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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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

88 (Table S2). The frequency of homozygous individuals for the mutated allele was generally low,
89 ranging between 0% and 13% (Table S2).
90 We tested the associations between each SNPs and plumage traits using the R *SNPassoc*
91 package v. 1.9.2 (Gonzáles et al. 2007), assuming different modes of inheritance (codominant,
92 dominant, recessive, overdominant, log-additive). 'False discovery rate' method was used to correct
93 p-values for multiple testing; corrections were applied separately to each plumage trait and to each
94 mode of inheritance.
95 Since mutation expression on phenotypic traits was unknown, we explored whether
96 individuals homozygous for the mutated allele have a different phenotypic expression compared to
97 wild-type individuals. To this end, we calculated the 95% non-parametric bootstrap CIs (based on
98 5000 replicates) for both plumage traits of each locus homozygous for the wild-type allele (Table
99 1). We then checked whether melanin-based trait values of homozygous mutated individuals were
100 included or not within the CIs of the phenotypic distribution of homozygous wild-type individuals.
101 The two plumage traits were not significantly associated ($r = -0.06$, $n = 94$, $p = 0.58$),
102 suggesting that ventral colouration and spottiness may be controlled by different pathways. SNP
103 variants were not significantly associated with ventral colouration (all $p > 0.22$) nor spottiness (all p
104 > 0.89). However, plumage trait values for some rare homozygous variants (homozygous mutated <
105 four individuals) were outside the 95% confidence intervals of the phenotypic distribution of wild-
106 type homozygous individuals (ventral plumage colouration: loci *MC1R* 78C>T; 291G>A; 513C>T;
107 717A>G; -50C>A; spottiness: loci *MC1R* 513C>T; 717A>G; Table 1), suggesting that rare SNP
108 variants may be associated with extreme melanin-based plumage phenotypes. Unfortunately, formal
109 statistical testing was not feasible due to the very small number of homozygous genotypes for the
110 mutated allele at these loci.
111 Plumage variation of male lesser kestrel might be partly age-related, as in other raptor
112 species (Dreiss et al. 2010; Lopez-Idiaquez et al. 2016). Since the exact age of individuals included
113 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.

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