Ducrest et al. 2008). *MC1R* is a G protein-coupled receptor expressed in feather melanocytes, whose upregulation leads to an increase in eumelanin over pheomelanin production (Ducrest et al. 2008). At both inter- and intra-specific levels, variation in melanin-based colouration may be determined by mutations at *MC1R*. However, melanin-based colouration may also be determined by variation at other genes, especially in species with a complex eu/pheomelanin pattern. These genes include those influencing the switch between the production of the two melanin pigments either upstream (e.g. *ASIP, POMC*), or downstream of *MC1R* (e.g. *TYR* and *TYRP1*, *tyrosinase-related protein 1*; Bourgeois et al. 2016, 2017) and involved in melanosome migration (e.g. *SLC24A5*).

The lesser kestrel (*Falco naumanni*) is a sexually dimorphic diurnal raptor whose adult males show large inter-individual variations in melanin-based traits involving reddish ventral plumage (light to dark reddish) and black spots on breast, belly and underwing coverts, which may vary between absent and very abundant (Figure 1). Previous studies reported an association between polymorphism of melanin-based plumage traits in Falconidae and genetic polymorphism at *MC1R* (Johnson et al. 2012) and *TYRP1* (Cortimiglia et al. 2017), a key gene for eumelanin production (Nadeau et al. 2007). In the present study, we measured the degree of plumage melanisation of 105 lesser kestrel males from a breeding population in

Southern Italy (40°44'N-16°32'E) and sequenced their *MC1R* and *TYRP1* genes to examine whether single nucleotide polymorphisms (SNPs) at these genes were associated with inter-individual variation in ventral plumage colouration and black spot patterning of underparts.

In May-July 2016, males were captured at the nest, individually marked (details in Podofillini et al. submitted) and photographed using a digital reflex camera (Canon EOS100D) including a colour reference chart (X-Rite ColorCheckerPassport) in each picture. Colour was standardised using Photoshop CS3 (v. 10.0) plugin InCamera (Bergman and Beehner 2008). To evaluate intensity of ventral plumage reddish colouration, we measured the median of red, green and blue components of pixels. Higher red/blue values were associated with darker reddish colouration. We used ImageJ software (v. 1.51) to isolate, count and measure the ventral and underwing spots by means of Auto Local Threshold plugin. Spot pattern was quantified by a principal components analysis on the average size of spots and on the areas covered by spots on vent and underwing. Only the first principal component was used in the statistical analysis since it explained >75% of the observed variance (hereafter 'spottiness'): higher spottiness values were associated with a greater spot coverage and larger spots.

Genomic DNA was extracted from blood samples collected upon capture using Qiagen BioSprint®96DNA Blood Kit. PCR primers designed on *Falco cherrug* and *F. peregrinus* genomic sequences and PCR conditions are described in Table S1. Sequences of *TYRP1* (8 exons, 1969 bp), *MC1R* 5'- untranslated region (UTR;444 bp) and *MC1R* (959 bp) were obtained by Sanger sequencing of the purified DNA fragments corresponding to the different exons (Accession numbers: MG423585-MG423616). The *MC1R* and *TYRP1* 5'-UTR regions were sequenced as they may affect translation and transcription, because they may contain binding sites for transcription factors (San-Jose et al. 2015). Sequences with forward and reverse primers were aligned and eye-checked using CodonCode Aligner (v. 7.0.1). Individuals were considered as heterozygous at a given locus if double peaks of half

size were present in both strands. For *TYRP1*, we found four SNPs in its intron 1, one in exon 2 5'-UTR, and four in exons 2, 4 and 5; for *MC1R*, we found three SNPs in the 5'-UTR and fourteen in the coding sequence, only one of which was non-synonymous (Table S2). The frequency of homozygous individuals for the mutated allele was generally low, ranging between 0% and 13% (Table S2).

We tested the associations between each SNPs and plumage traits using the R *SNPassoc* package v. 1.9.2 (Gonzáles et al. 2007), assuming different modes of inheritance (codominant, dominant, recessive, overdominant, log-additive). 'False discovery rate' method was used to correct p-values for multiple testing; corrections were applied separately to each plumage trait and to each mode of inheritance.

Since mutation expression on phenotypic traits was unknown, we explored whether individuals homozygous for the mutated allele have a different phenotypic expression compared to wild-type individuals. To this end, we calculated the 95% non-parametric bootstrap CIs (based on 5000 replicates) for both plumage traits of each locus homozygous for the wild-type allele (Table 1). We then checked whether melanin-based trait values of homozygous mutated individuals were included or not within the CIs of the phenotypic distribution of homozygous wild-type individuals.

The two plumage traits were not significantly associated ($r = -0.06$, $n = 94$, $p = 0.58$), suggesting that ventral colouration and spottiness may be controlled by different pathways. SNP variants were not significantly associated with ventral colouration (all $p > 0.22$) nor spottiness (all $p > 0.89$). However, plumage trait values for some rare homozygous variants (homozygous mutated < four individuals) were outside the 95% confidence intervals of the phenotypic distribution of wild-type homozygous individuals (ventral plumage colouration: loci *MC1R* 78C>T; 291G>A; 513C>T; 717A>G; -50C>A; spottiness: loci *MC1R* 513C>T; 717A>G; Table 1), suggesting that rare SNP variants may be associated with extreme

melanin-based plumage phenotypes. Unfortunately, formal statistical testing was not feasible due to the very small number of homozygous genotypes for the mutated allele at these loci.

Plumage variation of male lesser kestrel might be partly age-related, as in other raptor species (Dreiss et al. 2010; Lopez-Idiaquez et al. 2016). Since the exact age of individuals included in our study was unknown, we could not test for age effects on expression of plumage traits. However, in the subset of males recaptured during the subsequent year (2017), ventral plumage colouration was significantly darker (paired-samples t-test, $t_{20} = -2.93$, $p < 0.01$) and spottiness significantly lower ($t_{16} = 3.60$, $p < 0.01$). Trait expression was significantly positively correlated between years (ventral plumage: $r = 0.48$, $n = 21$, $p = 0.03$; spottiness: $r = 0.81$, $n = 17$, $p < 0.001$). Among-year changes explained ca. 7% of the variance in both plumage traits (variance of year effect calculated from mixed models with bird identity as random factor according to Nakagawa and Schielzeth 2013).

To date, bird studies have investigated the role of *MC1R* in determining discrete colour morphs, while the association between *MC1R* sequences variation and continuous traits received less attention (Bourgeois et al. 2016; San-Jose et al., 2015). Our findings suggest that inter-individual variation in continuous melanin-based plumage traits is not strongly explained by SNPs in the coding and UTR regions of these two candidate genes, despite some rare mutations were associated with extreme phenotypes. Large intra-specific variation in male melanin-based plumage colouration may thus mostly depend on local changes in expression of genes involved in the melanogenesis pathway, variations in other genes (e.g. Bourgeois et al. 2017) or epigenetic modifications of gene sequences, as well as a combination of these possibilities (San-Jose and Roulin 2017). Future studies might also evaluate whether aging operates as a regulator of the expression of melanogenesis pathway genes.

Acknowledgements

We thank C. Simon for assistance with laboratory work and L. M. San-Jose for useful suggestions. We also thank two anonymous reviewers for useful suggestions. Study from the Naumanni76 team (paper#03).

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Figure 1. Patterns of inter-individual variation in ventral plumage colouration and breast and underwing black spot-coverage. Standardized pictures show the extremes of ventral plumage colouration (A: light; B: dark) and spottiness (PCA scores) (C: low; D: high). Histograms show the variability of the two traits.

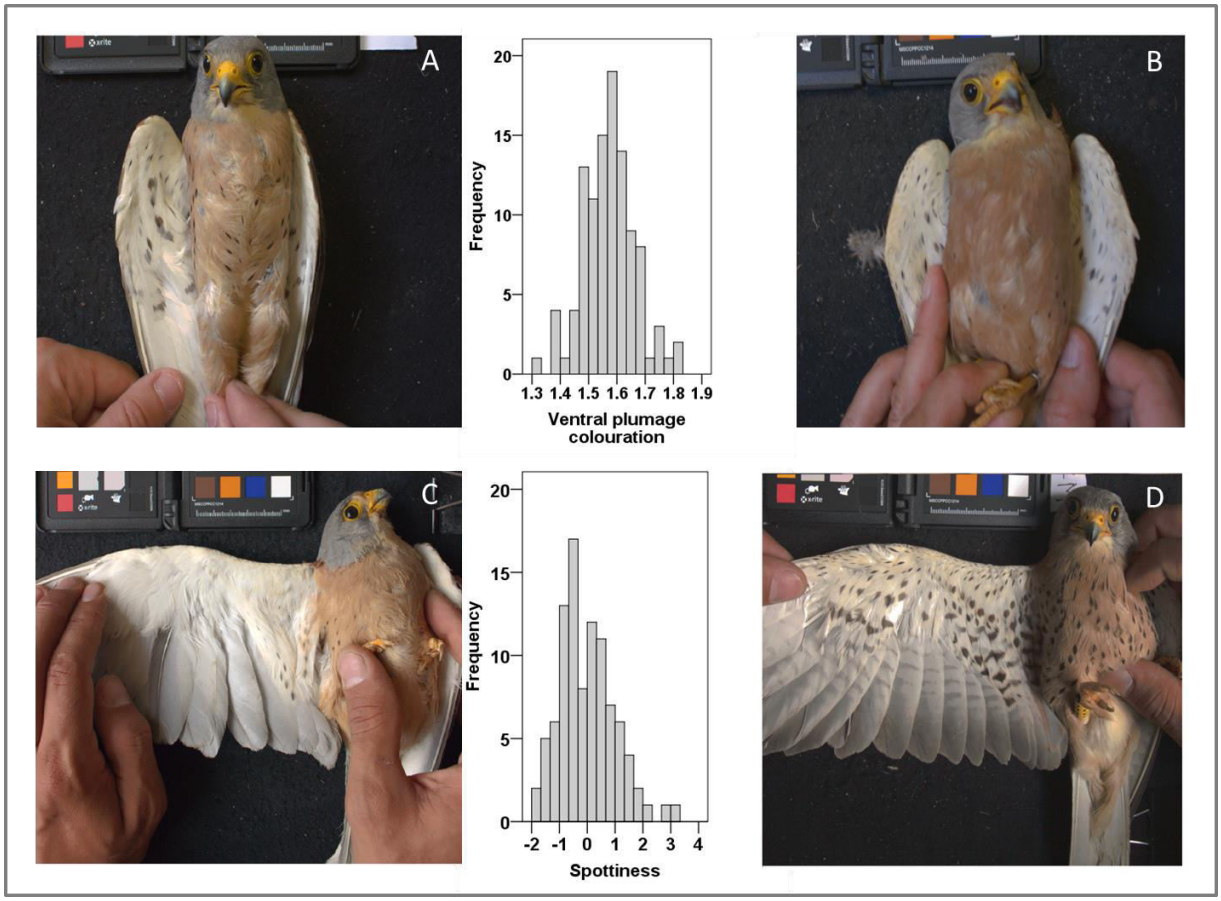

Table 1. Variation in ventral plumage colouration and spottiness of male lesser kestrels according to single-nucleotide genetic polymorphisms at *MC1R* and *TYRP1*. Mean value, sample size (round brackets) and non-parametric bootstrap 95% confidence interval (square brackets) of ventral plumage colouration and spottiness are reported for homozygous wild-type and homozygous mutated individuals (ventral plumage colouration: higher values denote darker reddish colouration; spottiness: higher values denote greater spot coverage and larger spots; see main text for details of scoring). Phenotypic data for homozygous mutated are shown as individual values or as the mean value (with confidence intervals) if sample size is >3. SNP names include the position of the mutation relative to the ATG start codon (i.e. -50 indicates that the SNP is located 50 bp before the ATG start codon). Homozygous mutated individuals that could be considered phenodeviant for a given trait (i.e. values that lay outside the 95% confidence interval) are highlighted in boldface.

	Ventral plumage colouration		Spottiness	
SNP	Homozygous wild-type	Homozygous mutated	Homozygous wild-type	Homozygous mutate
MC1R -50 C>A	1.58 (78) [1.56 – 1.60]	**1.38**	0.04 (78) [-0.24 – 0.26]	0.08
MC1R 78 C>T	1.58 (92) [1.55 – 1.59]	**1.40; 1.49**	-0.02 (92) [-0.25 – 0.14]	0.12; **0.21**
MC1R 291 G>A	1.58 (97) [1.56 – 1.59]	**1.51**	0.02 (97) [-0.24 – 0.15]	**-1.48**
MC1R 513 C>T	1.57 (101) [1.56 – 1.59]	**1.49**	-0.002 (101) [-0.22 – 0.19]	**-0.31**
MC1R 516 C>T	1.57 (96) [1.55 – 1.59]	1.58 (4) [1.45 – 1.71]	0.01 (96) [-0.26 – 0.15]	**-0.52**; 0.12
MC1R 717 A>G	1.57 (103) [1.56 – 1.59]	**1.70**	-0.02 (103) [-0.21; 0.20]	**1.83**
MC1R 735 C>T	1.58 (86) [1.56 – 1.60]	1.54 (13) [1.50 - 1.59]	0.06 (86) [-0.22 – 0.21]	0.33 (11) [-0.69 - 0.02]
MC1R 765 C>T	1.58 (95) [1.56 – 1.59]	1.55 (7) [1.43 - 1.66]	-0.02 (95) [-0.22 – 0.20]	0.16 (6) [-0.52 - 0.83]
TYRP1 exon 2 -66 G>A	1.57 (83) [1.55 – 1.59]	1.56; **1.74**	0.08 (83) [-0.18 – 0.26]	0.14
TYRP1 exon 2 -128 C>T	1.57 (87) [1.56 – 1.59]	1.56	0.04 (87) [-0.22 – 0.25]-	-[a]

a: spottiness data was missing for the single homozygous mutated individual at this locus (see Table S2).

Table S1. Primers used for PCR amplification and DNA sequencing. T_m is the annealing temperature used in PCR reactions; 'Frag. Size' is the expected size of the amplified PCR fragment in bp. For the PCR amplification of *MC1R* CDS, 10 ng of genomic DNA was performed using 1x Go Taq buffer (Promega, Dübendorf, Switzerland), 1x Q solution (Qiagen, Hombrechtikon, Switzerland), 200 µM dNTPs, 2.5 mM $MgCl_2$, 500 nM each primers and 0.5U of GoTaq (Promega) in 20 µl final volume with the following cycle conditions: 95°C 5min, followed by 36 cycles at 94°C 30 sec, 61°C 30 sec, 72°C 1 min, and 72°C 10 min. The PCR fragments were checked on 1.5 % agarose gel and sent to Microsynth or GATC (Microsynth, Balgach, Switzerland, GATC, Konstanz, Deutschland) for purification and Sanger sequencing. For *MC1R* 5'*UTR*, *TYRP1* exons, the PCR protocols were similar but Q solution was not used and the annealing temperatures (T_m) are reported in Table S1. Primers for exon 3 to 8 of *TYRP1* correspond to primers of exon 2 to 7 described in Cortimiglia et al. (2017).

Gene region	Forward primer (5'-3')	Reverse primers (5'-3')	T_m	Frag. size
MC1R CDS	GACCATGTCGACGCTGGC	GTCCCGCTGCCTACCAGGAG	61°C	955
*MC1*R-5'UTR	GCACTGCGGAAGCACTGGT	CAGATGAGCATGTCGATGAAGT TGT	60°C	444
TYRP1-exon 1	TTGGCCTGATCTTGGAAATG CT	TCAGCAGTTCCCGTCTGTGTTC	60° C	600
TYRP1-exon 2	AGGGTTGGGAAAAAGGGTT CA	AGGGAACTGCATGATGGTGGAT	62°C	450

Table S2. Location of the identified single nucleotide polymorphisms within *MC1R* and *TYRP1* genes. The position of amino acid in the polypeptide and corresponding amino acid name are also reported (with ATG start codon position being +1). Number of sequenced individuals (N), homozygous wild-type (N_0), heterozygous (N_1) and homozygous mutated (N_2) is shown. For mutation *MC1R* 612C>G or C>T, we excluded from the analyses the rare heterozygous 'CT' (N = 6 individuals).

	SNP position from start codon	Amino acid position	Amino acid	N	N_0	N_1	N_2	$\%N_2$
MC1R								
	-50 C>A	-	-	102	78	23	1	0.98
	-21 C>T	-	-	102	94	8	0	0.00
	-17 C>T	-	-	102	94	8	0	0.00
	78 C>T	26	Ala	105	92	11	2	1.90
	291 G>A	97	Leu	105	97	7	1	0.95
	310 C>T	104	Leu	105	98	7	0	0.00
	408 C>T	136	Ile	105	97	8	0	0.00
	438 C>T	146	Tyr	105	93	12	0	0.00
	513 C>T	171	Thr	105	101	3	1	0.95
	516 C>T	172	Val	105	96	5	4	3.81
	612 C>G or C>T	204	Leu	105	88	10	1	0.95
	627 C>T	209	Phe	105	98	7	0	0.00
	628 G>A	210	Ala>Thr	105	103	2	0	0.00
	681 C>T	227	Thr	105	99	6	0	0.00
	717 A>G	239	Thr	105	103	1	1	0.95
	735 C>T	245	Gly	105	86	5	14	13.33
	765 C>T	255	Phe	105	95	3	7	6.67
TYRP1 intron 1 exon2								
	-66 G>A	-	-	104	83	19	2	1.92
	-128 C>T	-	-	104	87	16	1	0.96
	-307 G>A	-	-	104	101	3	0	0.00
	-367 T>A	-	-	104	90	14	0	0.00
	-411 G>A	-	-	104	98	6	0	0.00

Part II

Protoporphyrin-based eggshell pigmentation

Chapter 5

Protoporphyrin-based eggshell pigmentation is associated with female plumage colouration and predicts offspring sex ratio in the barn swallow

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Under Review in Journal of Avian Biology

Protoporphyrin-based eggshell pigmentation is associated with female plumage colouration and predicts offspring sex ratio in the barn swallow

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Abstract

Inter- and intra-specific variation in eggshell colouration has long fascinated evolutionary biologists. Among species, such variation may accomplish different functions, the most obvious of which is camouflage and background matching. Within species, it has been proposed that inter-female variation in eggshell pigmentation patterns can reflect egg, maternal or paternal traits and hence may provide cues to conspecifics about egg, maternal or paternal phenotypic quality. However, the relationship between protoporphyrin-based eggshell pigmentation and egg or maternal/paternal traits appears to be highly variable among species. We investigated patterns of intraspecific variation in Eurasian barn swallow (*Hirundo r. rustica*) protoporphyrin-based eggshell pigmentation, and analysed its association with egg and clutch characteristics, maternal/paternal phenotypic traits and parental feeding effort. Eggshell pigmentation pattern was repeatable in clutches laid by the same females in different years, but it was not significantly associated with egg traits (including position in the laying sequence, size, yolk testosterone concentration and antioxidant capacity). It was weakly or non-significantly associated with female (health status, reproductive success, clutch characteristics, survival) and male traits (sexual ornaments), and did not significantly predict parental nestling feeding effort. However, females with darker breast plumage colouration (a melanin-based trait related to fitness) laid highly protoporphyrin-covered eggs, suggesting the presence of a previously unappreciated link between protoporphyrin biosynthesis and plumage melanisation. Moreover, the proportion of male offspring increased in clutches originating from highly protoporphyrin-covered eggs, suggesting that parents could acquire visual cues about their future brood sex composition before egg hatching. Our results support the idea that intraspecific signalling via eggshell pigmentation is a species-specific rather than a general feature of avian taxa.

Keywords: eggshell colouration, female quality, *Hirundo r. rustica*, melanin-based colouration, parental investment, protoporphyrin, SSECH.

Introduction

Birds are unique among amniotes to have evolved a pigmented eggshell showing huge among-species variation. Two different pigments determine eggshell pigmentation: biliverdin, an antioxidant pigment producing blue-green colouration, and protoporphyrin IX, a pro-oxidant pigment resulting in brownish colouration of egg maculation and spots (Kennedy & Vevers, 1976). Several non-mutually exclusive hypotheses have been proposed to explain the evolution of the large variability observed in eggshell pigmentation (Kilner et al., 2006; Cassey et al., 2010). Eggshell pigmentation can accomplish a variety of adaptive functions depending on the life-history traits of individual species, such as camouflage and crypsis (Sanchez et al., 2004; Stoddard et al., 2011; Lovell et al., 2013), resistance to bacterial penetration (Fargallo et al., 2014), structural compensation for eggshell thinning caused by calcium deficit (Solomon, 1997; Gosler et al., 2005), egg recognition (Davies & Brooke, 1989; Soler et al., 2000), and/or increasing detectability to parents in cavity-nesters (von Haartman, 1957; Solomon, 1997; Cherry & Gosler, 2010). In addition, the so-called 'Sexually Selected Eggshell Colouration Hypothesis' (SSECH) argues that in species with biparental care eggshell pigmentation might reliably signal female quality and/or the quality of eggs and of the future progeny to the social mate (Moreno & Osorno, 2003). According to this hypothesis, the social partner can modulate parental investment according to the information conveyed by eggshell pigmentation (Moreno & Osorno, 2003). The SSECH has received more attention for biliverdin-based than protoporphyrin-based egg colourations: since biliverdin is an antioxidant, females could show their antioxidant capacity by depositing a larger amount of blue-green pigment on their eggs (Moreno & Osorno, 2003; Soler et al. 2005; Moreno et al., 2006, 2008; Hargitai et al., 2008; but see Krist & Grimm, 2007; Hanley & Doucet, 2009; Honza et al. 2011). Studies that have tested the SSECH on species laying protoporphyrin-coloured eggs of passerine species have obtained conflicting results (Martínez-de la Puente et al., 2007; Sanz & García Navas, 2009; Holveck et al., 2012;

Krištofík et al., 2013; Giordano et al., 2015; Hargitai et al., 2016a,b; Poláček et al. 2016). Contrary to biliverdin, protoporphyrin is a pro-oxidant compound and its accumulation in the liver induces oxidative stress in females (Afonso et al., 1999). This is why a larger amount of protoporphyrin released on the eggshell (i.e. resulting in more, bigger and/or darker spots) may indicate two opposite individual states: 1) high female ability to sustain high levels of circulating pro-oxidants and thus a higher capacity of the antioxidant system, indicating a female of high quality and in good health (i.e. showing better maternal breeding performance in terms of larger maternal effects and/or larger parental care; Moreno & Osorno, 2003); or 2) female poor health state and low maternal investment, as high eggshell protoporphyrin may indicate female inability to remove this harmful molecule (Moreno & Osorno, 2003).

Protoporphyrin-based eggshell pigmentation may also reflect egg quality, but evidence is conflicting (Martínez-de la Puente et al., 2007; Sanz & García Navas, 2009; Holveck et al., 2012; Krištofík et al., 2013; Giordano et al., 2015; Hargitai et al., 2016a,b; Poláček et al. 2016). Sanz & García Navas (2009) showed that darker eggs were laid by high quality blue tit females (*Cyanistes caeruleus*) and that males modulated their provisioning rates relying on eggshell pigmentation pattern. Similarly, males invested more in offspring hatched from more pigmented clutches in the tree sparrow (*Passer montanus*) (Poláček et al. 2016). In this species, eggshell pigmentation reflected egg and offspring quality (i.e. darker eggs had bigger volume and offspring hatched from these were heavier) but did not provide any information about maternal phenotype (Poláček et al., 2016). In addition, a positive relationship between the distribution of the spots of protoporphyrin-based pigments on the eggshell and the antioxidant capacity of both yolk and female blood was reported in the great tit (*Parus major*) (Giordano et al. 2015). In contrast, blue tit females laying darker eggs were in poorer conditions because they had higher levels of stress protein (HSP70) and lower levels of circulating immunoglobulins (Martínez-de la Puente et al. 2007). Similar findings have been reported in another study on the great tit, where females laying darker eggs had more

circulating lymphocytes and a paler yellow breast plumage, both regarded as signals of 'poor quality', and that darker eggs had a lower yolk antioxidant concentration (Hargitai et al. 2016a).

Finally, female eggshell pigment deposition may depend on condition or ornament expression of their mates (Hargitai et al., 2008; Martínez-Padilla et al., 2010; Holveck et al., 2012). In a study of the collared flycatcher (*Ficedula albicollis*), females mated with 'low quality' males produced eggs with higher biliverdin content, suggesting compensatory egg maternal investment by females mated with unattractive males (Hargitai et al., 2008). Higher eggshell biliverdin levels may in fact be a proxy of yolk lutein content (Hargitai et al., 2008) or of other important egg components (i.e. testosterone, López-Rull et al., 2008; carotenoids and vitamin E, Navarro et al., 2011) that may boost offspring fitness from 'low quality' fathers. Moreover, in the Eurasian kestrel (*Falco tinninculus*) females mated with males in better conditions laid highly protoporphyrin-pigmented eggs (Martínez-Padilla et al., 2010).

In this study we investigated whether the protoporphyrin-based eggshell pigmentation pattern was related to the quality of the eggs and/or to the quality of the females in an European population of the barn swallow (*Hirundo r. rustica*), a socially monogamous passerine whose eggs vary in distribution, intensity, spot size and coverage of red-brownish eggshell protoporphyrin spots, which often concentrate around the blunt tip of the egg. Even if in the study subspecies (*H. r. rustica*) only females incubate eggs, males regularly visit and inspect nests during egg laying and incubation, implying that they have the possibility to evaluate egg characteristics.

The aims of our study were fourfold. First, we described the inter- and intra-clutch variability of barn swallow eggshell pigmentation and checked whether eggshell pigmentation reliably reflects individual characteristics by describing consistency among successive breeding seasons in the eggshell pigmentation pattern. Second, we tested whether the eggshell pigmentation pattern was related to egg quality. To this end, since maternal effects can occur

through variation in egg mass, macro-constituents (e.g. albumen content) and quantitatively minor components that may affect post-natal performance (Blount et al., 2002; Christians, 2002; Bonisoli-Alquati et al., 2007, 2008; Groothuis & Schwabl, 2008; Navara & Mendonça, 2008; Rubolini et al. 2012; Parolini et al., 2017a), we analysed the relationship between eggshell pigmentation and egg mass, yolk/albumen ratio, yolk Total Antioxidant Capacity (TAC) and yolk testosterone concentration. Third, we tested whether eggshell pigmentation pattern was related to female quality-related traits. According to previous studies (Hargitai et al., 2016a; Poláček et al., 2016), female characteristics were categorized as follows: 1) health status during the egg laying period (haematocrit, plasma TAC, ectoparasite load); 2) expression of plumage traits (tail length, melanin-based breast plumage colouration), which may indicate 'intrinsic' female quality (Romano et al., 2017a,b); 3) 'reproductive success traits' (total number of eggs laid per breeding season, seasonal hatching success); 4) 'clutch characteristics' (laying date, clutch size and nestling sex ratio); and 5) individual survival until the following year. Fourth, we tested whether females adjusted their eggshell pigment deposition according to male sexual ornament (tail length, melanin-based breast plumage colouration; Romano et al 2017a) expression and whether male partners modulated their level of parental investment (nestling feeding rates, either absolute or relative to female feeding rates) according to variation in eggshell pigmentation. We refrain from formulating predictions concerning the relationships between protoporphyrin-based eggshell pigmentation and egg/female/male traits because of the contrasting results previously obtained in other species.

Materials and methods

Study species and field procedures

We studied barn swallows breeding in 9 colonies (farms) located near Milan (Italy) during 2015-2016. The same colonies were followed since 2010. Females lay 1-3 clutches of 1-7 eggs (modal size: 5), one egg per day, and incubate them for approximately 14 days after the laying of the penultimate egg (Møller, 1994). Hatching occurs over 24–36 h (Saino et al., 2001) and both parents feed the offspring until the fledging at 18-20 ca. days (Møller 1994).

During both breeding seasons, we captured breeding adults using mist-nets. This procedure guarantees efficiently that very few breeding individuals (<5%) escape from captures (details in Saino et al., 1999). Barn swallows show extremely high breeding philopatry, implying that individuals that have bred in a site in their first reproductive year do not move to another site to breed the next one (Møller, 1994; Saino et al., 1999). Because of high philopatry and capture efficiency, individuals that were found for the first time in any given year at the colony can be considered at their first breeding season (yearlings: coded as age 0), while individuals that were captured at the colony where they already bred the previous year(s) were aged as old individuals (adults: coded as age 1). We have also captured adults during the breeding season of 2017 in order to collect information about the survival of individuals still alive in 2016. All the captured individuals were subjected to standard measurements, including the length of the outermost tail feathers, a well-known trait under sexual selection in our population (Møller, 1994; Romano et al., 2017a). We also plucked 2-3 feathers from the left paramedial breast plumage region to quantify its colouration by spectrometric analyses (see below). Breast plumage colouration is a sexually selected trait in the barn swallow (Safran et al. 2005; Romano et al., 2017a), even if its role in our study subspecies seems weak (Costanzo et al., 2017; Parolini et al., 2017b; Romano et al. 2017a). As a proxy of ectoparasite load, we counted the number of holes in wing and tail feathers

produced by chewing lice (Order Mallophaga; hereafter 'ectoparasite load'; Saino et al., 2012, 2014). Before releasing birds, we univocally marked them with coloured plastic rings in order to identify breeding pairs during observations at the nests (for details, see Saino et al., 1999), and we collected a small blood sample in a capillary tube (ca. 90 µl) by puncturing their brachial vein. The blood was kept cold until centrifugation (13,000 rpm for 10 minutes), which occurred on the same day of its collection. After centrifugation, we measured haematocrit (proportion of red blood cells over the total amount of blood), separated plasma and red blood cells and stored them at -80 °C until analyses of non-enzymatic total antioxidant capacity (see below).

All the nests in the farms were inspected at least once per week in order to record breeding events and to record clutch size and hatching success. The total number of laid eggs was computed as the sum of the size of all clutches laid by females during the entire breeding season. We chose this parameter as the best proxy for seasonal fecundity because nestling mortality is typically very low in our study areas (<5% of the nestlings in broods that produce any nestlings; our pers. obs. on the focal study population; Saino et al, 2017; see also Møller, 1994; Turner, 2006). Hence, clutch size strongly positively predicts seasonal breeding success (Møller, 1994). Clutch hatching success was computed as the number of hatched nestlings divided by the number of eggs in the clutch. The seasonal hatching success was computed as the number of the total hatched nestlings divided by the number of the total eggs laid in a breeding season.

At the completion of the first clutch of each females (+2 days at most), we took a photo of all eggs. The eggs were safely removed from the nest and placed in the soft bottom of a box with an ID tag and a ruler. We photographed each clutch in a standardized way using the camera of an iPhone 6 (Apple Inc., CA, USA) from a distance of 18 cm in order to evaluate eggshell pigmentation (see below). Moreover, in a subset of 20 clutches from 2015, we marked eggs according to the laying sequence in order to test for possible variation in

eggshell pigmentation according to the laying order. In addition, from those clutches we also collected eggs to be subjected to laboratory analyses (see below). In order to minimize the impact of egg collection on the reproductive output of single individuals and breeding colonies, we only collected the 4th laid egg on the day of laying. On the same day, all the eggs were weighted. Yolk was separated from the albumen, weighted, divided in two aliquots and stored at -20 °C until TAC and steroid analyses (see below).

As an estimate of male parental investment, we computed the absolute and relative number of male feeding visits in 49 nests during 2015. Parental nestling feeding effort was quantified on videos recorded with a Sony DCR-SR72E camera placed in front of the nest and fixed on a tripod (see Romano et al., 2015b for details on video-recording on entire barn swallow broods). For each nest, videos of two hours were recorded in the morning (between 6:00 a.m. and 10:00 a.m.) at two different ages: at 8 ± 1 (video 1) and 11 ± 1 (video 2) days after the hatching of the first egg(s) of the clutch. The number of feeding visits performed by both parents was counted on video recordings using VLC Media Player 2.2.1 (Free Software Foundation, Inc., Boston, MA). The number of feeding visits of both parents was correlated between the two trials (number of mother feeding visits: $r = 0.48$; number of father feeding visits: $r = 0.59$; all $p < 0.01$; $N = 49$). In the analyses, we therefore used the mean value of male and female feeding visits between the two trials and the ratio between these two mean values (relative male feeding rate).

On day 12 ± 2 days after hatching, we safely removed all nestlings from their nests to collect a small blood sample (ca. 40 µl) with capillary tubes, which was used for molecular sex determination after polymerase chain reaction (PCR) amplification of the sex-specific avian CHD-1 gene (Griffiths et al., 1998; Saino et al., 2008a). Offspring primary sex ratio (PSR) was expressed as the proportion of males over the total number of offspring number in the brood (for clutches where all eggs hatched).

Eggshell pigmentation scoring

Barn swallows lay eggs with a mostly white eggshell, which is finely maculated with protoporphyrin-based brownish spots. Gosler et al. (2000) proposed a qualitative visual criterion to describe great tits eggs, whose eggshell pigmentation is comparable with that of the barn swallow. The Gosler et al. (2000) criteria have been adopted to estimate the pattern of protoporphyrin-based eggshell pigmentation in other passerine species (Holveck et al. 2012; Hargitai et al., 2016a; Poláček et al., 2016). All the eggshells were scored on the pictures by the same observer (MC) using qualitative visual criteria, categorizing eggshell pigmentation according to four different components: *pigment distribution* (D), representing the pigment distribution along the surface of the egg, was scored from 1 (pigment concentrated on one tip of the egg, invariably the blunt end) to 5 (pigment equally distributed along the surface); *pigment intensity* (I), representing the colour of the pigmented area of the eggshell, was scored from 1 (pale pigment) to 5 (dark pigment); *spot size* (S) was scored from 1 (small spots) to 3 (large spots); *pigment coverage* (C), representing the amount of eggshell pigmented surface, was scored from 1 (little covered) to 5 (highly covered; Gosler et al., 2000; Hargitai et al., 2016a,b; Figure 1). Repeatability of eggshell pigmentation scores, tested on a random subset of eggs scored twice, was very high ($R_D = 0.87$; $R_I = 0.93$; $R_S = 0.82$; $R_C = 0.85$; all $p < 0.01$; $N = 105$). Since different eggshell pigmentation scores may convey different information (e.g. Martínez-de la Puente et al., 2007; Giordano et al., 2015; Hargitai et al., 2016a; Poláček et al., 2016) and the four scores of eggshell pigmentation were weakly or moderately correlated in our sample (Pearson's correlation coefficient r: pigment distribution and pigment intensity = -0.02; pigment distribution and pigment coverage = 0.27; pigment distribution and spot size = -0.13; pigment intensity and pigment coverage = -0.20; pigment intensity and spot size = -0.13; pigment coverage and spot size = 0.45; N = 568 eggs), we decided to consider the four scores as separate variables, as done in previous studies (e.g. Hargitai et al., 2016a,b). Moreover, because of the moderate intra-clutch repeatability of

eggshell pigmentation scores (ICC_D = 0.46, 95% CL = 0.38-0.55; ICC_I = 0.57, 95% CL = 0.49-0.64; ICC_S = 0.39, 95% CL = 0.30-0.48; ICC_C = 0.39, 95% CL = 0.30-0.48; N = 125 clutches; ICC values calculated according to Wolak et al., 2012) and because the inclusion of every single egg in the analyses would have implied to differently weigh each female based on her clutch size, in most analyses we used the clutch average of each eggshell pigmentation score.

Total antioxidant capacity of yolk and female plasma

We measured the non-enzymatic TAC in the plasma of a sub-sample of females (N = 65) and in the sample of homogenized yolk of collected eggs (N = 20) according to the colorimetric method developed by Erel (2004). Plasma was used as it is, while an appropriate amount of yolk (~ 0.1 g), was homogenized in 100 mM phosphate buffer pH 7.4, with 1 mM EDTA and 100 mM KCl, by an automatic homogenizer. After 10 min centrifugation at 13,000 rpm, a small aliquot of the supernatant was immediately processed for TAC analysis. Briefly, the colour of 2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{*+}$) bleaches depending on the concentration of antioxidants in the sample. The reaction is monitored spectrometrically at λ = 750 nm and the final absorbance is inversely related to TAC of samples. We calibrated the reaction by drawing a standard curve with serial dilution of Trolox and the results were expressed as μM Trolox equivalent for plasma and in mM Trolox equivalent/g yolk for egg yolk.

Yolk testosterone assay

Yolk testosterone was extracted from homogenized yolk samples (N = 20) with a double ether extraction followed by liquid column chromatography according to methods described by Schwabl (1993). Briefly, 50 mg of yolk was weighed and vortexed with 1000 μl of deionized water. Next, 3 mL of petroleum:diethyl ether (30:70 vol/vol) was added, the mixture was

vortexed for 30 sec and was allowed to settle for 20 min. Samples were then snap frozen and the supernatant was poured off and dried. The sample was reconstituted in 1 ml 10% ethyl acetate in isooctane and steroids were separated using celite column chromatography. Testosterone was eluted in 20% ethyl acetate in isooctane. Testosterone was quantified with a commercial enzyme immune-essay (EIA) kit (ENZO, NY, USA). Anti-testosterone had a crossreactivity of 100% with testosterone, 14.6% with 19-hydroxytestosterone, 7.20% with androstenedione, and <1% with all other steroids. Average recoveries were 97.3% and average intra-assay variation was 5.6%. Testosterone was analyzed over two assays and the inter-assay variation was 2.1%.

Spectrophotometric colour analyses

We quantified the melanin-based breast female plumage colouration by measuring the reflectance of one randomly chosen breast feather using an Avantes DH-2000 spectrometer in a dark chamber (for details see Saino et al., 2013a). A single feather per individual was chosen because its reflectance is highly correlated with that of the entire individual breast plumage region (Romano et al., 2015a). Reflectance spectra were processed to quantify colouration using tetrachromatic colour space model (Goldsmith, 1990) with TetraColorSpace program (Version 1a; Stoddard & Prum, 2008) run in MATLAB 7 (MathWorks, Natick, MA), assuming UVS cone type-retina and adopting the spectral sensitivity of the blue tit because it is the most closely related species to barn swallow for which spectral sensitivity information is implemented in TetraColorSpace program. Each colour vector in tetrahedral colour space was converted into spherical coordinates θ, φ and r (Stoddard and Prum, 2008). θ represents the red-blue-green hue component, φ the ultraviolet one while r accounts for saturation. In the barn swallow breast feather colour range, decreasing θ value and increasing *rA* value indicates breast darker colouration that is associated with higher concentration of melanins (Saino et al., 2013a). Because in our population associations between plumage

colouration and behavioural and physiological traits were observed on breast θ (Saino et al., 2013a; Romano et al., 2015a; Costanzo et al., 2017; Parolini et al., 2017b), to avoid inflating number of statistical tests and because the three colour components variables are highly correlates (Romano et al., 2015a; Corti et al., 2017) in all analyses we focused only on the θ. However, the same analyses carried out on *rA* provided qualitatively similar results (details not shown).

Statistical analyses

We tested the associations between the four eggshell pigmentation scores and female and egg quality in separate statistical models for each pigmentation score [pigment distribution (D), pigment intensity (I), spot size (S), pigment coverage (C)].

The association of the four eggshell pigmentation scores with laying order was tested in multinomial generalized linear mixed models (GLMMs), including clutch identity as random factor. The association between the four eggshell pigmentation scores and egg traits (one egg per clutch; see above) was tested in multinomial generalized linear models (GLMs). In the former models we considered as independent variable only the laying order, while in the latter ones predictors were egg mass (mg), yolk/albumen ratio, yolk TAC (mM Trolox equivalent/g yolk) and yolk testosterone (pg/mg). Multinomial models were fitted using the GLIMMIX procedure in SAS 9.1.3 (SAS Institute, 2006), assuming a multinomial error distribution and a cumlogit link function (using the cumulative logit link function, which is suitable for ordered scores; Schabenberger, 2006).

Eggshell pigmentation was recorded in 125 first clutches laid by 108 females (17 females were sampled for two years). Sample size slightly changed depending on the analysis because of practical reasons (e.g. insufficient plasma sample for TAC analyses). The association between the four eggshell pigmentation scores (average clutch value) and female quality was tested using linear mixed models (LMMs). In all models, female identity was

included as a random effect because some females were sampled for two years. Female age was included as covariate because all physiological and morphological traits can be affected by age (Møller, 1994; Turner, 2006). In 'health status' models we included as independent variables those describing female health status (haematocrit, ectoparasite load and plasma TAC; N = 66 clutches). Plasma TAC was measured on blood samples collected during captures and therefore in different moments of the breeding cycle with respect to individual laying date. In order to account for this possible confounding effects of changes in oxidative status during the breeding cycle (Rubolini et al 2012; Serra et al 2012), we included a new variable, 'relative TAC date', computed as the difference between the date of capture and the laying date of the first egg. Positive and negative values indicate that egg deposition was respectively before or after the capture date. In the 'intrinsic quality' models we included as independent variables those describing the female 'intrinsic' quality [female ornaments (breast θ, tail length), N = 110 clutches]. Since female plumage traits (tail length, breast θ) were significantly age-related (tail length increases and breast colouration becomes darker; see Results; Lifjeld et al., 2011; see also Møller, 1993, 2002), age was included in all models. Two-way interactions between tail length and breast θ with female age were also included in the 'intrinsic quality' models to investigate possible age-specific patterns of covariation between female traits and eggshell pigmentation. Interaction terms not attaining statistical significance were dropped from final models. In the 'reproductive success traits' models, we included as independent variables those describing female reproductive traits: in the first models we included the total number of eggs laid in a given breeding season (N = 112 clutches) while in the second we included the seasonal hatching success (proportion of hatched eggs on total laid eggs) weighed for the total number of laid eggs. In the latter models we did not consider the clutches where eggs were collected (N = 92 clutches). In the 'clutch characteristic' models we included as independent variables those describing female clutch traits [clutch size, hatching date and hatching success (proportion of hatched eggs on laid

eggs); N = 111 clutches]. In a subset of nests where all eggs hatched and no egg was collected (N = 44 clutches), we also included nestling PSR as independent variable. The association between female survival (survived = 1; non survived = 0) and the four eggshell pigmentation scores was tested in separate binomial GLMMs ('survival' models), with female identity as a random effect (accounting for repeated sampling of surviving females) and each pigmentation score as a predictor (N = 100 clutches).

The association between eggshell pigmentation pattern and male ornaments (tail length, breast θ) was tested, as for female quality, by LMMs including male age as covariate, as well as male and female identities as random factors, accounting for repeated sampling of individuals in different breeding seasons (N = 82 clutches). The two-way interactions between tail length and breast θ with male age were also included in initial models to test for age-specific patterns, and were dropped if not significant. Because male plumage ornaments (tail length, breast θ) were significantly age-related (see Results; Møller, 1988; Lifjeld et al., 2011), to assess their independent effect on eggshell pigmentation pattern we performed models with the same design separately for each variable (see Results).

The associations between parental investment and eggshell pigmentation pattern were tested in 49 nests for which we video-recorded parental nestling feeding behaviour. Using Gaussian GLMs, we investigated whether eggshell pigmentation scores (one separate model for each score) predicted male feeding visits, female feeding visits or relative male feeding rate (expressed as mean value of male and female feeding visits between the two trials). We added brood size as an additional independent variable in all models because parental feeding rates are known to increase with brood size (e.g. Costantini et al., 2014).

Gaussian GLMs were fitted using the glm R software function, while LMMs and the binomial GLMM of survival were run using the lme4 (Bates et al., 2012) and lmerTest (Kuznetsova et al., 2015) packages of the R software (vers. 3.4.0; R Development Core Team,

2012). GLMMs were not overdispersed (overdisperal parameters < 1.16). Overdispersion was calculated using the blmeco package (Korner-Nievergelt et al., 2015).

Results

Eggshell pigmentation variability

There was broad variation in each of the four eggshell pigmentation scores (Figure 2). Eggshell pigmentation scores showed a moderate but significant intra-clutch similarity (see Materials and methods for details). Moreover, eggshell pigmentation scores were highly correlated between eggs laid by the same females in different years ($r_D = 0.66$; $r_I = 0.74$; $r_S = 0.62$; $r_C = 0.75$; all $p < 0.01$; N = 17 females), indicating that females were highly consistent among years in their eggshell pigmentation pattern, which may thus represent a reliable indicator of female characteristics over the long term.

Relationship between eggshell pigmentation and egg traits

Eggshell pigmentation scores were not significantly associated with egg laying order (multinomial GLMMs; D: $F_{3,51} = 0.06$, $p = 0.98$; I: $F_{3,51} = 0.60$, $p = 0.62$; S: $F_{3,53} = 0.63$, $p = 0.60$; C: $F_{3,51} = 1.30$, $p = 0.28$). Moreover, eggshell pigmentation scores were not significantly predicted by egg mass, yolk/albumen ratio, yolk TAC or yolk testosterone (Table 1).

Relationship between eggshell pigmentation and female traits and survival

We did not find any significant association between eggshell pigmentation pattern and female health status (Table 2). Controlling for age-related changes in female plumage trait expression [mixed models of female traits with age as predictor and female identity as a random effect, tail length: estimate (SE) = 3.85 (0.84), $F_{4,65} = 20.84$, $p < 0.001$, N = 113; breast θ; estimate (SE) = -0.06 (0.01), $F_{1,82} = 33.60$, $p < 0.001$, N = 110], eggshell pigmentation scores were not significantly associated with tail length (Table 2). However, females with lower θ values (darker breast plumage colouration) laid eggs with higher pigmentation coverage (Table 2). Other eggshell pigmentation scores did not significantly predict breast θ (Table 2). Two-way

interactions between female traits and age were not significant and removed from the model (all p-values > 0.07).

In the 'reproductive traits' models, we found that adult females laid eggs characterized by a more homogeneous (higher pigment distribution) eggshell pigmentation. However we did not find any significant association between eggshell pigmentation pattern and the total number of laid eggs or the seasonal hatching success (Table 3).

We did not find any significant association between eggshell pigmentation pattern and female clutch characteristics (Table 4). However, PSR significantly increased in clutches originating from highly covered eggs (Figure 3) and characterized by bigger eggshell spots (i.e. larger pigmentation spot size; Table 4). Qualitatively similar results were found if the latter model was run using secondary sex ratio instead of PSR, disclosing also a positive association between more homogeneous eggs (larger pigment distribution) and secondary SR (N = 84 clutches) [LMM, estimate (SE) = 0.74 (0.29), $F_{1,49} = 6.52$, p = 0.014; other model details not shown].

We did not find any significant association between eggshell pigmentation pattern and female survival (Table 5).

Relationship between eggshell pigmentation, male ornaments and male parental investment

Controlling for age-related changes in male plumage ornament expression [mixed models of male traits with age as predictor and male identity as a random effect, tail length: estimate (SE) = 4.14 (1.01), $F_{1,48} = 16.62$, p < 0.001, N = 108; breast θ: estimate (SE) = -0.03 (0.01), $F_{1,82} = 7.36$, p = 0.008, N = 85], we found that females laid eggs with bigger spots when mated with males displaying paler breast plumage colouration (higher θ value; Table 6). Other eggshell pigmentation scores were not significantly associated with male ornaments (Table 6). Two-way interactions between male traits and age were non-significant and were removed from the models (all p-values > 0.17).

Males did not modulate their parental investment according to eggshell pigmentation, both in terms of absolute and relative number of feedings provided to their nestlings (Table 7). Female nestling feeding effort was also not significantly related to eggshell pigmentation (Table 7). In all models, parental effort strongly increased in relation to brood size, as expected (e.g. Costantini et al., 2014).

Discussion

In this study, we investigated patterns of protoporphyrin-based eggshell pigmentation variation in the Eurasian barn swallow, with the aim of describing the extent of intraspecific variability, the repeatability of eggshell pigmentation scores between different reproductive events, and of elucidating whether eggshell pigmentation may act as a reliable cue of egg and/or female traits that males could use to adjust their parental investment accordingly. We found broad variation in eggshell pigmentation, and we observed that females laid eggs with a similar pattern of eggshell pigmentation in different years, suggesting that eggshell pigmentation pattern can be a consistent and reliable indicator of female quality traits. However, we found few statistically significant associations between eggshell pigmentation pattern and egg and female characteristics, and we did not find evidence that eggshell pigmentation pattern could affect male brood provisioning behaviour. Below we briefly discuss the main findings of the study.

First, none of the eggshell pigmentation scores was significantly associated with any of the egg traits we considered, including yolk TAC and testosterone, two constitutively minor compounds that may mediate important maternal effects (see Introduction). Hence, there was no evidence that protoporphyrin-based eggshell pigmentation reliably reflected egg traits that could be exploited by male partners to evaluate the quality of the female investment in a clutch. Similarly, egg traits were not significantly related with female quality traits, with the single exception of breast melanin-based colouration (see below). Although we cannot rule out that eggshell pigmentation could significantly predict other fitness-related egg component (e.g. immunoglobulins or other immune factors), these findings are consistent with the lack of significant adjustment of male parental effort in nestling provisioning to eggshell pigmentation. Indeed, in a male's perspective, the information content of eggshell pigmentation appears negligible. For instance, males are exposed to different sources of information about their mate quality, both before and after laying, during incubation and the

hatching period (Stoddard et al. 2011). Males may thus adopt other indicators of female quality, which are easier to perceive and are more informative than eggshell pigmentation pattern, such as feeding behaviours or ornaments, in order to modulate their feeding investment. Similarly to our findings, Stoddard et al. (2011) reported that, in great tits, male parents did not adjust nestling provisioning visits to the eggshell pigmentation pattern that they have observed during incubation.

We found that females with darker melanin-based breast colouration laid highly spot-covered eggs. In the barn swallow, melanin-based breast plumage colouration is a well-known male ornament, with darker males being more sexually attractive than paler ones (Romano et al., 2017a). This is especially the case for the Nearctic subspecies *H. r. erythrogaster*, where males with darker breast plumage colour show a greater reproductive success than lighter conspecifics (Safran & McGraw, 2004; Safran et al., 2005). However, the sexual signalling role of breast plumage colour variation is less clear in our study subspecies (Costanzo et al. 2017; Parolini et al., 2017b). Despite this, melanin-based breast plumage colouration seems related to a suite of life-history traits, such as immune response (Saino et al., 2013b), viability (Saino et al., 2013c), and natal dispersal (Saino et al., 2014). The association we found between eggshell pigmentation coverage and darker melanin-based breast plumage colouration is likely to be indirect. For instance, genes controlling melanin-based colouration, that are well known to have broad physiological interactions (Ducrest et al. 2008), may have cascading effects on those physiological pathways that lead to an increase of protoporphyrin IX levels in the oviduct shell gland, hence promoting protoporphyrin deposition on the eggshell (higher pigment coverage score). However, the lack of association between eggshell pigmentation coverage, a proxy of a larger amount of protoporphyrin in the eggshell, and the other female traits we considered (tail length and age) do not lend support to the idea that higher eggshell pigmentation coverage could be a signal of high 'intrinsic' female quality.

The significant association between the proportion of male offspring in the clutch and higher eggshell pigment coverage is intriguing, as it suggests that male partners (and females as well) can acquire information about sex composition of their brood already during the pre-hatching period, which may provide them a cue to tune their parental decisions. Our findings, coupled with the observation that parents can obtain information about the sex of their future nestlings from the emissions of volatile compounds of the embryonated eggs before hatching (Costanzo et al., 2016), suggests that parents have the opportunity to acquire information on sex ratio of their own future brood by both visual and olfactory cues. For barn swallow, the production of sons implies a larger investment compared to daughters since male nestlings are not only more susceptible to harsh environmental conditions (Bonisoli-Alquati et al., 2008; Saino et al., 2008b), but also solicit parental care with a more intense begging activity compared to their sisters (Boncoraglio et al., 2008), which may translate into a greater parental workload. Whether such visual and olfactory information is actually exploited by parents to maximise their fitness is not known. It might be speculated that, by acquiring insights into the sex composition of the future brood already at the clutch stage, parents may be able to better modulate resource allocation to self-sustainment in the pre-hatching period in order to compensate for the expectedly greater energy expenditure required to care for a male-biased brood.

The combination of associations between eggshell pigment coverage and female melanin-based breast colouration, and between eggshell pigment coverage and sex ratio is consistent with a previous study on the same population carried out over different years, in which it was observed that, across a period of several years, sex ratio was predicted by darker maternal breast melanin-based colouration (Romano et al., 2015a). On the proximate side, these findings suggest a link between the physiological mechanisms (i.e. hormonal) linking plumage coloration, eggshell pigmentation pattern and ovulation of eggs of either sex, likely mediated by broad pleiotropic effects of the gene networks regulating melanin biosynthesis

(melanocortin system; for details see Ducrest et al., 2008). Such genes controlling plumage colouration, are indeed know to affect hormonal state, influencing sexual behaviour, aggressiveness, exocrine gland activity, stress response, immune function and energy homeostasis (Ducrest et al., 2008), which in turn are known to affect sex ratio of avian species (Roulin et al., 2010; Romano et al., 2015a) and possibly protoporphyrin-based egg pigmentation through as yet undescribed physiological links (see above). This result is supported also by the fact that females maintain the same eggshell pigmentation pattern throughout the years.

Finally, the significant association between spot size and breast θ suggests the possibility that females may adjust eggshell pigmentation according to male ornaments. However, this finding is difficult to interpret because of the lack of detectable associations between eggshell spot size and egg traits.

To summarize, in spite of the large variability in eggshell pigmentation and repeatable eggshell patterning across years by the same females, eggshell pigmentation seems to not be significantly related to egg quality and female quality in barn swallows. Notwithstanding, our study highlighted a significant association between female breast colouration and eggshell pigmentation, suggesting the existence of a previously unappreciated link between biosynthesis of protoporphyrin IX and plumage melanisation. In addition, together with female breast colouration, eggshell pigmentation pattern may constitute a reliable proxy of both primary and secondary brood sex ratio. Despite males not changing their level of brood parental investment according to eggshell pigmentation, acquiring visual cues about brood sex ratio before egg hatching may let them better prepare for provisioning a highly energy-demanding male-biased brood. Further experiments, whereby clutches differing in pigmentation are cross-fostered while male condition and foraging behaviour is accurately monitored, may provide a test of the hypothesis that cues about brood sex ratio are actually exploited by partners to tune their reproductive effort or condition to an expected greater

workload after hatching. Hence, we can safely conclude that eggshell pigmentation does not have any major function of signalling egg or female characteristics to mates in the study species, supporting the idea that intraspecific signalling through eggshell pigmentation is a species-specific trait rather than a general pattern across avian taxa.

Acknowledgements: We thank C.D. Possenti, S. Podofillini, and G. Bazzi for assistance with field and laboratory work.

Permit: The study was authorized by Regione Lombardia (Decreto n° 2959). The study was conducted in private lands, and land owners gave us the permission to access their farms. No approval by Animal Ethics Committee was required for the present experimental protocol.

Funding: The authors received no specific funding for this work.

Author contributions: MC, AR, NS, DR conceived the study; MC, AR, AC, NS, DR collected field data; MC, ABB, KJN, MP performed laboratory analyses; AC performed colour analysis; MC, AR, DR carried out the statistical analyses; MC, AR, DR drafted the manuscript. All authors gave final approval for publication.

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Figure 1. Variation of the protoporphyrin-based eggshell pigmentation pattern in barn swallow eggs according to visual criteria (see Gosler et al., 2000). Pigment intensity (I) scored from 1 (pale pigment) to 5 (dark pigment); pigment distribution (D) scored from 1 (pigments concentrated on one tip of the egg, invariably the blunt end) to 5 (pigment equally distributed along the surface); pigment coverage (C) scored from 1 (little pigmented) to 5 (highly pigmented) and spot size (S) scored from 1 (small spots) to 3 (large spots). Rows represent increasing values from top to bottom.

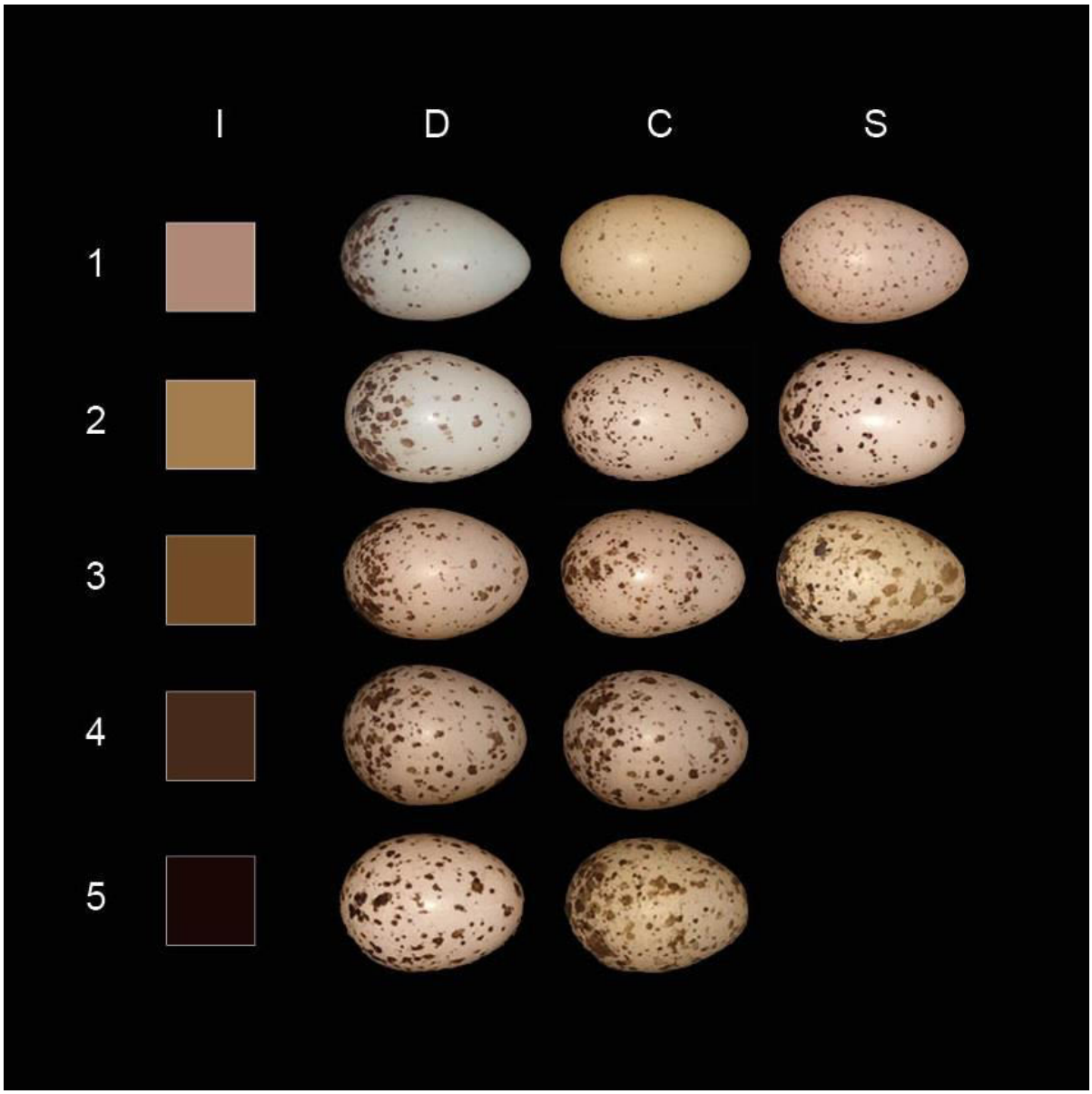

Figure 2. Frequency distribution of the four eggshell pigmentation scores.

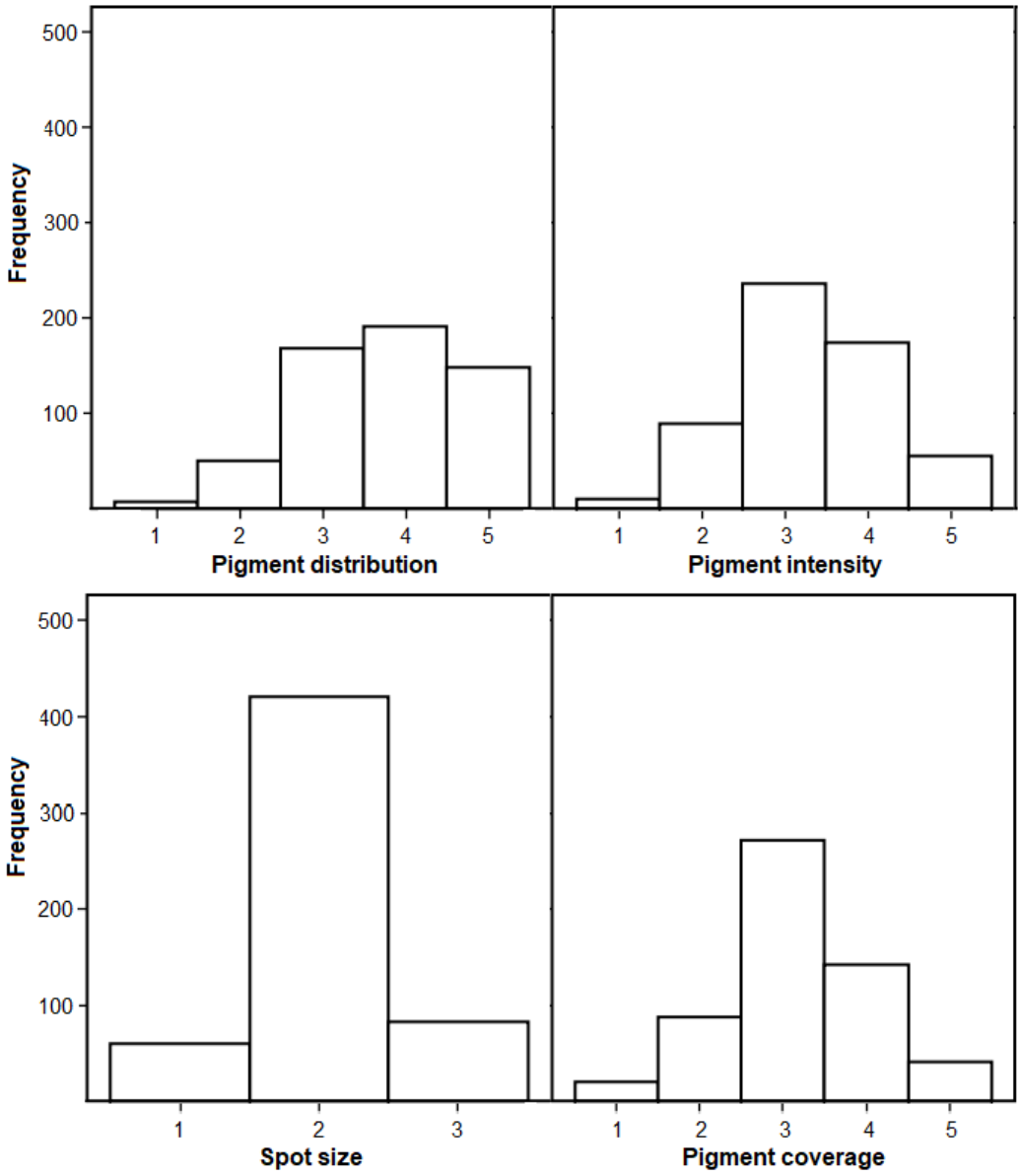

Figure 3. Association between eggshell pigment coverage score (from 1, little covered to 5, highly covered; average clutch value) and offspring primary sex ratio (PSR; proportion of males over the total number of offspring). The simple regression line is shown for illustrative purposes.

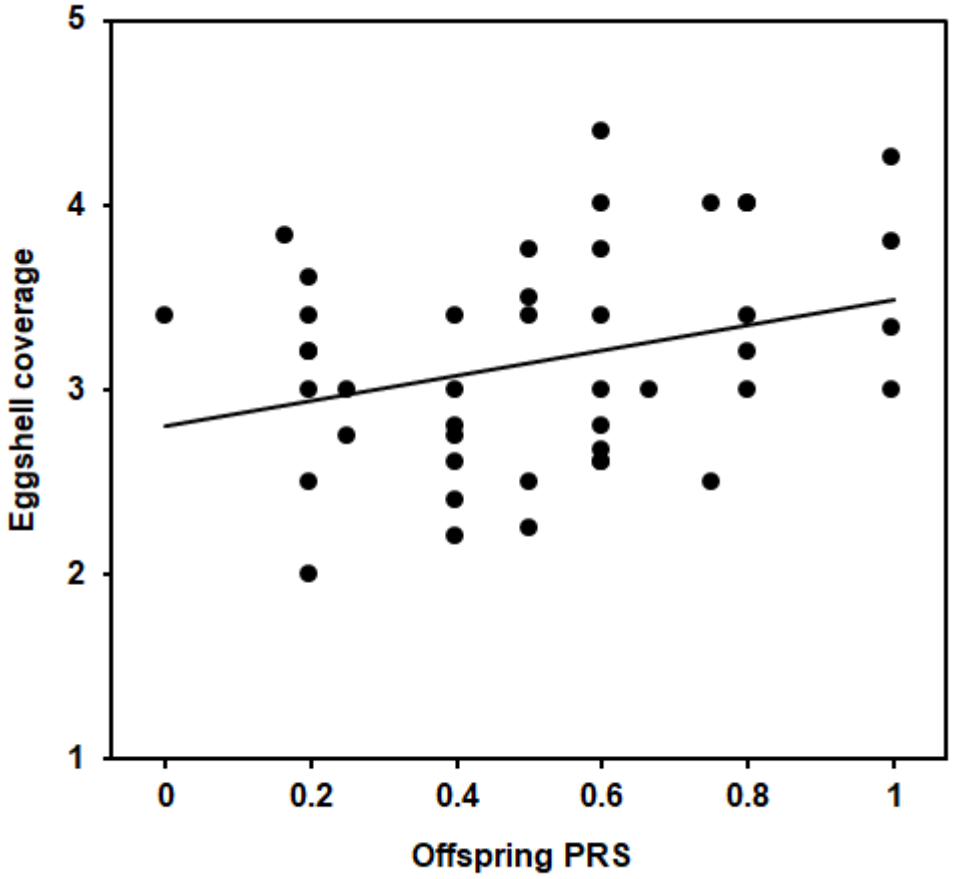

Table 1. Multinomial general linear models of the association between the four eggshell pigmentation scores and egg traits (N = 20 eggs).

		Estimate (SE)	df	F	p
Pigment Egg mass		0.01 (0.01)	1, 13	0.26	0.62
distribution	Yolk/albumen ratio	4.49 (9.53)	1, 13	0.22	0.65
	Yolk TAC	-0.14 (0.08)	1, 13	3.41	0.09
	Yolk testosterone	0.06 (0.05)	1, 13	1.61	0.23
Pigment	Egg mass	0.01 (0.01)	1, 13	0.29	0.60
intensity	Yolk/albumen ratio	0.77 (9.19)	1, 13	0.01	0.93
	Yolk TAC	0.03 (0.07)	1, 13	0.22	0.64
	Yolk testosterone	-0.06 (0.05)	1, 13	1.40	0.26
Spot size	Egg mass	-0.01 (0.01)	1, 14	1.78	0.20
	Yolk/albumen ratio	4.76 (13.01)	1, 14	0.13	0.72
	Yolk TAC	0.19 (0.11)	1, 14	2.64	0.13
	Yolk testosterone	-0.01 (0.07)	1, 14	0.01	0.94
Pigment	Egg mass	-0.01 (0.01)	1, 13	1.39	0.26
coverage	Yolk/albumen ratio	11.81 (9.50)	1, 13	1.55	0.24
	Yolk TAC	0.07 (0.07)	1, 13	0.88	0.37
	Yolk testosterone	0.02 (0.05)	1, 13	0.20	0.67

Table 2. Linear mixed models of the association between the four eggshell pigmentation scores (average clutch value) and female health status and 'intrinsic' quality, controlling for female age and timing of TAC sampling relative to start of egg laying (relative TAC date; see Methods). Female identity was included as a random factor in all models (see Methods), In the 'intrinsic quality' models, two-way interactions between tail length and breast θ with female age were removed because did not attain statistical significance (p-value always > 0.07). Statistically significant effects are marked with asterisks.

		Estimate (SE)	df	F	p
Health status (N = 66 clutches)					
Pigment distribution	Haematocrit	-2.12 (2.78)	1, 56	0.58	0.45
	TAC	-0.01 (0.01)	1, 52	1.19	0.28
	Relative TAC date	0.01 (0.01)	1, 52	1.39	0.24
	Ectoparasite load	-0.01 (0.02)	1, 55	0.09	0.76
	Age	0.30 (0.17)	1, 52	3.33	0.07
Pigment intensity	Haematocrit	2.86 (1.99)	1, 16	2.07	0.17
	TAC	-0.01 (0.01)	1, 17	0.94	0.35
	Relative TAC date	-0.01 (0.01)	1, 16	4.65	0.047*
	Ectoparasite load	-0.01 (0.01)	1, 16	4.08	0.06
	Age	0.04 (0.12)	1, 16	0.14	0.72
Spot size	Haematocrit	1.17 (1.46)	1, 34	0.64	0.43
	TAC	0.01 (0.01)	1, 29	0.70	0.41
	Relative TAC date	-0.01 (0.01)	1, 29	0.13	0.72
	Ectoparasite load	-0.01 (0.01)	1, 33	0.01	0.94
	Age	0.02 (0.09)	1, 30	0.05	0.83
Pigment coverage	Haematocrit	-1.88 (2.59)	1, 44	0.53	0.47
	TAC	0.01 (0.01)	1, 39	0.88	0.35
	Relative TAC date	0.01 (0.01)	1, 40	1.71	0.20
	Ectoparasite load	0.01 (0.01)	1, 44	0.01	0.93
	Age	-0.14 (0.15)	1, 40	0.25	0.36
Intrinsic quality (N = 110 clutches)					
Pigment distribution	Tail length	-0.02 (0.01)	1, 100	2.05	0.16
	Breast θ	-1.13 (1.15)	1, 105	1.25	0.27
	Age	0.14 (0.15)	1, 105	0.86	0.36
Pigment intensity	Tail length	-0.01 (0.01)	1, 105	0.04	0.84
	Breast θ	-1.10 (1.20)	1, 105	0.85	0.36
	Age	-0.08 (0.16)	1, 95	0.29	0.59
Spot size	Tail length	0.01 (0.01)	1, 100	0.05	0.83
	Breast θ	-0.28 (0.56)	1, 106	0.30	0.58
	Age	-0.05 (0.08)	1, 105	0.13	0.72
Pigment coverage	Tail length	-0.02 (0.01)	1, 101	1.74	0.20
	Breast θ	-2.11 (1.05)	1, 106	4.02	0.047*
	Age	-0.25 (0.14)	1, 104	3.10	0.08

Table 3. Linear mixed models of the association between the four eggshell pigmentation scores (average clutch value) and female reproductive success traits (see Methods). Female identity was included as a random factor in all models (see Methods). In all models, we controlled for female age (details not shown for brevity).

		N	Estimate (SE)	df	F	p
Pigment distribution	Seasonal N of eggs laid	112	-0.06 (0.03)	1, 108	3.64	0.06
	Hatching success	92	-0.05 (0.46)	1, 75	0.14	0.90
Pigment intensity	Seasonal N of eggs laid	112	0.03 (0.03)	1, 107	0.71	0.40
	Hatching success	92	0.76 (0.45)	1, 61	3.04	0.09
Spot size	Seasonal N of eggs laid	112	-0.02 (0.01)	1, 109	1.66	0.20
	Hatching success	92	-0.19 (0.23)	1, 83	0.76	0.39
Pigment coverage	Seasonal N of eggs laid	112	-0.02 (0.03)	1, 109	0.38	0.54
	Hatching success	92	-0.40 (0.39)	1, 70	1.05	0.31

Table 4. Linear mixed models of the association between eggshell pigmentation scores (average clutch value), female clutch characteristic and nestling primary sex ratio (PSR), controlling for female age. Female identity was included as a random factor in all models (see Methods). Statistically significant effects are marked with asterisks.

		Estimate (SE)	df	F	p
Clutch characteristics (N_{clutch} = 111)					
Pigment distribution	Clutch size	0.12 (0.11)	1, 99	0.96	0.33
	Hatching date	0.01 (0.01)	1, 104	0.77	0.38
	Hatching success	0.63 (0.42)	1, 100	2.24	0.14
	Age	0.26 (0.18)	1, 105	2.14	0.15
Pigment intensity	Clutch size	-0.02 (0.12)	1, 74	0.02	0.87
	Hatching date	0.01 (0.01)	1, 78	0.29	0.59
	Hatching success	0.65 (0.41)	1, 77	2.50	0.12
	Age	0.02 (0.18)	1, 85	0.03	0.87
Spot size	Clutch size	-0.10 (0.06)	1, 97	2.93	0.09
	Hatching date	0.01 (0.01)	1, 102	0.09	0.76
	Hatching success	-0.14 (0.20)	1, 99	0.54	0.46
	Age	-0.01 (0.08)	1, 104	0.01	0.94
Pigment coverage	Clutch size	0.04 (0.11)	1, 86	0.14	0.71
	Hatching date	-0.01 (0.01)	1, 92	0.41	0.52
	Hatching success	0.23 (0.38)	1, 89	0.38	0.54
	Age	-0.25 (0.16)	1, 96	2.48	0.12
Primary SR (N_{clutch} = 44)					
Pigment distribution	Clutch size	0.08 (0.18)	1, 39	0.23	0.63
	Hatching date	0.01 (0.01)	1, 37	1.50	0.23
	Age	0.75 (0.28)	1, 37	7.43	0.010*
	PSR	0.61 (0.40)	1, 32	2.31	0.14
Pigment intensity	Clutch size	0.01(0.16)	1, 13	0.01	0.93
	Hatching date	0.01 (0.01)	1, 39	1.05	0.31
	Age	0.05 (0.31)	1, 37	0.02	0.88
	PSR	-0.07 (0.27)	1, 4	0.06	0.82
Spot size	Clutch size	0.08 (0.09)	1, 39	0.81	0.37
	Hatching date	0.01 (0.01)	1, 39	0.79	0.38
	Age	-0.10 (0.14)	1, 39	0.56	0.46
	PSR	0.49 (0.21)	1, 39	5.57	0.023*
Pigment coverage	Clutch size	0.13 (0.15)	1, 29	0.71	0.41
	Hatching date	-0.01 (0.01)	1, 39	0.02	0.89
	Age	-0.20 (0.25)	1, 39	0.62	0.44
	PSR	1.02 (0.29)	1, 8	12.29	0.009*

Table 5. Generalized linear mixed models of the association between female survival (survived = 1; non survived = 0) and the four eggshell pigmentation scores (average clutch value). Female identity was included as a random factor in all models (see Methods). Models were not overdispersed (overdispersion < 1.16).

	Estimate (SE)	***Z***	**p**
Survival (N = 100)			
Pigment distribution	0.31 (0.28)	0.11	0.91
Age	0.20 (0.42)	0.48	0.63
Pigment intensity	0.38 (0.27)	1.38	0.17
Age	0.26 (0.42)	0.61	0.54
Spot size	-0.76 (0.60)	-1.26	0.21
Age	0.16 (0.42)	0.38	0.71
Pigment coverage	-0.06 (0.31)	-0.18	0.86
Age	0.20 (0.41)	0.47	0.64

Table 6. Linear mixed models of the association between eggshell pigmentation scores (average clutch value) and male ornaments (tail length and breast plumage colouration) controlling for male age (N = 82 clutches). Male and female identity were included as random factors in all models (see Methods). Two-way interactions between tail length and breast θ with male age were removed because did not attain statistical significance (p-value always > 0.17). Statistically significant effects are marked with asterisks.

		Estimate (SE)	**df**	**F**	**p**
Pigment distribution	Tail length	-0.01 (0.01)	1, 77	0.18	0.67
	Breast θ	-0.35 (1.32)	1, 10	0.07	0.80
	Age	0.05 (0.15)	1, 9	0.12	0.74
Pigment intensity	Tail length	-0.02 (0.01)	1, 77	2.57	0.11
	Breast θ	-0.82 (0.75)	1, 68	1.20	0.28
	Age	0.11 (0.07)	1, 43	3.05	0.09
Spot size	Tail length	0.01 (0.01)	1, 77	3.46	0.07
	Breast θ	1.49 (0.64)	1, 37	5.47	0.025*
	Age	0.04 (0.07)	1, 35	0.38	0.54
Pigment coverage	Tail length	0.01 (0.01)	1, 71	0.45	0.50
	Breast θ	-0.88 (1.41)	1, 70	0.39	0.54
	Age	-0.23 (0.16)	1, 69	2.18	0.14

Table 7. Linear models of the association between eggshell pigmentation scores (average clutch value) and parental feeding effort, measured as mean value of male feeding visits between two trials and relative male feeding rate (N = 49 broods).

		Estimate (SE)	t	p
Mean male feeding visits	Pigment distribution	0.22 (1.05)	0.21	0.84
	Brood size	4.00 (0.86)	4.66	<0.001
	Pigment intensity	-0.08 (1.12)	-0.07	0.95
	Brood size	4.05 (0.83)	4.85	<0.001
	Spot size	-0.81 (1.80)	-0.45	0.66
	Brood size	4.05 (0.83)	4.87	<0.001
	Pigment coverage	0.11 (1.16)	0.09	0.93
	Brood size	4.03 (0.87)	4.65	<0.001
Mean female feeding visits	Pigment distribution	0.13(0.93)	0.14	0.89
	Brood size	3.24 (0.76)	4.25	<0.001
	Pigment intensity	0.07 (0.99)	0.07	0.95
	Brood size	3.27 (0.74)	4.43	<0.001
	Spot size	-0.48 (1.60)	-0.30	0.77
	Brood size	3.26 (0.74)	4.43	<0.001
	Pigment coverage	1.27 (1.01)	1.26	0.21
	Brood size	3.01 (0.75)	4.00	<0.001
Relative male feeding rate	Pigment distribution	0.01 (0.07)	0.18	0.86
	Brood size	0.07 (0.06)	1.33	0.19
	Pigment intensity	-0.04 (0.07)	-0.55	0.59
	Brood size	0.08 (0.05)	1.40	0.17
	Spot size	0.04 (0.12)	0.34	0.73
	Brood size	0.08 (0.05)	1.42	0.16
	Pigment coverage	0.004 (0.08)	0.05	0.96
	Brood size	0.08 (0.06)	1.35	0.18

Chapter 6

Parental quality and protoporphyrin-based eggshell pigmentation in a biparental raptor species: a food supplementation experiment

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In Submission

Parental quality and protoporphyrin-based eggshell pigmentation: a food supplementation experiment in a biparental raptor species

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Abstract

According to the 'sexually selected eggshell colouration' hypothesis, intraspecific variation in avian eggshell pigmentation could be a post-mating sexually selected signal, as it may convey information about the quality of females and their eggs to mates, and/or reflect differential allocation by females according to male quality. We evaluate whether protoporphyrin-based eggshell pigmentation is a reliable signal of female quality, clutch and egg traits and whether it reflects mate quality traits in the biparental lesser kestrel (*Falco naumanni*). In this species, males perform mate feeding, and their provisioning effort is a key determinant of female condition during the pre-laying/laying period. More pigmented eggshells were assumed to reflect a greater egg investment by females because high protoporphyrin eggshell release could only be sustained by females in prime health state. We experimentally manipulated the female's perception of male quality by providing females with extra food during egg laying. Food supplementation did not significantly affect parental body condition. However, females mated with males showing brighter grey head plumage (an indicator of male condition) laid more pigmented eggs, suggesting that females may adjust eggshell pigmentation according to male quality. Eggshell pigmentation was not significantly affected by food supplementation, but eggs of larger clutches were more pigmented. Within clutches, more pigmented eggs had greater hatching success. Our findings support the hypothesis that protoporphyrin-based eggshell pigmentation reflects clutch and egg quality and that eggshell pigmentation reflect high female egg investment according to perceived male quality in a species where mate feeding behaviour affect female condition.

Keywords: eggshell pigment intensity, food supplementation, hatching success, lesser kestrel, male quality, laying order.

Introduction

The large inter- and intraspecific variation in eggshell pigmentation patterns has long been fascinating evolutionary biologists. At the inter-specific level, several hypotheses have been proposed to explain such variation. Interspecies differences in egg pigmentation may indeed be related to egg camouflage and crypsis (Solis and de Lope 1995; Sanchez et al. 2004; Stoddard et al. 2011), eggshell strength (Gosler et al. 2005), brood parasitism (Stokke et al. 2002), egg recognition (Davies and Brooke 1989; Soler et al. 2000), and/or function to increase detectability of eggs by parents in cavity-nesters (von Haartman 1957; Solomon 1997; Cherry and Gosler 2010). Two main pigments are responsible for eggshell pigmentation: the antioxidant blue-green biliverdin and the pro-oxidant reddish-brown protoporphyrin IX, the latter usually arranged in maculation and spots over the eggshell (Kennedy and Vevers 1976).

Since release of pigments on the eggshell may entail costs for laying females (Moreno and Osorno 2003; Soler et al. 2008), it was hypothesized that, for species where both sexes contribute to parental care, inter-female variation in eggshell pigmentation patterns could function as a post-mating sexually selected signal about the quality of the female and/or her eggs, and that males may this signal to modulate their parental investment accordingly ('Sexually Selected Eggshell Colouration Hypothesis', SSECH). Due to the different features of biliverdin and protoporphyrin pigments, biliverdin-based eggshell pigmentation has received more attention than protoporphyrin-based pigmentation in the last decades (Moreno and Osorno 2003; Soler et al. 2005; Moreno et al. 2006, 2008; Hargitai et al. 2008; Giordano et al. 2015; but see Krist and Grimm 2007; Hanley and Doucet 2009; Honza et al. 2011). While the antioxidant nature of biliverdin makes predictions about the relationship between female/egg quality and eggshell pigmentation straightforward (higher eggshell antioxidants are expected to be related to high quality females/eggs), protoporphyrin is a pro-oxidant.

Hence, its accumulation on the eggshell may indicate two opposite individual states of the laying female: (1) a higher ability to sustain elevated levels of circulating pro-oxidants and thus a higher capacity of her antioxidant system to withstand the oxidative cost associated with the deposition of this pigment on the eggshell ('high quality hypothesis') or (2) her inability to remove these harmful compounds from her organism, indicating poor physiological condition ('low quality hypothesis'; Moreno and Osorno 2003). To date, support for each of these two hypotheses is mixed. For instance, high quality blue tit (*Cyanistes caeruleus*) females laid more protoporphyrin-pigmented eggs and males increased provisioning efforts when rearing clutches hatched from more pigmented eggs (Sanz and García Navas 2009). Similarly, in tree sparrows (*Passer montanus*) more protoporphyrin-pigmented eggs had larger volume and males invested more in offspring hatched from those eggs (Poláček et al. 2017). By contrast, female great tits (*Parus major*) laying more protoporphyrin-pigmented eggs had relatively more circulating lymphocytes and a paler yellow plumage, both of which are indicators of poor health and low individual quality (Hargitai et al. 2016).

Variation in protoporphyrin-based eggshell pigmentation may also reflect variation in the quality of the egg and, hence, the reproductive value of the clutch. However, previous studies testing this hypothesis have obtained conflicting results (Holveck et al. 2012; Krištofík et al. 2013; Hargitai et al. 2016). Indeed, Holveck et al. (2012) found no association between eggshell spot pattern and yolk carotenoid concentration in blue tits, but they found that eggs with larger and more concentrated spots contained more antibodies. Krištofík et al. (2013) reported that more pigmented reed warbler (*Acrocephalus scirpaceus*) eggs were more resistant to microbial infections, while Hargitai et al. (2016) reported that great tit eggs with darker spots had a lower yolk antioxidant concentration, suggesting a lower egg quality.

Finally, variation in protoporphyrin-based eggshell pigmentation may be related to the quality of the mate, whereby females may invest differentially in eggshell pigmentation when

mated with males differing in quality. Differential eggshell pigmentation investment according to male traits has been documented in blue tits (Sanz and García-Navas 2009) and in the Eurasian kestrel (Martínez-Padilla et al. 2010), suggesting that females laid more protoporphyrin-pigmented eggs if paired with prime-condition males, whereas no effects of male traits on protoporphyrin-based eggshell pigmentation was found in barn swallows (*Hirundo rustica*) (Corti et al. submitted). However, in the collared flycatcher (*Ficedula albicollis*), whose females lay blue-green eggs, females mated with 'low quality' males laid eggs with higher biliverdin content, suggesting that females compensated for lower quality of their mates by laying higher quality eggs (Hargitai et al. 2008).

Many raptor species show mate-feeding behaviour during the breeding season, whereby female condition during pre-laying and laying periods largely depends on male feeding efforts, and thus from mate quality (Newton 1979; Lundberg and Alatalo 1992; Martínez-Padilla et al. 2010; Village 2010). The quantity/quality of food brought by their mate can be used by females in mate choice decisions (Mougeot et al. 2002), or it can directly affect female egg laying effort and egg quality (Donázar et al. 1992). In species with mate-feeding behaviour, female allocation decisions to reproduction may thus be expected to covary with direct indicators of male quality, such as male provisioning ability, either via an effect on female body condition or because it can function as an indicator of male quality. Hence, besides being possibly related to 'intrinsic' (i.e. genetic, such as colouration) indicators of male quality, eggshell pigmentation may depend on male provisioning ability. In this context, Martínez-Padilla et al. (2010) investigate patterns of protoporphyrin eggshell deposition in food-supplemented and non-supplemented pairs of the Eurasian kestrel (*Falco tinnunculus*). Eggshell pigmentation was associated with higher hatching success and egg mass in food-supplemented females, although food supplementation did not significantly affect eggshell protoporphyrin deposition. Moreover, male (rather than female) body condition was the strongest predictor of eggshell pigmentation (Martínez-Padilla et al. 2010),

suggesting that eggshell pigmentation is more related to male intrinsic quality rather than to (experimentally altered) provisioning ability.

We report the results of a food supplementation experiment of the lesser kestrel (*Falco naumanni*), a diurnal raptor whose eggs broadly vary in the intensity of reddish-brown protoporphyrin-based eggshell pigmentation (Figure 1). The lesser kestrel is sexually dimorphic, showing marked sex differences in melanin-based plumage traits: males show grey head, rump and tail feathers and black spots on breast and underwing coverts, while females are mostly brownish (Tella et al. 1997). Both sexes present broad inter-individual variation in plumage traits, as females could display male-like plumage traits, with brownish to greyish rump and tail colouration, while males show variable brightness in grey head feathers and a high variability in breast and underwing spots, which may vary between absent and very abundant (Tella et al. 1997; Corti et al. submitted; our unpublished data). We aim at investigating the associations between protoporphyrin-based eggshell pigmentation and parental quality, clutch and egg traits. Since parental quality may affect protoporphyrin-based eggshell pigmentation, by means of a food supplementation experiment, whereby some pairs were food-supplemented during egg laying while others did not receive food and served as controls, we aimed at altering the perception of male quality by females. Indeed, in the lesser kestrel, male mate-feeding behaviour seems to have a crucial role in determining female condition during pre-laying and laying period, allowing females to lay earlier and/or larger clutches (Donazár et al. 1992). Moreover, male contribution to parental care is high, since males incubate eggs and feed nestlings (Cramp 1998).

First, we investigated whether food supplementation affected female and male body condition, and eggshell pigmentation. We then tested the association between protoporphyrin-based eggshell pigmentation and parental 'intrinsic' quality considering different plumage traits and parental body condition. To our knowledge, the role of plumage colour traits as reliable indicators of 'intrinsic' quality has never been investigated in the lesser kestrel.

However, in females, we assumed that a greater degree of plumage eumelanization (i.e. a greyer rather than browner plumage colouration, see Prota 1992; Hearing 1998; Fargallo et al. 2007) indicated females of higher 'intrinsic' quality (Ducrest et al. 2008; Roulin and Ducrest 2011), similarly to the closely-related Eurasian kestrel, where females with greyer rump plumage colouration showed better body condition and increased innate immunity (Vergara et al. 2009; Parejo et al. 2011). In males, we measured the brightness of head feathers as a potential signal of male 'intrinsic' quality to females (males in better body condition displayed brighter head plumage; see Materials and methods).

Secondly, we explored the associations between protoporphyrin-based eggshell pigmentation and clutch and egg traits, such as clutch size, laying date, nestling sex ratio, egg mass, laying order and egg hatching success.

According to previous work, we expected: 1) that food-supplemented females laid eggs with greater protoporphyrin-based eggshell pigmentation, both because of improved body condition (and consequent reduction of the costs of protoporphyrin eggshell deposition) and because food supplementation should simulate higher foraging efforts by their mates, potentially inducing a greater egg investment by the female; 2) a positive association between protoporphyrin-based eggshell pigmentation and parental traits denoting high 'intrinsic' quality; hence, we expected more pigmented eggs to be laid by greyer females, by females in better body condition, by females paired with better body condition males and/or with males displaying brighter head plumage; 3) a positive association between intensity of protoporphyrin-based eggshell pigmentation and egg/clutch traits associated with high fitness, such as clutch size, egg size and/or egg hatching success.

Materials and methods

Study species and field procedures

The lesser kestrel is a small (ca. 120 g) diurnal raptor, feeding on invertebrates, lizards and small mammals (Rodríguez et al. 2010). During the pre-laying and laying period, females are mostly fed by males (Donázar et al. 1992). Females lay one clutch of 2-8 eggs (modal size 4) per breeding season, incubated by both parents for ca. 30 days (Cramp 1998). Extra-pair fertilization occur at a relatively low rate (Negro et al., 1996).

The present study was carried out in April-July 2016 in the breeding colony of the city of Matera (Southern Italy; 40°67' N, 16°60' E). We performed a food supplementation experiment involving 100 nestboxes. The content of nestboxes was checked every 2 days. Starting from laying of the first egg of each clutch, in a sample of nestboxes we simulated high food availability by putting 3 dead laboratory mice (*Mus musculus*, ca. 20 g each) at the rear of the internal chamber (supplementary fed group) every 2 days until clutch completion. After clutch completion, the dose was reduced to 1 mice every 2 days, and this rate of supplementation was continued until 10 days after hatching of the first egg of a clutch. Nestboxes were randomly sequentially assigned to either the supplementary fed (N = 50) or the unfed control (N = 50) group and observation of pellets containing white fur inside the nestboxes confirmed consumption of the extra food.

When a new egg was found, it was weighted using a digital scale (± 0.1 g) and marked with a non-toxic marker according to the laying sequence. At clutch completion, we took a digital photo of all the eggs. Eggs were safely removed from the nestbox and placed in the soft bottom of a box with a unique clutch identification tag. We photographed each clutch on a standard black background using a Canon EOS 100D camera, including in each picture a colour standard (X-Rite ColorChecker Passport) according to Stevens et al. (2007). Camera was set to take pictures in RAW format.

Starting from the last 10 days of incubation, we captured adults in nestboxes. They were individually ringed with a metal and a PVC plastic ring. Body mass was recorded using a digital scale (± 0.1 g) and keel length was measured using dial calliper (± 0.1 mm). We then took pictures of the back of each female, positioning each individual on a dark background. The eumelanin-based grey colour intensity of the female rump (see Fargallo et al. 2007; eu- to pheomelanin ratio in kestrel grey feathers is 7.1) was scored on a four-class scale by the same observer (MC) based on pictures (see Fargallo et al. 2007 for a similar procedure): 1 (brownish colouration), 2 (brownish-grey colouration), 3 (greyish colouration) and 4 (dark greyish colouration) (hereafter rump grey plumage score). In order to quantify the brightness of male head plumage (see below), we collected approximately five feathers from the nape region.

At ca. 9 days after hatching, we safely removed all nestlings from their nestboxes to collect a small blood sample (ca. 200 µl) in capillary tubes. Blood samples were used for molecular sex determination after polymerase chain reaction (PCR) amplification of the sex-specific avian CHD-1 gene (Griffith, 1998). Offspring primary sex ratio (PSR) was expressed as the proportion of males over the total number of offspring in the brood (for clutches where all eggs hatched). We collected information on PSR for 31 broods (14 from the supplementary fed group and 17 from the unfed controls).

Eggshell pigmentation analyses

Egg images were analysed using the inCamera plug-in for Adobe Photoshop CS3 and their colour was corrected/standardized according to Bergman and Beehner (2008). To evaluate eggshell pigment intensity, we selected the entire eggshell area using the 'Magnetic Lasso' tool (a minimum of 200 pixels). Colour measurements were made by recording the median value of red (R), blue (B) and green (G) of the selected area using the histogram palette. Since kestrel eggs are predominantly covered with reddish-brown protoporphyrin pigment, we were

primarily interested in differences in the red component (see Martínez-Padilla et al. 2010). Because the actual value of each colour channel is only informative when compared to the values of other colour channels, as an index of reddish eggshell pigment intensity we computed the ratio of R to B (Vortman et al. 2011, 2015; hereafter, eggshell pigment intensity).

Spectrometric colour determination of male head feathers

We measured the spectral reflectance of the five head feathers collected for each male. We took three measurements from each feather, in proximity of the feather tip, along the inner web, and close to the rachis. We averaged the resulting spectra for each individual. The spectral reflectance was measured with an Ocean Optics S2000 spectrometer. The light optical-fibre probe was held at a 90° angle at a distance of 5 mm from the feather surface through a black PVC tube mounted on the ferrule tip, which also has the function to avoid any disturbance from ambient light. The reflectance spectra were recorded with the Ocean Optics OOIBase32 software with standards set to white and dark before each measurement session. We used an integration time of 300 ms, the mean of 5 spectra for each measurement, and a boxcar smoothing function of 11 pixels. Spectral analyses were performed using the *pavo* package of the R software (v. 3.3.2). We considered only wavelengths between 300 and 700 nm, as they correspond to the visible spectrum of birds that includes the UV component. Brightness and ultraviolet saturation (UV Chroma) were computed as the mean reflectance over the visible spectrum of birds and the relative contribution of the UV spectra (R300-400) on the total spectra, respectively. In the sample of males included in this study, brightness of male head plumage was significantly and positively predicted by male body condition in a linear model controlling for food supplementation [estimate: 12.88 (6.15 SE), $F_{1,52} = 4.39$, $p = 0.041$, N = 55 males], while this was not the case for UV Chroma (details not shown for brevity).

Statistical analysis

We excluded data from clutches with incomplete egg laying (clutch size < three eggs) or failed clutches (clutches with no hatched eggs). We obtained digital egg images from 75 clutches (37 from supplementary fed group and 38 from unfed one). Sample size slightly changed depending on the analysis due to practical reasons (e.g. lack of nestling blood samples, no adults captured). In order to investigate the possible differences in eggshell pigment intensity between the first egg (that was laid before food supplementation started) and all the other eggs of the clutch we ran a linear mixed model (LMM) including eggshell pigment intensity as independent variable and a factor "first vs. other eggs" (1 = first egg and 0 = the other eggs of the clutch) and food supplementation as predictors (and the two-way interaction), clutch identity being included as a random effect. Since we did not find any significant difference between eggshell pigment intensity of first laid egg vs. other eggs within the same clutch ($F_{1,259} = 0.73$, $p = 0.39$, N = 75 clutches, N = 314 eggs), we included first egg data in all models. Moreover, because of the moderate intra-clutch repeatability of eggshell pigment intensity (intra-class correlation coefficient = 0.43, 95% CI = 0.31 to 0.55, N = 75 clutches; calculated according to Wolak et al. 2012) and because the inclusion of every single egg in the analyses would have implied to differently weigh each male and female based on his/her clutch size, in most analyses we used the mean clutch eggshell pigment intensity.

We computed body condition of both males and females as the ratio between body mass and keel length. Body condition was computed only for individuals captured during incubation because: 1) exploratory analyses showed a progressive and linear decline of female (but not male) body mass during incubation, which however decreased abruptly and non-linearly from hatching onward (our unpublished data); and 2) the sample size of birds captured during incubation was larger. Overall, body condition was computed for 40 females

(25 from supplementary fed nestboxes and 15 from unfed nestboxes) and 55 males (28 from supplementary fed nestboxes and 27 from unfed nestboxes).

We first assessed, separately for each sex, whether food supplementation (0 = unfed; 1 = supplementary fed) affected body condition during incubation by a linear model with body condition as the dependent variable, and food supplementation and days since start of egg laying (to control for changes in body mass during incubation) as predictors.

Effects of male and female traits on eggshell pigment intensity were investigated separately for each sex by means of linear models including body condition (residuals of the linear model accounting for decline of body mass during incubation and treatment), rump grey plumage score and food supplementation as predictors in the female model, while in the male one we included body condition, brightness of head plumage and food supplementation. We also included UV Chroma of head plumage in the male model to control for UV reflectance, as raptors are sensitive to UV light (Lind et al. 2013).

To investigate the association between eggshell pigmentation and clutch traits, we ran a linear model of mean clutch eggshell pigment intensity where laying date (date of laying of the first egg of a clutch), clutch size, nestling PSR and food supplementation were included as predictors. We also ran the same model while excluding PSR in order to investigate the possible association between mean clutch eggshell pigment intensity and clutch traits with a larger sample size (PSR was available for only a subset of the clutches).

We evaluated the associations between egg traits and eggshell pigment intensity by means of a LMM of eggshell pigment intensity including laying date, clutch size, laying order, egg mass (see below) and food supplementation as predictors, with clutch identity as a random intercept effect. Because nestboxes were monitored three times a week, in some cases we could not determine the laying order of all eggs in a clutch. Hence, when two newly laid eggs were found in a same nestbox during the same monitoring session, we coded each egg using the mean of the expected values of the two eggs in the laying sequence (i.e. in the case

of uncertainty between the second and the third egg, we coded both eggs as 2.5). In addition, in the exploratory analyses we observed that eggshell pigment intensity varied with laying order in a non-linear fashion, which was best described by a quadratic term of laying order (details not shown for brevity). We thus included both the linear and the quadratic term of laying order in the analysis of eggshell pigment intensity of individual eggs. Because mixed models do not account for non-independence of measurement of predictors within a given level of data aggregation (e.g. clutch), to separate between- from within-clutch effects of egg mass on eggshell pigment intensity we used the approach suggested by van de Pol and Write (2009). Original egg mass values were replaced by two new variables: the mean clutch value of egg mass, computed separately for each clutch (egg mass $_{MEAN}$) and the difference between the egg mass value of each single egg and the mean of its clutch (egg mass $_{DEV}$). Positive egg mass $_{DEV}$ values thus indicate eggs whose mass is higher than the mean of its clutch.

The effect of eggshell pigment intensity on egg hatching success was assessed by a binomial generalized linear mixed model (GLMM) whereby hatching success (0 = not hatched, 1 = hatched) was included as dependent variable and egg laying date, clutch size, laying order, eggshell pigment intensity, egg mass and food supplementation as predictors. Clutch identity was included as a random intercept effect. To disentangle within- from between-clutches effects of eggshell pigment intensity on egg hatching success, we computed eggshell pigment intensity $_{MEAN}$ and eggshell pigment intensity $_{DEV}$, as specified above for egg mass.

Two-way interactions between predictors and food supplementation were included in all initial models and dropped altogether if not significant. In all models, predictors have been centred on their mean values. LMMs and GLMMs were fitted using the R *lmer* package (v. 1.1-13).

Results

Mean clutch eggshell pigment intensity, adult traits and food supplementation

Controlling for days since onset of egg laying, body condition of both males and females was not significantly affected by food supplementation, although the effect was marginally non-significant and in the predicted direction for females (i.e. food supplemented females tended to show a better body condition) [females: food supplementation, estimate = 0.02 (0.01 SE), $F_{1,37} = 3.31$, p = 0.08; days since onset of egg laying, estimate = -0.01 (0.01 SE), $F_{1,37} = 5.44$, p = 0.025; males: food supplementation, estimate = 0.01 (0.09 SE), $F_{1,52} = 0.15$, p = 0.70; days since onset of egg laying, estimate = 0.01 (0.01 SE), $F_{1,52} = 0.08$, p = 0.79]. Mean clutch eggshell pigment intensity was not significantly predicted by female body condition (expressed as residuals from the above-mentioned linear model controlling for food supplementation and days since onset of egg laying) and rump grey plumage score (Table 1). Mean clutch eggshell pigment intensity was significantly and positively predicted by male head plumage brightness, but not by male body condition or other predictors (Table 1, Figure 2), indicating that females mated with males showing brighter head plumage laid more pigmented eggs.

Mean clutch eggshell pigment intensity, clutch traits and food supplementation

Mean clutch eggshell pigment intensity was not significantly related to any of the clutch traits and was not significantly affected by food supplementation (Table 2). However, in the model excluding the non-significant PSR effect, a significant two-way interaction between laying date and food supplementation emerged (Table 2; Figure 3). The interaction showed that among unfed control females late-laid clutches were significantly darker than early laid ones [estimated slope: 0.01 (0.01 SE), $t_{74} = 2.11$p = 0.039], whereas this was not the case among supplementary fed females [estimated slope: -0.01 (0.01 SE), $t_{74} = -1.61$, p = 0.11].

Eggshell pigment intensity, egg traits and food supplementation

Eggshell pigment intensity varied in quadratic fashion with egg laying order, intermediate-laid eggs of a clutch being more pigmented than first- and last-laid ones (Table 3; Figure 4). Controlling for significant laying order effects, clutch size significantly and positively predicted eggshell pigment intensity (Table 3): larger clutches were thus composed of more pigmented eggs on average than smaller clutches. Similarly to the clutch trait model reported in Table 2, a significant two-way interaction between laying date and food supplementation was found (Table 3) (slopes were similar to those of mean clutch eggshell pigment intensity, details not shown for brevity). In addition, a significant two-way interaction between mean clutch egg mass and food supplementation emerged (Table 3): specifically, among supplementary fed females, heavier eggs were significantly more pigmented (estimated slope: 0.05 (0.02 SE), $t_{70} = 2.45$, $p = 0.017$), whereas this was not the case among unfed females (estimated slope: -0.03 (0.02 SE), $t_{70} = -1.40$, $p = 0.17$). This effect did not emerge in the clutch level model shown in Table 2, probably because that model did not account for laying order effects on eggshell pigment intensity.

Egg hatching success was significantly negatively predicted by laying order and positively by eggshell pigment intensity$_{DEV}$ (Table 4): hatching success was lower in late-laid eggs, and more pigmented eggs within-clutches hatched more frequently than paler ones. Moreover, egg hatching success was differentially associated with clutch size according to food supplementation (Table 4): parameter estimates revealed that among supplementary fed females, egg hatching success was significantly higher in larger clutches (estimated slope: 1.15 (0.53 SE), $Z = 2.17$, $p = 0.030$), whereas this was not the case among unfed females (estimated slope: -0.31 (0.57 SE), $Z = -0.54$, $p = 0.59$).

Discussion

In this study we investigated the role of protoporphyrin-based eggshell pigmentation as a signal of female quality, clutch and egg traits, and paternal traits, in the lesser kestrel, a species where males perform mate feeding and where both sexes incubate eggs and contribute to parental care when raising offspring. Based on previous evidence, we aimed at manipulating parental body condition as well as the female perception of male quality by providing extra food to parents during the egg laying period, and assessed the effects of food supplementation on protoporphyrin-based eggshell pigmentation. Food supplementation did not significantly improve parental body condition, and affected eggshell pigmentation differentially according to laying date and egg mass. However, females mated with males showing brighter head plumage (which was positively correlated with male body condition) laid more pigmented eggs. Moreover, eggshell pigmentation varied with laying sequence (i.e. intermediate-laid eggs were more pigmented than first- and last-laid ones), and more pigmented eggs within clutch had a greater probability of hatching. Below we focus on food supplementation effects on eggshell pigment intensity.

Male lesser kestrels feed their females during pre-laying and through laying period, sustaining the egg laying efforts of their mates (Donázar et al. 1992). Female condition during these periods depends mainly on male efforts to provide food resources (Newton 1979). Hence, by manipulating the amount of food received by females during egg laying [under the assumption that females are able to compare provisioning efforts of their mates compared to other available partners in the population, or that the female is able to evaluate mate provisioning in relation to available resources (Mougeot et al. 2002)], we expected to alter the perceived food provisioning ability of males, and thus their perceived ability to provide direct fitness benefits to their female mates. Our findings indicate that food supplementation affected eggshell pigment deposition depending on egg laying date and egg mass. The

significant interactions between food supplementation and laying date and between food supplementation and the mean egg mass in a clutch suggested that our experiment was able to alter the natural covariation between eggshell protoporphyrin pigmentation and laying date and egg mass. The absence of a direct food supplementation effect on eggshell pigment deposition is consistent with previous evidence from the closely-related Eurasian kestrel (Martínez-Padilla et al. 2010) and do not fully support the idea that females base their eggshell pigment allocation decision according to mate food provisioning ability. Rather, the significant interaction with laying date hints at the possibility that dietary variation (and hence environmental effects) could influence eggshell protoporphyrin-based pigmentation patterns (Jagannath et al. 2008; Hargitai et al. 2017). Indeed, food supplementation could have altered not only food abundance but also diet composition of supplementary fed females. Diet composition of lesser kestrel varies during the breeding season (Rodríguez et al. 2010), and this effect may be reflected in natural variation in eggshell pigmentation among unfed controls, but clearly this could differ among mice-supplemented females. Hence, eggshell pigmentation could be a marker of inter-individual variation in diet even on a very short term (we started food supplementation at laying of the first egg, and thus the average supplemented females were fed for a total of ca. 6 days and they received ca. 9 mice each during egg laying). The effect of the interaction between food supplementation and egg mass on eggshell pigment intensity is more difficult to explain: we may hypothesize that it reflects a complex interaction between food supplementation and female condition, which affected patterns of eggshell pigment deposition and differed between food-supplemented and unfed control females.

Remarkably, females mated with males showing brighter head plumage laid significantly more pigmented eggs. The observation that female eggshell pigment deposition was more related to male compared to female quality traits is consistent with a similar study carried out on the closely-related Eurasian kestrel, whereby male body condition was the only

trait significantly explaining variation in female eggshell pigment deposition (Martínez-Padilla et al. 2010). Since head plumage brightness reflects male quality, this finding may suggest a differential investment in eggshell pigmentation by females mated with males of likely higher 'intrinsic' quality, irrespective of their food provisioning ability (because of the lack of direct food supplementation effects on eggshell pigment deposition). Moreover in lesser kestrel males, head plumage brightness could be an age-related trait, as suggested by its significant negative correlation with breast and underwing spottiness ($r = -0.27$, $p = 0.018$, $N = 78$), a plumage trait that decreased according to age in our study population (Corti et al. submitted). Hence, since age is an important trait related to individual 'intrinsic' quality (high quality individuals are expected to be better survivors), we may speculate that a greater investment by females in eggs laid for high-quality males could potentially provide fitness benefits.

This interpretation relies on the assumption that darker eggs are of higher quality, an assumption which received some support by our findings: females that laid eggs with high eggshell pigment intensity also laid larger clutches and, within clutches, more pigmented eggs showed higher hatching success, irrespective of laying order-related variation in egg hatching success (early-laid eggs in the laying sequence were more likely to hatch than late-laid ones). Whether a greater hatching success of more pigmented eggs is a direct consequence of eggshell protoporphyrin or a consequence of eggshell pigment-related variation in eggshell structural properties or egg compositions (in terms of macro- or micronutrients affecting egg hatching success) remains to be elucidated. Overall, food supplementation, which likely affected egg composition, did not markedly affect egg hatching success. However, controlling for clutch size, a significant effect of food supplementation on egg hatching success emerged, whereby, in the supplementary fed group (but not among controls) eggs belonging to larger clutches had higher hatching success. This finding may suggest that food supplementation

boosted further egg investment by females whose energy investment in reproduction was already high (i.e. laid larger clutches).

Finally, we detected a significant laying-sequence related variation in eggshell pigment deposition, with early- and late-laid eggs being less pigmented than intermediate eggs in the laying sequence. Previous studies investigating laying-sequence related variation in egghsell pigmentation showed variable patterns, some studies showing an increase of pigment deposition along the laying sequence (Gosler et al. 2005), other showing a decrease (López de Hierro and De Neve 2010; De Coster et al. 2013) or no significant association with laying order (Corti et al. submitted). The adaptive significance (if any) of egg-laying sequence related variation in eggshell pigment deposition remains to be elucidated, and the most likely explanation is that such a pattern simply reflects changes in female condition and/or hormonal state during egg laying. Moreover, it may also reflect laying-sequence related variation in egg composition in terms of micro- or macro-nutrients. Analyses of within clutch covariation between egg composition and eggshell pigmentation (see Rubolini et al. 2011) may provide a clue to such covariation effects.

In conclusion, our results, together with those obtained by Martínez-Padilla et al. (2010) in a closely-related species, suggest that lesser kestrel females differentially invest in eggshell pigmentation according to an intrinsic quality trait of their mates (head plumage brightness) rather than to their own condition/quality or to perceived mate ability to provide direct fitness benefits (i.e. food) during egg laying. Hence, male intrinsic quality traits may have an important role in shaping eggshell pigment deposition by their mates. This supports the idea that eggshell pigment deposition results from post-mating sexual selection by females in bird species where female condition is strongly affected by male mate-provisioning effort and where male paternal contribution, possibly reflected by their intrinsic quality, is of capital importance for achieving high fitness.

Acknowledgements

We thank the Comune di Matera, Ufficio Scuole, the Director of the Istituto Comprensivo 'G. Minozzi', M. R. Santeramo, and the employees of the Provincia di Matera for authorizing and tolerating our work and our presence on the breeding colonies, as well as for constant support during field activities. We thank R. Ambrosini for useful suggestions. Study from the Naumanni76 team (paper #04)

Compliance with ethical standards

Data of this study have been collected following standard minimally invasive protocols. In order to take blood samples, nestlings were taken out of their nestbox and kept inside a plastic container covered with a cotton bag in a safe position and then placed back into the nestbox as soon as possible. Blood was taken after puncturing the brachial vein with sterile needles and the puncturing site was accurately disinfected. After handling, nestlings and adults were observed to behave normally, not showing any sign of stress-related behaviours. Capture and handling were carried out by Istituto Nazionale per la Protezione e la Ricerca Ambientale (ISPRA) under the authorization of Law 157/1992 [Art.4 (1) and Art. 7 (5)].

Disclosure of potential conflict of interest

The authors declare that they have no conflict of interest.

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Figure 1. Variation of protoporphyrin-based eggshell pigmentation intensity in two representative 5-egg clutches (top: highly pigmented; bottom: little pigmented; colour is standardized as described in the Methods section). Numbers above eggs indicate the laying order.

Figure 2. Association between male head plumage brightness and mean clutch eggshell intensity. Regression line and 95% confidence intervals range are also reported.

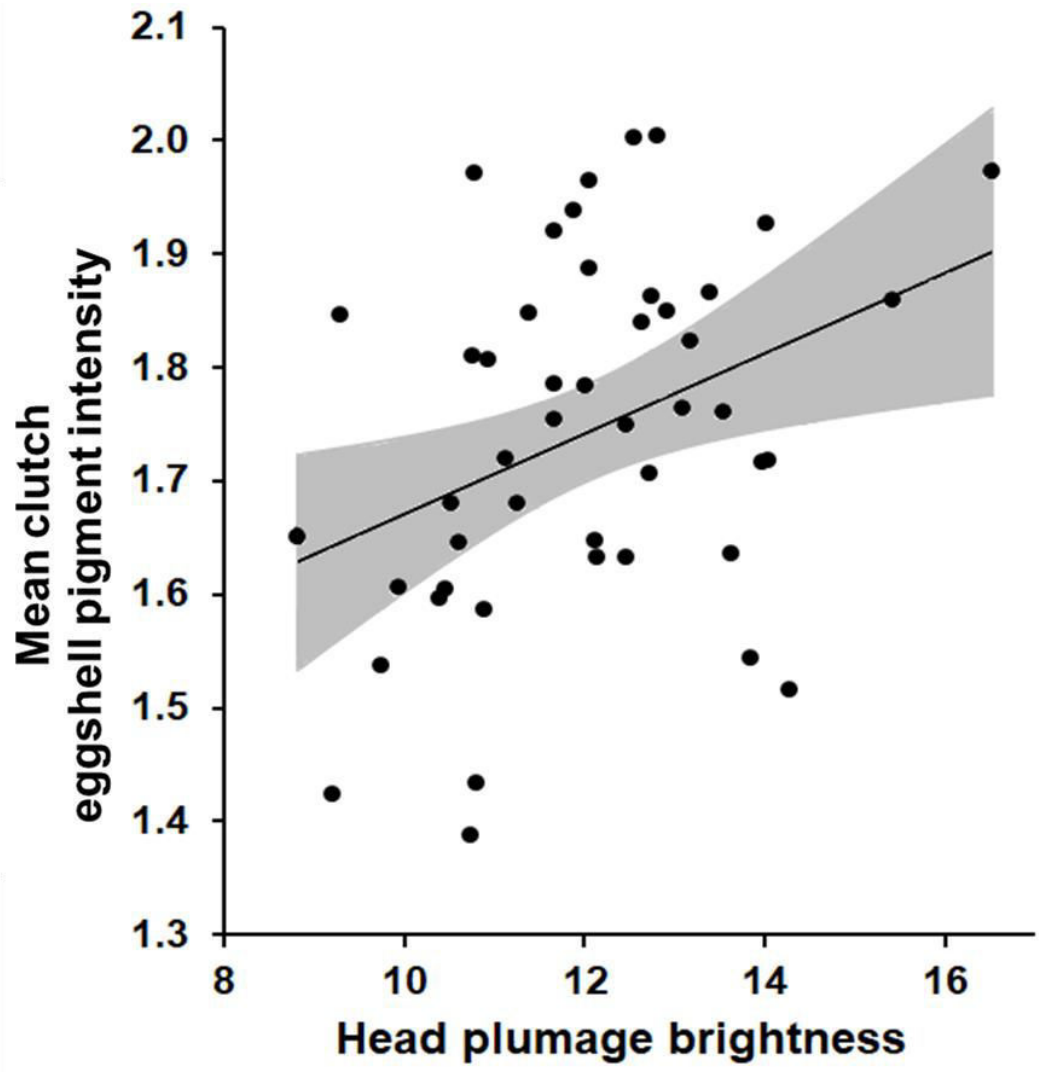

Figure 3. Effect of food supplementation on eggshell pigment intensity according to egg laying date and food supplementation. Supplementary fed pairs: filled dots and broken line [estimated slope: -0.01 (0.01 SE), $t_{74} = -1.61$, $p = 0.11$]; unfed pairs: open dots and solid line [estimated slope: 0.01 (0.01 SE), $t_{74} = 2.11 p = 0.039$; estimated slopes derived from the linear model reported in Table 3].

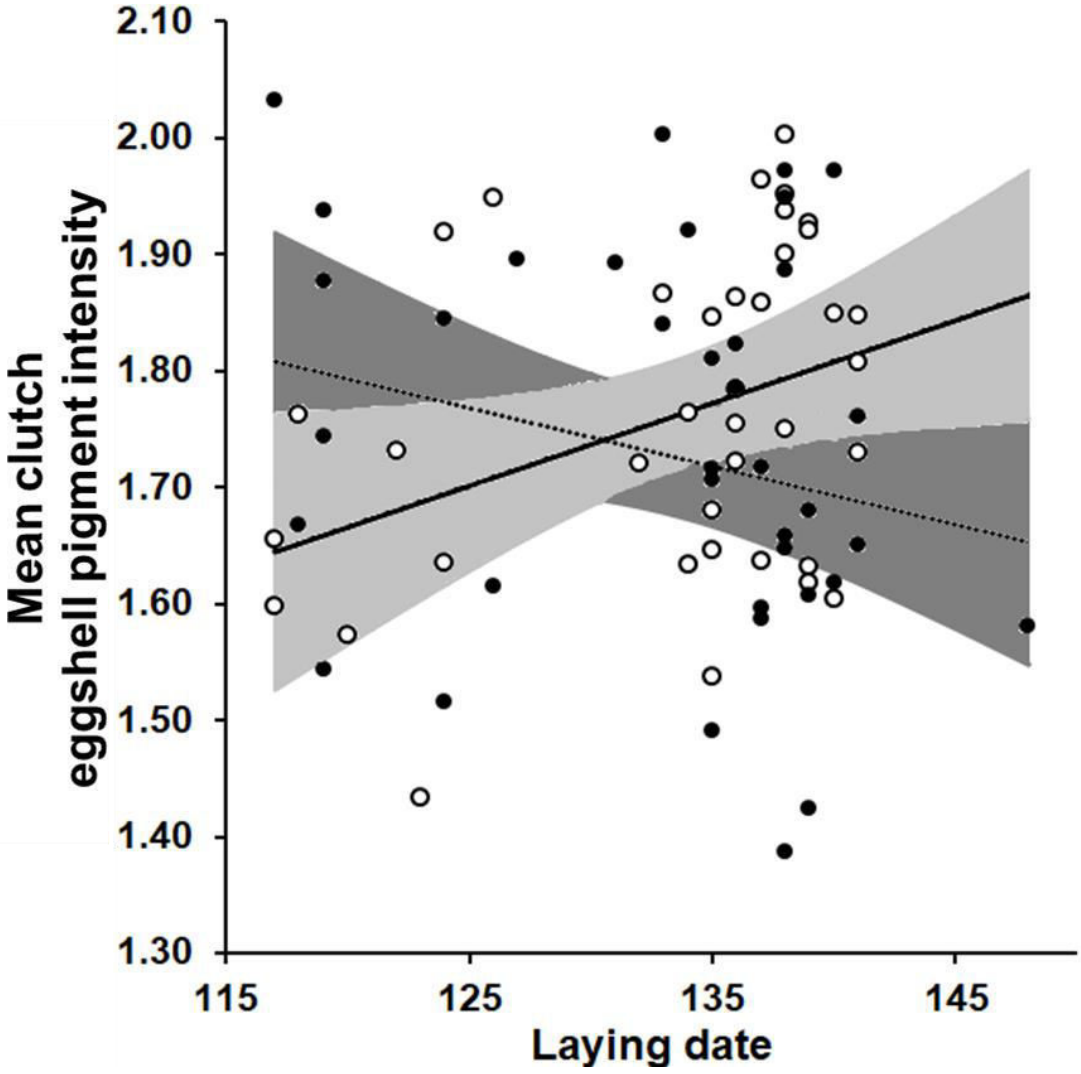

Figure 4. Association between eggshell pigment intensity and laying order. Eggshell pigment intensity varied in a quadratic fashion through laying sequence, with intermediate eggs being more pigmented than first- and last-laid ones. Grey triangles and error bars report mean and SE of eggshell, pigment intensity per each position in the laying sequence. 95% confidence intervals of the regression line are also shown. To better represent overlapping datapoints, dots were randomly scattered around each laying order position.

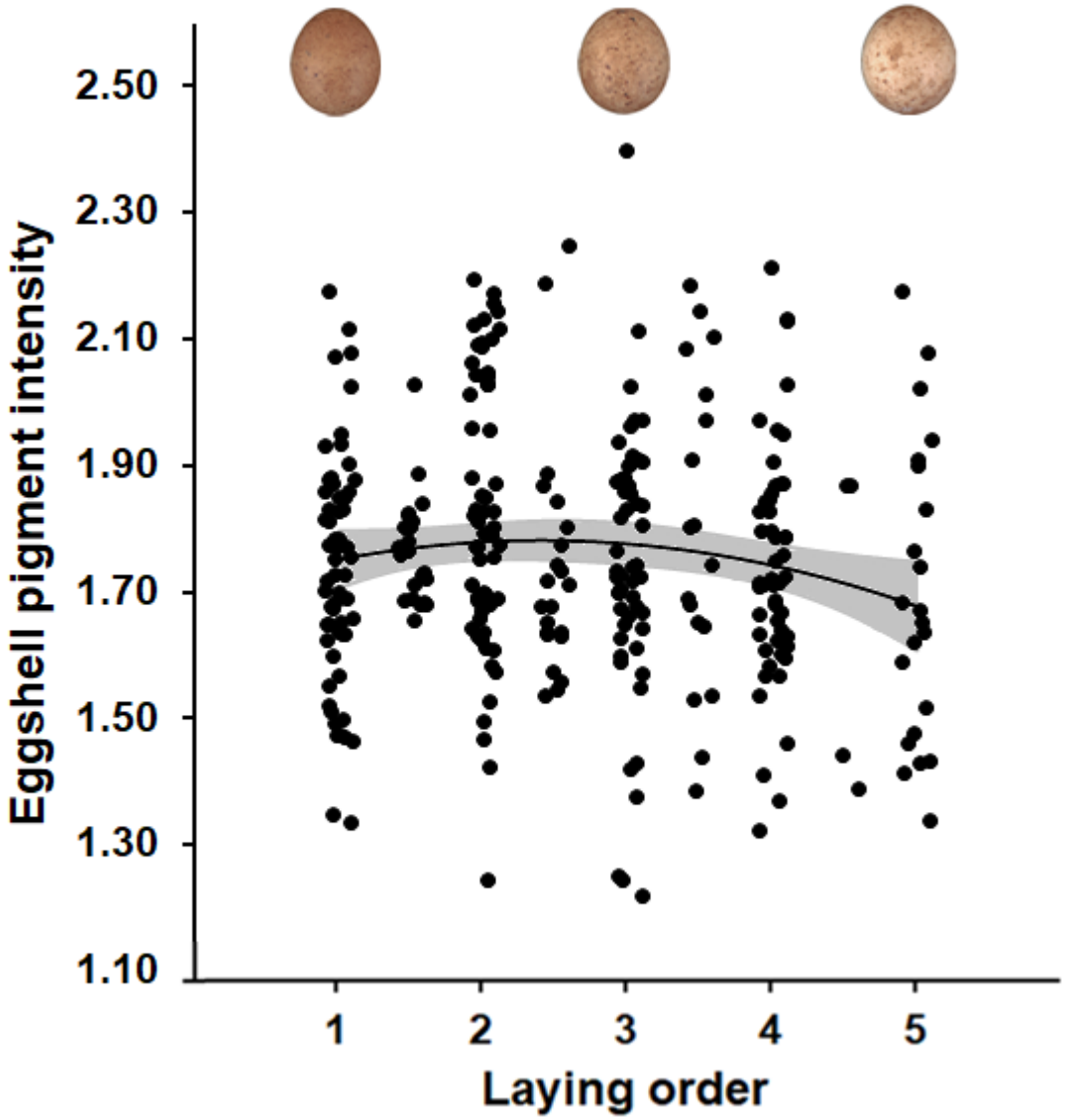

Table 1. Effects of female and male condition and plumage traits on mean clutch eggshell pigment intensity. Female rump grey plumage colouration was scored in four classes (1 corresponding to brownish plumage colouration and 4 to dark greyish colouration). In the female model, body condition was expressed as the residuals of a linear model accounting for the significant decline of body mass during incubation and the marginally non-significant treatment effect (see Results).

Predictors	**Estimate (SE)**	***t***	**p**
Female traits (n = 31 females)			
Body condition (residuals)	-0.82 (0.82)	-1.00	0.33
Rump grey plumage score	-0.03 (0.03)	-1.17	0.25
Male traits (n = 47 males)			
Body condition	0.42 (0.68)	0.62	0.54
Head plumage brightness	0.03 (0.01)	2.09	0.043
Head plumage UV Chroma	-3.04 (2.48)	-1.22	0.23
Food supplementation	-0.04 (0.04)	-0.92	0.37

Table 2. Linear models reporting the effects of clutch traits on mean clutch eggshell pigment intensity. Models including and excluding the non-significant effect of primary sex ratio (PSR) are reported.

Predictors	**Estimate (SE)**	***t***	**p**
Model including PSR (N = 31 clutches)			
Laying date	0.01 (0.01)	0.51	0.61
Clutch size	0.05 (0.04)	1.16	0.26
PSR	0.02 (0.12)	0.18	0.86
Food supplementation	-0.05 (0.06)	-0.84	0.41
Model excluding PSR (N = 75 clutches)			
Laying date	0.01 (0.01)	0.44	0.66
Clutch size	0.03 (0.03)	1.33	0.19
Food supplementation	-0.04 (0.04)	-1.17	0.24
Food supplementation × laying date	-	-0.84	0.011

Table 3. Linear mixed model of the effects of egg traits on eggshell pigment intensity (sample size: 75 clutches and 314 eggs). Clutch identity was included as a random effect.

Predictors	**Estimate (SE)**	***F***	***d.f***	**p**
Laying date	0.01 (0.01)	2.29	1, 71	0.13
Clutch size	0.06 (0.03)	5.68	1, 76	0.020
Laying order	0.08 (0.03)	4.87	1, 240	0.028
Laying order^2	-0.02 (0.01)	6.48	1, 241	0.011
Egg mass $_{DEV}$	0.01 (0.01)	0.87	1, 250	0.35
Egg mass $_{MEAN}$	0.01 (0.01)	0.59	1, 70	0.44
Food supplementation	-0.04 (0.03)	1.14	1, 69	0.29
Food supplementation × laying date	-	5.39	1, 70	0.023
Food supplementation × egg mass $_{MEAN}$	-	7.51	1, 70	0.008

Table 4. Binomial mixed model testing the association between hatching success and egg and clutch traits (sample size: 75 clutches and 314 eggs), with clutch identity as a random effect. To disentangle between- vs. within-clutch effects of eggshell pigment intensity and egg mass on egg hatching success, both variables were expressed by two new variables (according to van de Pol and Write, 2009): the mean clutch value of each variable, computed separately for each clutch (labeled $_{MEAN}$), and the difference between the value of each single egg and the mean value of its clutch (labeled $_{DEV}$). Positive $_{DEV}$ values thus indicate eggs that were more pigmented/heavier than the mean of their clutch. The model was not overdispersed (overdispersion = 0.83).

Predictors	**Estimate (SE)**	**Z**	**p**
Laying date	-0.01 (0.03)	-0.21	0.83
Clutch size	0.45 (0.40)	1.11	0.27
Laying order	-0.47 (0.18)	-2.60	0.009
Eggshell pigment intensity$_{DEV}$	2.83 (1.37)	2.06	0.039
Eggshell pigment intensity$_{MEAN}$	0.85 (1.65)	0.51	0.61
Egg mass $_{DEV}$	0.20 (0.29)	0.68	0.49
Egg mass $_{MEAN}$	0.07 (0.22)	0.33	0.74
Food supplementation	-0.08 (0.50)	-0.16	0.87
Food supplementation × clutch size	-	1.98	0.048

Chapter 7

Synthesis and concluding remarks

The present thesis was aimed at expanding our knowledge about the possible signalling role of melanin-based plumage colouration and protoporphyrin-based eggshell pigmentation in two bird species, the barn swallow and the lesser kestrel using both correlational and experimental approaches.

Melanin-based plumage colouration

In **Chapter 2** we showed that parents modulated their investment according to the ventral plumage colouration of their offspring. Plumage darkening resulted in an increasing in food provisioning and in a body mass gain in male, but not in female offspring. Because darker ventral plumage colouration is associated with larger reproductive success in adult barn swallow males (Safran et al., 2005; Vortman et al., 2011; Romano et al., 2016), these results suggest that parents modulate their effort in favour of the offspring with the greatest expected fitness returns. Our study therefore provides evidence that parents invest differentially in offspring according to a trait expressed in early life, which is relevant to intra-sexual competition for access to mates at sexual maturity. To the best of our knowledge, only a previous study performed on eastern bluebird (*Sialia sialis*) has investigated parental favouritism according to plumage colouration, showing a preferential post-fledging defence behaviour toward the most ornamented sons (Barrios-Miller & Siefferman, 2013) and a preferential food allocation toward brighter coloured ones (Ligon & Hill, 2010). Our results are consistent and complementary to those obtained in the eastern bluebird, providing further evidence of parental ability to differentially invest in sons and daughters according to an early developmental trait, and adding new knowledge about the mechanism of parental favouritism for offspring that can be predicted to be more fitness-rewarding.

Although the association between individual quality and melanin-based ventral plumage colouration may be driven by the pleiotropic effects of the melanocortin system, in **Chapter 3** none of the three behaviours used as proxies of behavioural stress response was significantly predicted by nestling ventral plumage colouration, indicating that in the juveniles of this species melanin-based colouration does not convey reliable information on individual ability to cope with stressful events to conspecifics. The observed lack of covariation may be due to the reciprocally antagonistic effects of the modulators of eu- and pheomelanogenesis and to their role as modulators of the HPA stress response (Xiao et al., 2003; Charmandari et

al., 2005; Racca et al., 2005; Ducrest et al., 2008). Our results suggested that if the link between melanin-based colouration and HPA stress response relies only on compounds implicated in eumelanogenesis, the pleiotropic hypothesis might not apply to plumage colouration deriving from both melanin forms or where pheomelanins are the dominant driver of phenotype variation. My study further showed that the three behaviours were correlated with each other both at the individual- and brood-level, indicating that differences between nestlings are part of different behavioural strategies. Moreover, the within-brood repeatability of behavioural proxies suggests that common environment and/or genetic effects are important determinants of behavioural stress response.

In the study presented in **Chapter 4** I investigated whether variation in DNA sequences of *MC1R* and *TYRP1* affects the marked inter-individual variability in melanin-based plumage traits displayed by male lesser kestrels. No SNPs in *MC1R* nor in *TYRP1* genes were found to be related to melanin-based plumage traits. That results is consistent with the hypothesis that the large inter-individual variation of melanin-based plumage traits, which is consistent between years and partially age-related, might be determined by other genes of the melanogenesis pathway (e.g. Xu et al., 2013; Wang et al., 2014; Bourgeois et al. 2016), and/or local changes in the expression of those genes (Manceau et al., 2011), epigenetic modifications which do not alter the DNA sequences as well as a combination of these different factors (San-Jose & Roulin, 2017). Indeed, we showed that homozygous individuals for rare SNP variants displayed melanin-based plumage phenotypes, indicating that the investigated genes may play a role in controlling plumage variation but the limited size of mutated individuals in our samples and/or the possible recessive nature of the mutation did not allow to identify a statistically significant association.

Protoporphyrin-based eggshell pigmentation

The studies presented in **Chapter 5** and **6** tackled the issue of the possible signalling role of protoporphyrin-based eggshell pigmentation in both the barn swallow and the lesser kestrel.

In **Chapter 5** I found that, despite the large variability in eggshell pigmentation and the repeatable eggshell patterning across years by the same females, eggshell pigmentation seems not to be significantly related to egg traits, including position in laying sequence, egg size, yolk antioxidant capacity and yolk testosterone concentration, nor to female and male traits or to parental feeding effort. Nevertheless, females with darker ventral plumage colouration laid highly protoporphyrin-covered eggs, suggesting the presence of a previous

unappreciated link between protoporphyrin biosynthesis and plumage melanisation. In addition, together with female ventral colouration (Romano et al., 2015), eggshell pigmentation pattern may be a reliable proxy of both primary and secondary sex ratio, as hinted by the significant association between eggshell coverage and the proportion of male offspring. Despite males not modulating their parental investment according to eggshell pigmentation, they could acquire visual information about the sex composition of their future brood before hatching in order to optimally prepare for greater parental workload due to the expected high energy demands of raising a male-biased brood (Boncoraglio et al., 2008; Bonisoli-Alquati et al., 2008; Saino et al., 2008).

In **Chapter 6** we found that food supplementation did not significantly affect parental body condition, but affected eggshell pigmentation differentially according to laying date and egg mass. The significant interaction found between eggshell pigment intensity with laying date possibly hints that a dietary variation (caused by the food supplementation) could have influenced protoporphyrin-based eggshell pigmentation. Indeed, food supplementation could have altered not only food abundance but also diet composition of supplementary fed females. Diet composition of lesser kestrel varies during the breeding season (Rodríguez et al., 2010), and this effect may be reflected in natural variation in eggshell pigment intensity among unfed controls, but clearly this could differ among mice-supplemented females. The effect of the interaction between food supplementation and egg mass on eggshell pigment intensity could reflect a complex interaction between food supplementation and female condition, which affected eggshell pigmentation and differed between food-supplemented and unfed control females.

Furthermore, females mated with potential high quality males laid more pigmented eggs, suggesting that females may adjust eggshell pigmentation according to male quality. In addition, we found that females laying eggs with high eggshell pigment intensity also laid larger clutches and, within clutches, more pigmented eggs showed higher hatching success suggesting that darker eggs could be of higher quality. Finally, we detected a significant laying-sequence variation in eggshell pigment intensity, with intermediate-laid eggs more pigmented than first- and last-laid ones.

Concluding remarks

This thesis provides novel results about the role of melanin-based plumage colouration as a signal of individual quality in birds. Although in barn swallow melanin-based colouration is associated with many life-history traits, the melanocortin pleiotropic hypothesis showed a gap regarding how plumage traits deriving from both melanin forms or where pheomelanins are the dominant driver of phenotypic variation would fit into the hypothesis proposed by Ducrest et al., (2008). Further studies are required to disentangle the role of mixed melanin plumage in melanocortin pleiotropic hypothesis.

Moreover, this thesis provides evidence that intra-specific variation in mixed-melanin plumage traits should be further investigated considering the whole melanogenesis pathway, its expression and its epigenetic modifications as well as the role of regulator factor such as the age of individuals.

Finally, since in barn swallow protoporphyrin-based eggshell pigmentation does not have any major function of signalling egg or female characteristics to mates, while in lesser kestrel it might be a side effect of potential male quality, following along with the contrasting scenarios offered by previous studies the findings of this thesis support the idea that intra-specific signalling through eggshell pigmentation is a species-specific trait rather than a general pattern across avian taxa.

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Acknowledgements

I wish to express my most sincere gratitude to my supervisor Prof. Diego Rubolini, whose particular suggestions were source of critical stimulus in observing nature (and data) in a different way. I would also like to thank him for the opportunity to work in the world of behavioural ecology, for helping me to publish my researches in high-level international scientific journals and for his precious support during the three Ph.D years.

I am sincerely grateful to the colleagues I worked with: Marco Parolini, Alessandra Costanzo, Roberto Ambrosini, Nicola Saino, Manuela Caprioli, Luca Gianfranceschi, Jacopo G. Cecere and Matteo Griggio, who gave me very much appreciated support and suggestions, tutoring me in different situations, from data collection to lab work and statistical analyses as well as in the interpretation of the obtained results. I am also grateful to Alexandre Roulin and Anne-Lyse Ducrest-Roulin, who warmly hosted me in Lausanne, for their much appreciated teachings about plumage polymorphism.

I wish also to tribute a special thank to Andrea Romano and Gaia Bazzi, who supported me in many ways during my activities transmitting me all their knowledge and passion. I also reserve special thanks to Cristina Possenti and Stefano Podofillini, without whom my work would have been way much harder and way less amusing.

I would like to further thank all the students and field assistants who have greatly helped me in collecting data on the field: Ilaria Scartabellati, Letizia Mombelli, Davide Corbani, Francesco Drago, Elisa Alari, Elena Seghezzi, Marta Belloni, Barbara Scarpellini Andrea Curcio and Jessica Bellingardi. Moreover, I sincerely thank all farm owners who have allowed the access to their properties, the Comune di Matera, Ufficio Scuole, the Director of the Istituto Comprensivo ‘G. Minozzi’, M. R. Santeramo, and the employees of the Provincia di Matera who made this research possible. Finally, I thank my families, Cecilia Graziosi and Marzio, who supported me during these years.

UNIVERSITÀ DEGLI STUDI DI MILANO

PhD ESciences Environmental

Ph.D. Course in Environmental Sciences

PhD Student Final Report

PhD Student:	**CORTI Margherita**
PhD Course Cycle:	XXX
Scientific Tutor:	Prof. RUBOLINI Diego
Thesis Project Title:	Colouration as an intraspecific signal in birds
Project performed at:	University of Milan; Department of Environmental Science and Policy; Laboratory of Behavioural and Evolutionary Ecology.

Research Period Abroad:			
05.12.2016 – 16.02.2017	Department of Evolution and Ecology (DEE), Biophore, Université de Lausanne, 1015 Lausanne, Swiss	Local Supervisor: Prof. Alexandre Roulin	Research Project Title: Large melanin-based plumage variation in lesser kestrel (*Falco naumanni*) males is not explained by sequences variation in *melanocortin-1-receptor* (*MC1R*) and *tyrosinase-related protein 1* (*TYRP1*) genes

List of Scientific Publications

Romano A., Bazzi G., Caprioli M., **Corti M.**, Costanzo A., Rubolini D., Saino N. (2016). 'Nestling sex and plumage color predict food allocation by barn swallow parents'. Behavioral Ecology 27: 1198-1205.

Corti M., Romano A., Bazzi G., Costanzo A., Podofillini S., Saino N., Rubolini D. (2017). 'Behavioural stress response and melanin-based plumage colouration in barn swallow nestlings'. Behaviour 154: 853-874.

Saino N., Rubolini D., Ambrosini R., Romano A., Parolini M., Canova L., **Corti M.**, Costanzo A. (2017).'Sex- and age-dependent morphology and selection on wing shape in the barn swallow (*Hirundo rustica*)'. Journal of Avian Biology 48: 1441-1450.

Corti M., Romano A., Costanzo A., Bentz A.B., Navara K.J., Parolini M., Saino N., Rubolini D. 'Protoporphyrin-based eggshell pigmentation is associated with female plumage colouration and predicts offspring sex ratio in the barn swallow'. Under Review in Journal of Avian Biology.

Corti M., Podofillini S., Cecere J.G., Griggio M., Gianfranceschi L., Ducrest-Roulin A., Roulin A., Saino, N., Rubolini D. 'Sequence variation in *MC1R* and *TYRP1* genes and their relationship with melanin-based plumage trait expression in lesser kestrel (*Falco naumanni*) males'. Accepted in Journal of Ornithology.

Costanzo A., Romano A., Ambrosini R., Parolini M., Rubolini D., Caprioli M., **Corti M.,** Canova L., Saino N. 'Personality of barn swallows covaries with melanic coloration and predicts survival independently of spatio-temporal ecological variation'. Submitted.

Podofillini S., **Corti M.,** Costa I., Pirrello S., Griggio M., Serra L., Saino N., Cecere J.G., Rubolini, D. 'Parental quality and protoporphyrin-based eggshell pigmentation in a biparental raptor species: a food supplementation experiment'. In submission.

Podofillini S., Cecere J.G., **Corti M.,** Griggio M., Rubolini D. 'Contextual effects of extra food supply on breeding parameters and parent quality in the lesser kestrel (*Falco naumanni*)'. In prep.

List of attended Meetings and Congresses

II Behavioural Ecology Meeting, B.E.M. Chioggia (VE), 25th – 26th February 2016.

I Congresso Nazionale Congiunto S.It.E. (Società Italiana di Ecologia) -UZI (Unione Zoologica Italiana) -SIB (Società Italiana di Biogeografia). Università degli Studi di Milano-Bicocca. Milano, 30th August -2nd September 2016.

III Congresso Nazionale Fauna Problematica. Palazzo del Ridotto, Cesena (FC), 24th - 26th November 2016.

XIX Convegno Italiano di Ornitologia, C.I.O. Torino, 26th September – 1st October 2017.

Meeting and Congress Contributions

Corti M., Romano A., Bazzi G., Caprioli M., Costanzo A., Rubolini D., Saino N. 'Nestling sex and plumage color predict food allocation by barn swallow parents'. I Congresso Nazionale Congiunto SITE-UZI–SIB, Milano 30th Ago – 2nd Sep 2016.

Corti M., Drago F., Chiatante A., Giunchi D., Rubolini D. 'Stima di distribuzione ed abbondanza del piccione domestico (*Columba livia* var.*domestica*) nell'area urbana del Comune di Piacenza'. III Congresso Nazionale Fauna Problematica, Cesena 24th-26th Nov 2016.

Podofillini S., **Corti M.**, Costa I., Cecere J.G., Rubolini D., Griggio M. 'Analysis of eggshell maculation in the lesser kestrel'. XIX CIO (Convegno Italiano di Ornitologia), Torino, 26th Sep – 1st Oct 2017 .

Corti M., Drago F., Chiatante A., Giunchi D., Rubolini D. 'Distribution and abundance estimation of the domestic pigeon (*Columba livia* var. *domestica*) in the urban area of Piacenza'. XIX CIO (Convegno Italiano di Ornitologia), Torino, 26th Sep – 1st Oct 2017 .

Ambrosini R., Musitelli F., Grigolo C. P., Rubolini D., **Corti M.**, Costanzo A., Franzetti A., Gandolfi I. 'Microbioma cloacale, sopravvivenza e successo riproduttivo nella rondine *Hirundo rustica'*. XIX CIO (Convegno Italiano di Ornitologia), Torino, 26th Sep – 1st Oct 2017 .

List of attended Seminars

Møller A.P. , Dec 2014 - 'Ecology and Evolution at Chernobyl and Fukushima'.

Møller A.P., Dec 2014 - 'How to write a paper'.

Bandi C., Feb 2015 - 'Gendercide symbionts…e altre storie di sesso, simbiosi e parassitismo'.

Bandi C., Mar 2015 - 'Simbiosi, parassitismo e malattie infettive: un approccio evoluzionistico'.

Maugeri M., Mar 2015 - 'Variabilità e cambiamenti climatici in Italia negli ultimi due secoli'.

Bollati V., Mar 2015 - 'L'epigenetica ambientale come interfaccia tra ambiente ed espressione genica'.

Rubolini D., Mar 2015 - 'Unraveling climate change effects on igration birds: a comparative approach'.

Manenti R., Nov 2015 - 'Cave colonisation by the fire salamander (*Salamandra salamandrea*): zoological, ecological and evolutionary insight' .

Della Torre C., Nov 2016 - 'Do Carbon based nanoparticles act as carrier for benzo(α)pirene? An investigation on *Danio rerio* embryos .

Romano A., Apr 2016 - 'Evolution of sex allocation strategies in a sexually selected passerine bird'.

Mueller J.C., July 2016 - 'Genetic architecture and mapping of traits'.

Green M. D., Nov 2016 - 'Amphibian population ecology an environmental changes'.

Epis S., Nov 2016 - 'Of symbionts, arthropods and vector-borne disease'.

Ciccatelli A., Mar 2017 - 'Phytoremediation of contaminated environments and matrices: Advantages, Limitations, Present Status'.

Galimberti A., May 2017 - 'DNA barcoding: a universal tool for biodiversity characterization: theoretical and practical aspects'.

List of attended PhD Courses

Feb – Apr 2015 - English course organized by the PhD course in Environmental Sciences (Prof. Carson).

Sep 2015 - Statistic course organized by the PhD course in Environmental Sciences (Prof. Roberto Ambrosini).

Sep 2015 - Corso di matematica: ottimizzazione in più variabili (Prof. Paola Morando)

Oct 2015 - Genetica e conservazione delle popolazioni (Prof. Ettore Randi).

Mar 2016 - Comunicare la scienza (Dr. Michele Bellone).