Redness Variation in the Eurasian Scops-Owl Otus scops is Due to Pheomelanin But is Not Associated with Variation in the Melanocortin-1 Receptor Gene (MC1R)

Authors: Avilés, Jesús M., Cruz-Miralles, Ángel, Ducrest, Anne-Lyse, Simon, Céline, Roulin, Alexandre, et al.

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Research Papers

REDNESS VARIATION IN THE EURASIAN SCOPS-OWL *OTUS SCOPS* IS DUE TO PHEOMELANIN BUT IS NOT ASSOCIATED WITH VARIATION IN THE MELANOCORTIN-1 RECEPTOR GENE (*MC1R*)

LA VARIACIÓN EN EL GRADO DE ROJISMO EN EL AUTILLO EUROPEO *OTUS SCOPS* ES DEBIDA A FEOMELANINAS PERO NO SE ASOCIA CON VARIACIÓN EN EL GEN RECEPTOR DE LA MELANOCORTINA 1

Jesús M. Avilés[1] *, Ángel Cruz-miralles[2], Anne-Lyse Ducrest[3], Céline Simon[3], Alexandre Roulin[3], Kazumasa Wakamatsu[4] and Deseada Parejo[2]

Summary.—Melanin-based colorations in birds constitute a paradigm for the study of the molecular basis of phenotypic variation. Variation in the melanocortin-1 receptor (*MCR1*) gene, a key regulator of melanin synthesis in feather melanocytes, can lead to changes in the production of melanin and hence in feather colour. Here we investigate the proximate mechanisms behind colour plumage polymorphism in the Eurasian Scops-owl *Otus scops*, a species showing pronounced variation in the degree of redness. Although eumelanin pigment was three times more abundant than pheomelanin pigments, the degree of plumage redness was more strongly associated with the amount of pheomelanin than eumelanin pigments. We detected only one synonymous substitution and one non-synonymous substitution in *MC1R* which were, however, not associated with variation in plumage coloration. Redness variation in Eurasian Scops-Owls is primarily due to variation in pheomelanin, and to genes or regulatory elements other than *MCR1*.—Avilés, J.M., Cruz-Miralles, A., Ducrest, A.-L., Simon, C., Roulin, A., Wakamatsu, K. & Parejo, D. (2020). Redness variation in the Eurasian Scops-owl *Otus scops* is due to pheomelanin but is not associated with variation in the melanocortin-1 receptor gene (*mc1r*). *Ardeola*, 67: 3-13.

Key words: eumelanin, *MC1R*, melanism, *Otus scops*, pheomelanin, polymorphism.

[1] Departamento de Ecología Funcional y Evolutiva, EEZA-CSIC, Carretera de Sacramento s/n, 04120 La Cañada de San Urbano, Almería, Spain.

[2] Departamento de Anatomía, Biología Celular y Zoología, Facultad de Ciencias, Universidad de Extremadura, Avenida de Elvas, s/n, 06006 Badajoz, Spain.

[3] Department of Ecology and Evolution, University of Lausanne, Biophore Building, CH-1015 Lausanne, Switzerland.

[4] Department of Chemistry, Fujita Health University School of Medical Sciences, Toyoake, Aichi 470-1192, Japan.

* Corresponding author: javiles@eeza.csic.es

RESUMEN.—Las coloraciones basadas en melanina en las aves constituyen un paradigma para el estudio de las bases moleculares de la variación fenotípica. La variación en el gen del receptor de melanocortina-1 (*MCR1*), un regulador clave de la síntesis de melanina en los melanocitos de las plumas, puede provocar cambios en la producción de melanina y, por lo tanto, en el color de las plumas. Aquí investigamos los mecanismos próximos detrás del polimorfismo del plumaje de color en los autillos europeos *Otus scops*, una especie que muestra una marcada variación en el grado de rojismo. Aunque la cantidad de eumelanina era tres veces más abundante que la de feomelanina, el grado de rojismo del plumaje se asoció más fuertemente con la cantidad de feomelanina que con la eumelanina. Sólo detectamos una sustitución sinónima y una sustitución no sinónima en el MC1R que, sin embargo, no se asociaron con la variación en la coloración del plumaje. La variación de rojismo en el autillo europeo se debe principalmente a la variación en el contenido de feomelanina, y a genes o elementos reguladores de estos distintos del MCR1.—Avilés, J.M., Cruz-Miralles, A., Ducrest, A.-L., Simon, C., Roulin, A., Wakamatsu, K. y Parejo, D. (2020). La variación en el grado de rojismo en el autillo europeo *Otus scops* es debida a feomelaninas pero no se asocia con variación en el gen receptor de la melanocortina 1. *Ardeola*, 67: 3-13.

Palabras clave: eumelanina, feomelanina, *MC1R*, melanismo, *Otus scops*, polimorfismo.

INTRODUCTION

Understanding the molecular basis of phenotypic variation due to natural and sexual selection is a central goal of evolutionary biology and the study of melanin plumage colorations in birds has constituted a classic model system for its study (reviewed in Hubbard *et al.,* 2010; Roulin and Ducrest, 2013). Melanin pigments serve a wide range of functions in birds, including physical or anti-parasite protection, and their variable deposition in feathers is responsible for most non-structural brown, black and grey colour plumage variation (McGraw *et al*., 2005; McGraw, 2006; Galván and Wakamatsu, 2016) useful for camouflage or signalling. Melanin consists of two main forms, eumelanin (hereafter EM, responsible for grey-black colorations) and pheomelanin (hereafter PM, determining reddish-brown colour variation) (McGraw, 2006), the ratio between these two pigments determining how plumage coloration is finally perceived (e.g. McGraw *et al.,* 2005; Gasparini *et al*., 2009; Fargallo *et al*., 2018). The production of EM and PM is regulated by the activation of the melanocortin-1 receptor gene (*MC1R* hereafter) (Robbins *et al.,* 1993). *MC1R* encodes a seven-transmembrane domain G-protein-coupled receptor expressed primarily in melanocytes of developing feathers (Mundy, 2005). High *MC1R* activity leads to high levels of production of EM, whereas low *MC1R* activity associates with increased production of red or yellow PM (Robbins *et al*., 1993). Studies on the genetic basic of pigmentation have shown that variability at the *MC1R* locus can explain major dark/light colour polymorphism across a wide range of avian species (e.g. Theron *et al.,* 2001; Mundy *et al.,* 2004; Doucet *et al.,* 2004; Uy *et al.,* 2009; Gangoso *et al.,* 2011), although there are a growing number of exceptions (e.g. MacDougall-Shackleton *et al.,* 2003; Cheviron *et al.,* 2006; Dobson *et al*., 2012; Derelle *et al*., 2013; Farrell *et al*., 2015; Abolins-Abols *et al*., 2018). The association between variation at the *MC1R* and continuous melanin-based colour variations has received far less attention (see however Bourgeois *et al*., 2012; San-Jose *et al*., 2015; Corti *et al*., 2018).

The Eurasian Scops-owl *Otus scops* is a Strigiform species that is largely described as colour polymorphic, given the occurrence of two (dark-reddish and grey) main morphs (Del Hoyo *et al.,* 1999; Galeotti *et al.,* 2009).

However, intermediate morphs are frequent (Galeotti *et al.,* 2009; Parejo *et al.,* 2018), and spectrophotometric analyses have shown that colour variation in Eurasian Scops-owls is continuous (Parejo *et al.,* 2018). Recent findings from a wild population in southern Spain have revealed that the three morphs coexist within sex and age classes, and that the proportion of the three morphs is relatively stable, showing similar frequencies over eight studied years (Parejo *et al*., 2018). However, a temporal increase in the degree of redness of Italian Eurasian Scops-owls has been reported over the last century based on museum skin specimens (Galeotti *et al*., 2009). Although Eurasian Scops-owl colour variation is mostly defined by a graded change in body redness (Parejo *et al.,* 2018), which resembles melanin-based redness variation in the polymorphic Tawny Owl *Strix aluco* (Gasparini *et al.,* 2009), the absolute or relative role of EM and PM in determining plumage variation in Eurasian Scops-owls has not been investigated.

The main aim of this work was to study the proximate mechanisms behind colour variation in Eurasian Scops-owls. Firstly, we determined the role of melanin pigments in determining colour morph variation. Secondly, we sequenced *MC1R* to examine whether single-nucleotide polymorphisms of this gene are associated with plumage colour morph variants.

Materials and methods

Fieldwork

The study was performed from 2010 to 2017 in the Hoya of Guadix-Baza, Granada, southeastern Spain (37°18'N, 3°11'W). The area is an extensive agricultural landscape with scattered Holm Oaks *Quercus ilex* in which nest-boxes made of cork are located (see details in Rodríguez, Avilés, and Parejo, 2011; Parejo, Avilés, and Rodríguez, 2012; Parejo *et al*., 2018).

In the context of a long-term monitoring program of the Scops-owl population we routinely captured incubating females as well as males bringing food to the offspring (Parejo *et al*., 2018). In total 142 individuals were ringed with individually numbered metal rings and sexed by presence/absence of a brood patch. Captured adults were photographed for morph assignment. We extracted 225 ml of blood from each bird by brachial venipuncture for genetic analyses, and plucked three to five feathers from the same part of the head for melanin determination.

Colour scoring

We took two standardised photos of each individual: one head-on, showing the head and breast plumage; and the other from behind, showing the back and wings. All photos were taken about 50cm from the animal and always in shady areas around the nest, to homogenise light conditions. Photos were then used to score plumage coloration by focusing on the extent of redness on the head, breast and wings-back. Each body area was scored 1-3 according to whether they were predominantly greyer or browner (Parejo *et al*., 2018). We have previously shown that scores of the three body areas are highly correlated within individuals and that scores assigned by different observers to the same individual are highly repeatable (Parejo *et al*., 2018). Hence scores of the three body areas were summed to get an individual score for every bird (ranging from 3 to 9). Individuals were then classed as grey (score < 5.5), intermediate (5.5 ≤ score ≥ 7) or red-brown morph (score > 7) (see Supplementary material, Appendix 1, Figure A1). Based on recapture of a subset of individuals of known age, we have previously shown

that the morph score and morph classification are unaffected by age in Eurasian Scops-owls (Parejo *et al*., 2018). Hence, the possibility that age-related differences in plumage maturation might affect our results can be discarded.

Melanin concentration in feathers

We measured melanin composition and concentration in head feathers of 25 adult Eurasian Scops-owls. PM and EM concentration was estimated as described by Wakamatsu *et al*. (2002) and Ito *et al*. (2011). Feather samples (13-15mg) were homogenised with Ten-Broeck homogeniser at a concentration of 10 mg/mL H_2O. 100µL (1mg) aliquots were subjected to Soluene-350 solubilisation (Ozeki *et al*., 1996), alkaline hydrogen peroxide oxidation (Ito *et al*., 2011) and hydroiodic acid hydrolysis (Wakamatsu, Ito, and Rees, 2002). High-performance liquid chromatography (HPLC) was used to quantify EM and PM contents through specific degradation products, PTCA and TTCA for EM and PM by alkaline H_2O_2 oxidation of EM and PM, respectively, and 4-AHP by reductive hydrolysis of PM with hydriodic acid. EM content was estimated using a conversion factor of 25 for PTCA. For the conversion of TTCA in benzothiazole-type pheomelanin (BZ-PM) and 4-AHP in benzothiazine-type pheomelanin (BT-PM), we used factors of 36 and 7, respectively (Ito *et al*., 2011; d'Ischia *et al*., 2013).

MC1R genotyping

Genomic DNA was extracted from blood using the DNeasy Tissue kit (Qiagen, Hombrechtikon, Switzerland) and the Biosprint robot 96 (Qiagen). A 921 bp fragment of the *MC1R* gene was amplified using the following primers *MC1R*_4Fw (5'-GACCATGTCGACGCTGGC-3') and *MC1R*_955Rev (5'-GTCCCGCTGCCTACCAGGAG-3') designed on Barn Owl *Tyto alba* (San-Jose *et al*., 2015) and Tawny Owl (Emaresi *et al*., 2013) *MC1R* DNA sequences. The amplicon starts at 15 bp downstream of the translation start site and stops 8 bp upstream of the stop codon; thus only 23 bp of the coding sequence are missing. PCRs were performed in 20μL containing 2.5mM MgCl2, 0.2mM dNTPs, 4μL of GoTaq Reaction buffer 5×, 4μL of Q solution (Qiagen, Hombrechtikon, Switzerland), 500nM of each primer and 0.1U of GoTaq DNA polymerase (Promega, Dübendorf, Schweiz) and 10ng of genomic DNA. The cycle conditions were the following: 95°C for 5min followed by 35 cycles at 94°C for 30s, 61°C for 30s and 72°C for 60s and then a final extension at 72°C for 10min. The amplicons of 142 individuals were then PCR purified and sequenced in both directions at Microsynth (Microsynth, Balgach, Switzerland). Sequences were analysed with CodonCode Aligner 8.02.

Statistical Analysis

Analyses were performed using SAS 9.4 software (SAS Institute Inc., Cary, NC).

Initially we ran a general linear model (GLM SAS procedure) to study whether pigment concentration depended on melanin type (i.e. EM versus PM which is the sum of BZ-PM and BT-PM) and adult sex as fixed terms. We also entered the interaction term between sex and melanin type to test whether the relative importance of EM versus PM pigments in explaining the degree of reddish coloration differed between the two sexes. Then we ran a multiple linear regression model to study the relationships between amounts of EM and total PM pigments as predictors of degree of redness. Standard model validation graphs (Zuur, 2009) revealed that model assumptions of homoge-

neity of variance and normality of residuals were fulfilled. P values smaller than 0.05 were considered significant.

Ethical note

Data collection complies with the current laws of Spain and the fieldwork was authorised by the Consejería de Medio Ambiente y Ordenación del Territorio of the Junta de Andalucía (projects CGL2011-27561/BOS and CGL2014-56769-P; licence code: P06-RNM-01862). The study protocol was reviewed and approved by the ethical committee of the CSIC.

RESULTS

Melanin content in Eurasian Scops-owl feathers

In all feathers we found both EM (mean ± SE concentration 49.45 ± 2.49 μg/mg) and two types of PM: benzothiazine-type (BT-PM; mean ± SE concentration 11.95 ± 0.76 μg/mg) and benzothiazole-type (BZ-PM; mean ± SE concentration 4.53 ± 0.42 μg/mg) (Supplementary material, Appendix 1, Table A1, Figure A2). EM was more abundant than PM (melanin type effect: $F_{1,46} = 151.56$, $P < 0.0001$), and the pattern did not differ between male and female owls (sex*melanin type interaction: $F_{1,46} = 0.0008$, $P = 0.97$; sex effect: $F_{1,46} = 0.83$, $P = 0.35$).

EM was significantly and positively correlated with BZ-PM ($r_p = 0.46$, $P = 0.021$, N = 25), but not with BT-PM ($r_p = -0.05$, $P = 0.78$, N = 25) and the total amount of PM in feathers ($r_p = 0.34$, $P = 0.09$, N = 25).

Melanin content in relation to coloration

The degree of redness was positively associated with the amount of PM but unrelated to the amount of EM in feathers (Multiple regression: $F_{2,21} = 3.51$, $P = 0.04$; $R^2 = 0.25$; PM (Beta (SE): 0.53 (0.201), $t_{21} = 2.64$,

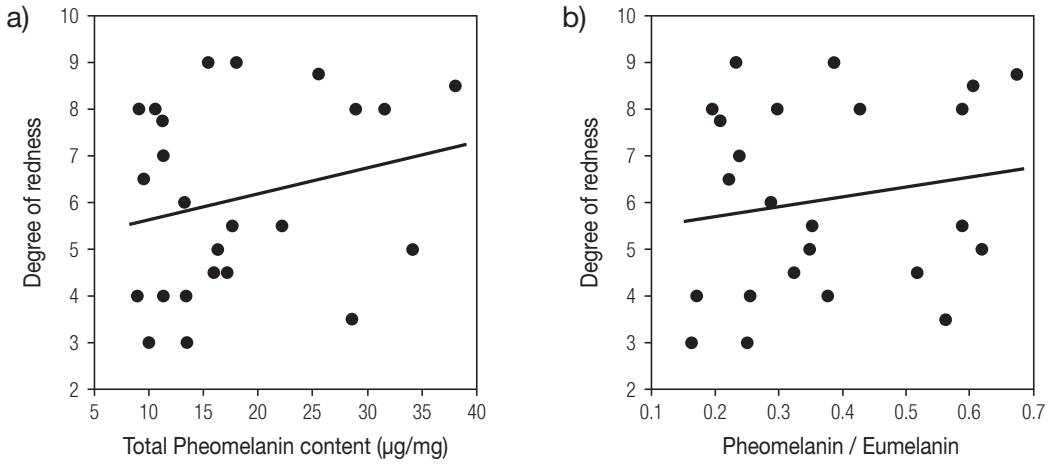

FIG. 1.—Relationships between degree of redness and a) PM content (i.e. BZ-PM + BT-PM) and b) PM / EM ratio in feathers of 25 Eurasian Scops-owls.
[Relaciones entre el grado de rojismo y a) el contenido de PM (i.e. BZ-PM + BT-PM) y b) la ratio PM / EM en plumas de 25 autillos.]

$P = 0.015$; EM (Beta (SE): −0.16 (0.20), $t_{21} = 0.81$, $P = 0.42$). Correlation analyses also revealed that individuals with a greater degree of redness had a higher PM/EM ratio ($r_p = 0.41$, $P = 0.04$, N = 25).

MC1R sequence and colour morphs

Among the 142 adult owls there were only two variable sites at the *MC1R* coding sequence: one synonymous substitution at site 111 (GAC codon mutated to GAT, encoded for the amino acid Aspartic acid, c.111C > T, D37D) and one non-synonymous amino acid substitution at site 70 (GCC codon mutated to ACC or even TCC, the encoded amino acids being either Alanine, Threonine or Serine, respectively, c.70G > A,T, A24T/S). Amino acid polymorphism at site 70 was shared by grey, intermediate and rufous-brown morphs (Supplementary material, Appendix 1, Table A2). The owls were monomorphic at all other sites known to determine major variation in melanin coloration in birds (Supplementary material, Appendix 1, Table A3).

Discussion

Melanin-based coloration in Eurasian Scops-owls

Our results suggest that variation in redness in male and female Eurasian Scops-owls is due to variation in melanin content. The amount of EM pigment was three times greater than that of PM pigment in the owls' head feathers, a ratio similar to that reported for the orange-red breast plumage of Eastern Bluebirds *Sialia sialis* (McGraw, Safran, and Wakamatsu, 2005) or Barn Swallows *Hirundo rustica* (McGraw, Safran, and Wakamatsu 2005), but remarkably larger than that reported for other polymorphic owls such as the Tawny Owl (ratio EM/PM 1.08, Gasparini *et al.,* 2009) or the Barn Owl (ratio EM/PM 0.13, Roulin *et al.,* 2008).

Although EM pigments were more abundant in feathers than PM pigments, when we considered EM and PM concentration separately as predictors of colour variation, we found that reddishness was indicative of high PM pigment deposition, but that it was unrelated to EM pigment concentration in feathers. In addition, EM and PM were not significantly inter-correlated across feathers suggesting that major colour variation in redness in Eurasian Scops-owls is primarily due to variation in PM pigment deposition in feathers. These findings would add to previous studies on melanin pigment content in Tawny Owls (Gasparini *et al*., 2009) and Barn Owls (Roulin *et al*., 2013) showing that graded changes in reddishness are related to changes in PM deposition in feathers.

Why reddish coloration is correlated with the amount of PM pigments stored in feathers and not with EM pigments is intriguing, and may be due to a differential functional role of EM and PM. Melanins are known to increase the resistance of avian feathers to abrasion and wear (Bonser, 1995; Mackinven and Briskie, 2014), and although it is unknown whether EM or PM differ in their mechanical proprieties, PM-rich feathers are assumed weaker than EM-rich ones (e.g. Galván and Solano, 2016). Eurasian Scops-owls are secondary cavity nesters that perch in dense vegetation and hunt on the ground (Del Hoyo, Elliott, and Sargatal, 1999). Hence, feathers with a high amount of EM may have primarily evolved in Eurasian Scops-owls to resist abrasion.

Regarding PM, a growing body of evidence has provided support for the idea that PM-based plumage colorations may function as honest signals of quality of the bearer which are constrained by physiological trade-offs or social interactions (reviewed in Roulin, 2016; Arai *et al*., 2017; Galván,

2018). PM production depends on the amount of cysteine and glutathione (GSH). GSH plays a critical role protecting cells from oxidative damages, in nutrient metabolism or in regulating immune function (Kosower and Kosower, 1978). Hence, there could be a physiological trade-off between anti-oxidative defence and PM expression, so that only high-quality individuals are able to express a high degree of reddishness (Galván *et al*., 2015). Indeed, it has been suggested that PM-based colour traits have a higher potential to evolve as honest signals of quality than EM-based colour traits due to the higher costs of PM production (Galván and Solano, 2016). In Eurasian Scops-owls, we have recently shown that two fitness surrogates (i.e. number of fledglings and the average fledgling mass at day 21) are not associated with female redness (Parejo *et al*., 2018), which would suggest that redness plumage variation would not reliably indicate differences in female quality. However, many aspects of individual quality (e.g. physiology) were not considered in that study. Moreover, the possible link between coloration and individual quality needs to be experimentally assessed in order to provide a sound test of a signalling function for PM coloration in Eurasian Scops-owls.

MC1R and colour variation in Eurasian Scops-owls

We have found that variation in the coding sequence of the *MC1R* fails to explain variation in the degree of redness of plumage in Eurasian Scops-owls. Although we did not sequence a short (23 bp) portion of the entire *MC1R* and cannot discard the possibility of a regulatory mutation near *MC1R*, we considered all SNP sites in this locus known to promote melanin colour variation (e.g. Theron *et al*., 2001; Gangoso *et al*., 2011; Mundy *et al*., 2004; Uy *et al*., 2009; Araguas *et al*., 2018). Hence, it seems unlikely that colour variation in Eurasian Scops-owls was determined by a non-synonymous mutation at the *MC1R* locus. This result is not unexpected given that about 150 genes have been identified to be involved in coloration and/or pattern designs in animals (Hubbard *et al*., 2010), and that different genes could encode for EM and PM. Future studies on the genetic basis of the PM-based polymorphism of Eurasian Scops-owls should consider studying coloration in relation to variability in other genes involved in melanogenesis, such as *MITF*, *ASIP*, *TYR*, *SLC45A2* and *TYRP1* that were not considered here (e.g. Chang *et al*., 2006; Gunnarsson *et al*., 2007; Linnen *et al*., 2009; Minvielle *et al*., 2010; Lehtonen *et al*., 2012; Bourgeois *et al*., 2016). In this regard, recent findings have shown that PM based polymorphism in the Reunion Grey White-eye *Zosterops borbonicus* was controlled by a single locus on chromosome 1 with two large-effect alleles (Bourgeois *et al*., 2017). In addition, other mechanisms, such as variation in expression of genes involved in melanogenesis and/or epigenetic effects at the *MC1R* locus, may better explain such continuous colour polymorphism (Emaresi *et al*., 2013; San-Jose *et al*., 2015; Galván, 2018). Finally, it is possible that differential regulation of a few genes rather than mutations in coding regions of the expressed genes could account for differences in coloration of Eurasian Scops-owls, such as recently shown in Dark-eyed Juncos *Junco hyemalis* (Abolins-Abols *et al*., 2018).

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Supplementary Electronic Material

Additional supporting information may be found in the on-line version of this paper. See volume 67(1) in www.ardeola.org

Figure A1. Colour variation and pheomelanin content in Eurasian Scops-owls.

Figure A2. Light microscopy of Eurasian Scops-owl feathers.

Table A1. Raw melanin pigment content in feathers of 25 adult Eurasian Scops-owls.

Table A2. Colour morph score and *MC1R* sequence variation for 137 adult Eurasian Scops-owls.

Table A3. MC1R sequence data for the 142 Eurasian Scops-owl individuals included in the study.

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