UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

POLIMORFISMO DE COLOR EN EL AUTILLO EUROPEO *Otus scops*: LIGANDO COLORACIONES MELÁNICAS, COMPORTAMIENTO Y FISIOLOGÍA EN AVES

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PROGRAMA DE DOCTORADO EN MODELIZACIÓN Y EXPERIMENTACIÓN EN CIENCIA Y TECNOLOGÍA (R007)

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Conformidad de los directores

Fdo. Deseada Parejo Mora Fdo. Jesús M Avilés Regodón

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En Badajoz, a

Fdo. Ángel Cruz Miralles

2021

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Jesús M. Avilés, Ángel Cruz-Miralles, Anne-Lyse Ducrest, Céline Simon, Alexandre Roulin, Kazumasa Wakamatsu, Deseada Parejo

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Entidad financiadora: Ministerio de Ciencia e Innovación. Programa Estatal de Fomento de la Investigación Científica y Técnica de Excelencia.

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Informe de los Directores de Tesis

Dra. Deseada Parejo Mora, como directora, y Dr. Jesús M Avilés Regodón como co-director de la tesis que lleva por título: "Polimorfismo de color en el Autillo europeo *Otus scops*: ligando coloraciones melánicas, comportamiento y fisiología en aves", de la que es autor el Doctorando y Licenciado en Ciencias Ambientales, Ángel Cruz Miralles, emiten el siguiente informe sobre la categorización de los artículos incluidos. Este informe responde al obligado cumplimiento del art. 46 apartado 2 de la normativa de la UNEX en relación a los depósitos de tesis doctorales por compendio de publicaciones. Este informe se elevará a la comisión académica del programa de Doctorado en modelización y experimentación en ciencia y tecnología (R007), para su aprobación.

En la presente tesis doctoral se incluyen los siguientes artículos:

1. **Determinants of color polymorphism in the Eurasian scops owl *Otus scops*.**

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Todos los coautores de los artículos mencionados aprueban el uso de estos para la realización de esta Tesis Doctoral. Las revistas elegidas para la publicación de los artículos son todas relevantes en sus categorías y los artículos son accesibles para la comunidad académica. Por lo tanto, el director y el co-director consideran que la presente Tesis Doctoral cumple con las condiciones exigidas para optar al grado de doctor.

En Badajoz a

Fdo. Deseada Parejo Mora Fdo. Jesús M Avilés Regodón

Directora de Tesis Codirector de Tesis

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RESUMEN

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RESUMEN

Uno de los mayores retos de la ecología evolutiva es entender cómo se origina y se mantiene la variación fenotípica en las poblaciones naturales. El estudio del polimorfismo de color con base genética es un paradigma clásico para entender los patrones de diversidad fenotípica y genética que se dan en la naturaleza. Esta tesis ahonda en el conocimiento de los mecanismos que promueven la variación fenotípica a través del estudio del polimorfismo de color en una población de autillo europeo. El presente trabajo demostró que el autillo europeo es una especie polimórfica con tres morfos presentes en los dos sexos y en todas las edades, si bien está coloración varía de manera continua desde las formas grises a las marrones-rojizas. La distribución de frecuencias de las variantes de color en la población no cambió durante los ocho años de estudio. El grado de rojismo del plumaje se asoció con la cantidad de feomelanina en las plumas, aunque la cantidad de eumelanina en estas fue tres veces superior. No se encontró relación entre el gen *MC1R* y la variación en la coloración del plumaje. Por tanto, 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*. Además, encontramos que los machos marrones-rojizos tardaron más tiempo en retornar al nido tras una molestia y que tenían niveles más altos de corticosterona en plumas que los grises. En las hembras, sin embargo, el comportamiento y los niveles de corticosterona en plumas no se asociaron con el color del plumaje. Las asociaciones encontradas entre el color, comportamiento y corticosterona en las plumas de los machos, pero no en las hembras, podrían sugerir la existencia de un fenotipo feomelánico integrado dependiente del sexo en el autillo europeo. Las hembras con coloraciones extremas no mostraron preferencias con respecto al color de los machos con los que se emparejan, mientras que las hembras intermedias prefirieron machos de coloración intermedia y mostraron una mayor supervivencia. Por tanto, el emparejamiento parece favorecer a los machos intermedios, porque todas las hembras incluyen machos intermedios entre sus parejas, y la supervivencia parece favorecer a las hembras intermedias. A pesar de esto, la proporción de individuos intermedios no aumentó durante el estudio lo que podría deberse a fluctuaciones temporales y/o espaciales a mayor escala en la selección sobre el color. Los insectos constituyeron el 89,9% de la biomasa aportada a los pollos durante el desarrollo temprano, siendo el orden de los ortópteros (69,7% de las presas), y en particular los saltamontes de la

familia *Acrididae*, la presa más abundante traída al nido mayoritariamente por los machos. Los machos marrones-rojizos cebaron menos saltamontes que los grises en condiciones de luna llena (i.e. alta luminosidad). Sin embargo, la eficiencia de alimentación de los machos no estaba relacionada con la luz de la luna. El número de saltamontes aportado por las hembras dependió de su coloración, pero no de la luz de la luna: las hembras marrones-rojizas cebaron menos saltamontes que las grises. Se evidencia la existencia de una segregación trófica dependiente del color basada en la variación de la luz de la luna en el autillo. En su conjunto estos resultados sugieren que el polimorfismo de color que se da en el autillo es complejo y explicable por varios mecanismos de selección natural y sexual que podrían actuar simultáneamente. La observación de una variación continua en la coloración en el autillo podría derivarse del efecto conjunto de la selección disruptiva que llevaría a favorecer las formas más marrones y grises del continuo, mientras que la mayor supervivencia y probabilidad de emparejamiento favorecería a los individuos intermedios.

INTRODUCCIÓN

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INTRODUCCIÓN

POLIMORFISMO

En la naturaleza existe una enorme variabilidad de formas, tamaños y colores tanto entre especies diferentes como entre individuos de la misma especie. El conjunto de los atributos o rasgos mediante los que se diferencian los distintos organismos constituyen su fenotipo que es dependiente de la estructura genética, pero que también está influenciado por las condiciones ambientales a las que está sometido a lo largo de su ontogenia, incluyendo los procesos epigenéticos (Johannsen 1911). El fenotipo engloba, por tanto, todas las características de un organismo y comprende múltiples niveles de organización, yendo desde el comportamiento (p.e. territorialidad) y los rasgos de historia vital (p.e. la edad de la primera reproducción), hasta la morfología (p.e. tamaño corporal) y la fisiología (p.e. niveles basales de hormonas).

En los organismos la variación genética puede surgir por diferentes mecanismos, y produce la aparición de formas alternativas de un mismo gen, denominadas alelos. Aproximadamente el 5-15% de los genes examinados en humanos son polimórficos (i.e. con varios alelos), y en muchos casos los distintos alelos pueden provocar cambios importantes en la expresión del fenotipo (Gillespie 1991) dando lugar a los polimorfismos visibles.

Ford (1940, 1945) definió el polimorfismo como “la ocurrencia conjunta en el mismo hábitat de dos o más formas genéticas distintas de una especie, en una proporción tal que la más rara de ellas no puede mantenerse por mutación recurrente”. En la década siguiente, Huxley (1955) propuso el término “morfo” para referirse a los distintos morfotipos o variantes del fenotipo que presentan las especies, sugiriendo que además de la existencia de morfos discretos, podía existir una continuidad entre los mismos, a modo de gradiente, aunque esta era rara entre las especies. Este último autor además incluyó la necesidad de reproducción cruzada entre morfos para considerar una especie como plenamente polimórfica, redefiniendo el polimorfismo como: “la coexistencia en una población de dos o más formas que se reproducen entre ellas, claramente distintas y genéticamente determinadas, la menos abundante de las cuales está presente en números demasiado grandes como para deberse únicamente a la mutación recurrente". Las discrepancias surgidas a la hora de considerar como especies polimórficas solo aquellas que

presenten morfos discretos o incluir también a aquellas que muestren una variación continua, se desvanecen a lo largo de la historia, de forma que actualmente se consideran también polimórficas las especies que muestran variación continua en la expresión de la coloración (e.g. McGraw et al. 2004, 2005; Hofmann et al. 2016). En la actualidad, la definición más aceptada de polimorfismo es la que propusieron Cavalli-Sforza y Bodmer (1971): "El polimorfismo genético es la ocurrencia en la misma población de dos o más alelos en un locus, cada uno con frecuencia apreciable", considerándose como frecuencias apreciables aquellas superiores al 1%. Siendo esta frecuencia mucho mayor que la que se observa en la naturaleza para la tasa de mutación de un alelo simple (Sheppard 1975).

Entender las causas y las consecuencias de las variaciones fenotípicas en la naturaleza ha supuesto un desafío para los biólogos evolutivos desde los tiempos de Darwin (Darwin 1859), debido a que estas diferencias constituyen el sustrato sobre el que opera la selección natural. En este contexto, dado que los morfos tiene una importante componente genética, es fundamental identificar los mecanismos evolutivos que mantienen el polimorfismo para entender los patrones de diversidad fenotípica y genética que se dan en la naturaleza (Mundy 2005; Hoekstra 2006).

El estudio del polimorfismo puede ayudar a entender el potencial de las especies para adaptarse a los desafíos del cambio global. Las especies polimórficas presentan variantes capaces de explotar diferentes nichos dentro de las poblaciones (Galeotti et al. 2003) y, por tanto, podrían ser más aptas para adaptarse a cambios ambientales a lo largo del tiempo (Forsman et al. 2008). La condición polimórfica podría también aumentar las probabilidades de colonización exitosa de nuevos ambientes promoviendo la expansión del rango de distribución (Forsman et al. 2008). Esto podría llevar a una alta tasa de especiación en las especies polimórficas si con posterioridad a la expansión se dieran restricciones al flujo génico entre las poblaciones (Gray and McKinnon 2007; Hugall and Stuart-Fox 2012).

EJEMPLOS DE POLIMORFISMOS

El polimorfismo está ampliamente extendido en la naturaleza, dándose tanto en el reino animal (Lamotte 1959; Shine et al. 1998; Forsman 1999; Hoffman and Blouin 2000; Kruger and Lindstrom 2001; Galeotti et al. 2003; Stuart-Fox et al. 2004; Hoekstra et al. 2005; Gosden et al. 2011) como en el vegetal (Marshall and Jain 1969; Mogford 1974a; Kay 1978; Warren and Mackenzie 2001; Ross-Ibarra et al. 2008).

Un ejemplo clásico de polimorfismo se da en el grupo sanguíneo en humanos. Los grupos sanguíneos se clasifican según los antígenos que se expresan en los eritrocitos, existiendo 3 grupos mayoritarios (Landsteiner 1901) y otros 33 muy escasos (Storry et al. 2016). Los tres grupos mayoritarios son el A, B y 0, que presentan los antígenos A, B o no tienen antígenos, respectivamente. Los grupos mayoritarios A, B y 0 se han mantenido en las poblaciones humanas a pesar de la selección natural dado que cada una de ellos es más ventajoso que el resto ante algunas enfermedades, como por ejemplo el cólera, la sífilis o la malaria (Stanley 2009).

Otro ejemplo clásico es el del polimorfismo en la coloración de la mariposa de los abedules (*Biston betularia*) (Ford 1976; Majerus 1998). Esta polilla, que presenta dos morfologías claramente diferenciadas, una blanca moteada y otra negra, descansa durante el día posada en los troncos de los abedules. La frecuencia de estos morfos en las poblaciones donde se estudió depende de su capacidad de camuflaje frente a sus principales depredadores, las aves. En condiciones naturales los morfos oscuros eran menos frecuentes que los claros ya que los últimos eran más crípticos en los troncos sobre los que crecían líquenes. Sin embargo, el oscurecimiento de la corteza de los abedules y la desaparición de los líquenes como consecuencia de la contaminación ambiental producida por la industrialización en el siglo XIX, trajo consigo un incremento en la frecuencia de las polillas oscuras que en las nuevas condiciones presentaban un mejor camuflaje (Kettlewell 1958; Cook 2003). Hay ejemplos de polimorfismos de color en casi todos los grupos animales. Así, por ejemplo, entre los anfibios encontramos algunas especies de ranas como *Fejervarya limnocharis, Pelophylax ridibundus* y *Lithobates sylvaticus*, que presentan dimorfismo en base a la presencia de una franja pigmentada dorsal (Moriwaki 1953; Browder et al. 1966; Berger and Smielowski 1982). Los morfos de estas

especies eluden la depredación según su grado de mimetismo en el ambiente, además presentan distinta resistencia a la desecación (Nevo 1973) y distinto uso del hábitat (Tarkhnishvili et al. 1999). En aves, el chingolo gorjiblanco (*Zonotrichia albicollis*) presenta dos morfos diferenciados en relación a la coloración de sus cabezas, observándose individuos con franjas blancas y otras con franjas marrones, que se mantienen gracias al emparejamiento selectivo con el morfo opuesto (Lowther 1961). En plantas también se da el polimorfismo de color. Por ejemplo, las plantas fanerógamas del género *Cirsium* exhiben flores moradas y blancas, así como flores con tonalidades intermedias entre ambas. Este polimorfismo floral se mantiene a través de mecanismos de selección dependiente de la frecuencia que ejercen distintas especies de polinizadores sobre las variantes de color (Mogford 1974a, b).

LA PARADOJA DEL MANTENIMIENTO DEL POLIMORFISMO

En su Teoría de la Evolución (1859) Darwin propuso que las poblaciones naturales evolucionan como consecuencia de la selección natural, la cual solo permite la supervivencia de los organismos más aptos y por tanto solo estos serán capaces de reproducirse y así transmitir sus genes a su descendencia (Futuyma 2013). Según esta teoría, el polimorfismo no debería existir de forma estable dentro de las poblaciones y solo podría originarse como un estadio transitorio, hasta que una sola variante prevaleciese. ¿Por qué persisten entonces los polimorfismos en la naturaleza? Para resolver esta paradoja, se han propuesto al menos 6 mecanismos diferentes, tres ligados a procesos de selección natural (Galeotti et al. 2003; Roulin 2004a) como: (1) la selección apostática; (2) la selección disruptiva, y (3) la ventaja del heterocigoto o heterosis; dos a procesos de selección sexual como (4) la selección intrasexual y (5) el emparejamiento selectivo o selección intersexual. Por último, además, algunos autores apuntan que el polimorfismo también podría ser mantenido por selección neutra (6) (Tabla 1).

A continuación, se describe brevemente la base de estos mecanismos y los agentes de selección que subyacen. Paralelamente se lleva a cabo una revisión exhaustiva sobre la literatura de polimorfismos en la naturaleza con el objeto de cuantificar la importancia relativa de este fenómeno en diferentes taxones y de los posibles mecanismos que favorecen su persistencia en las poblaciones. Para ello, se realizó una revisión sistemática de la literatura (a fecha de 12 de mayo de 2021), buscando en la Web of Knowledge con las siguientes palabras clave (búsqueda

1: ‘Polymorphism' y ‘disruptive selection’; búsqueda 2: ‘Polymorphism' y ‘heterozygous advantage’; búsqueda 3: ‘Polymorphism' y ‘apostatic selection’; búsqueda 4: ‘Polymorphism' y ‘sexual selection’; búsqueda 5: ‘Polymorphism' y ‘male-male competition’; y búsqueda 6: ‘Polymorphism' y ‘neutral selection’). La búsqueda bibliográfica arrojó 6402 publicaciones, de ellas 320 se conservaron después de consultar los resúmenes y resultados para verificar que se ofrecían evidencias sobre el mantenimiento del polimorfismo por alguno de estos mecanismos. De los artículos seleccionados, además del mecanismo que explicaría el mantenimiento del polimorfismo, se extrajo la información sobre el tipo de polimorfismo al que se refiere y la especie en la que se da (Tabla 1).

Tabla 1. Número de artículos científicos que explican el mantenimiento de polimorfismos dentro de las poblaciones según el mecanismo que lo haría posible y para las distintas clases taxonómicas. En aquellos casos en los que dos o más mecanismos actuaban de forma conjunta han sido contabilizados tantas veces como el número de mecanismos implicados.

	Selección apostática								Selección disruptiva								Selección intersexual							
	Color	Genético	Morfología	Comportamiento	Fisiología	Historia vital	Sistema inmune	**Total**	Color	Genético	Morfología	Comportamiento	Fisiología	Historia vital	Sistema inmune	**Total**	Color	Genético	Morfología	Comportamiento	Fisiología	Historia vital	Sistema inmune	**Total**
Aconoidasida	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Actinobacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alphaproteobacteria	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Gammaproteobacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sphagnopsida	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Monocotyledoneae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Magnoliopsida	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	2	0	0	0	0	2
Liliopsida	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	3	0	0	0	0	3
Pinopsida	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0
Chromadorea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Echinoidea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Asteroidea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cephalopoda	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
Gastropoda	4	0	0	0	0	0	0	4	16	0	0	0	0	0	0	16	2	0	0	0	0	0	0	2
Bivalvia	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
Malacostraca	1	0	0	0	0	0	0	1	4	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
Arachnida	1	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0
Insecta	1	0	0	0	0	0	0	1	19	7	2	1	1	1	0	31	5	3	1	0	1	0	0	10
Chondrichthyes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Actinopterygii	2	0	1	0	0	0	0	3	14	0	33	2	0	0	0	49	10	0	1	1	0	0	0	12
Amphibia	1	0	0	0	0	0	0	1	5	0	1	7	0	2	0	15	0	0	0	1	0	0	0	1
Reptilia	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	6	2	0	0	0	0	0	0	2
Ave	4	0	0	0	0	0	0	4	20	0	7	2	0	0	0	29	4	1	1	0	0	0	0	6
Mammalia	0	0	0	0	0	0	0	0	1	2	0	0	1	0	0	4	1	0	0	0	0	0	0	1
	14	0	1	0	0	0	0	15	87	13	46	12	2	3	0	163	24	4	8	2	1	0	0	39

	Selección intrasexual								Heterosis								Selección neutra							
	Color	Genético	Morfología	Comportamiento	Fisiología	Historia vital	Sistema inmune	**Total**	Color	Genético	Morfología	Comportamiento	Fisiología	Historia vital	Sistema inmune	**Total**	Color	Genético	Morfología	Comportamiento	Fisiología	Historia vital	Sistema inmune	**Total**
Aconoidasida	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	5
Actinobacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
Alphaproteobacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gammaproteobacteria	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	4
Sphagnopsida	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
Monocotyledoneae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	2
Magnoliopsida	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	9	0	0	0	0	0	10
Liliopsida	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2	0	5	0	0	1	0	0	6
Pinopsida	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chromadorea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
Echinoidea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1
Asteroidea	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	1
Cephalopoda	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gastropoda	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bivalvia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Malacostraca	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Arachnida	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Insecta	0	1	0	1	0	0	0	2	1	4	0	0	0	0	0	5	1	8	3	0	2	0	0	14
Chondrichthyes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
Actinopterygii	5	0	0	0	0	0	0	5	0	2	0	0	0	0	0	2	0	4	1	0	0	0	0	5
Amphibia	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	2
Reptilia	4	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1
Ave	1	0	0	0	0	0	0	1	1	4	0	0	0	0	0	5	1	5	0	0	0	0	0	6
Mammalia	0	0	0	0	0	0	0	0	0	18	0	0	1	0	1	20	0	22	0	0	1	0	1	24
	9	1	0	1	0	0	0	11	4	7	0	0	0	0	0	11	7	41	4	0	3	0	0	55

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MECANISMOS PARA EL MANTENIMIENTO DEL POLIMORFISMO

La selección apostática (1) es un mecanismo de selección negativa dependiente de la frecuencia que postula que los morfos menos abundantes serán menos reconocibles por depredadores y presas potenciales (Tabla 2), lo que les proporcionará ventajas frente a los morfos más frecuentes (Clarke 1969). Esto ocurriría porque tanto depredadores como presas desarrollan imágenes de sus presas y depredadores comunes pero no de las menos frecuentes (Tinbergen 1960; Rohwer and Paulson 1987; Allen 1988; Bond and Kamil 1998, 2002; Fowlie and Kruger 2003). La selección apostática también puede darse entre los parásitos de cría y sus hospedadores, se ha sugerido que las especies hospedadoras elaborarían una imagen del morfo más frecuente del parásito, pero serán menos hábiles en detectar el parasitismo por parásitos con fenotipos más raros (Honza et al. 2006). Hasta la fecha, la evidencia empírica sobre la existencia de selección apostática es muy escasa, siendo de todos los mecanismos propuestos el que con menos frecuencia explicaría el polimorfismo (Tabla 1). Dado que la selección apostática se basa en la detección de diferencias que se perciben por el canal visual, la mayoría de polimorfismos estudiados en el contexto de la selección apostática son de color (ver sin embargo Hori 1993) (Tabla 1).

La selección disruptiva (2), por su parte, se basa en que la selección favorece a los individuos de una población con morfologías extremas en perjuicio de las intermedias (Galeotti et al. 2003) (Tabla 2). Cada uno de los morfos alternativos presentaría ventajas adaptativas frente al resto en unas condiciones ambientales concretas (Mather 1955; Rueffler et al. 2006). Por tanto, un prerrequisito para la selección disruptiva es la existencia de heterogeneidad ambiental ya sea espacial o temporal (Hedrick 2006). Dado que las fuerzas selectivas varían en el espacio y en el tiempo, los fenotipos extremos obtienen unos valores de fitness globalmente similares, lo que permite que los extremos se perpetúen en la población (Ford 1945; Mather 1955; Skulason and Smith 1995; Galeotti et al. 2003; Bond and Kamil 2006). La selección disruptiva (2) puede explicar el polimorfismo cuando los morfos se especializan en distintas estrategias de forrajeo (Furness 1987; Skulason and Smith 1995; Bolnick et al. 2003) o presas (Skulason and Smith 1995; Karell et al. 2021) dentro de una población. La mayor parte de los polimorfismos analizados (46%, Tabla 1) parecen estar explicados por un mecanismo de selección disruptiva y la mayoría de ellos se refieren también a polimorfismos visuales (e.g. Cain and Currey 1963; Caldwell 1982; Whitney et

al. 2018). No obstante, la selección disruptiva puede explicar también polimorfismos comportamentales como diferencias en la preferencia por tipo de presas (Collins and Holomuzki 1984; Svanbäck and Persson 2009; Levis et al. 2017), patrones migratorios (Hermes et al. 2015), reproductivos (Mendoza-Cuenca and Macías-Ordóñez 2010) y diferencias en rasgos de historia vital (Healy 1974) u otros polimorfismos no visibles de tipo fisiológico (Zhurkevich and Fomicheva 1976; Baerwald et al. 1999; Shumate et al. 2011) (Table 1).

Tabla 2. Hipótesis propuestas para explicar el mantenimiento de los polimorfismos, predicciones y tipo de estudios que apoyan cada hipótesis.

Hipótesis	Predicciones	El mecanismo explica:
A) Por selección natural		
1) Selección apostática	Los morfos menos abundantes son ventajosos frente a los morfos más frecuentes.	Pocos casos, todos en polimorfismos visibles y la mayoría vinculados a la coloración.
2) Selección disruptiva	Los morfos extremos tienen ventaja frente a los intermedios. Entre los morfos extremos el más ventajoso depende de la variación en las condiciones ambientales en el tiempo (Segregación temporal) y el espacio (Segregación espacial).	Muchos casos (≈46%), la mayoría en polimorfismos visibles y muchos de ellos de color.
3) Heterosis	Los individuos heterocigóticos para un alelo tendrían ventajas frente a los homocigóticos.	Muchos casos, polimorfismos de diversos tipos, pero la mayoría en polimorfismos no visibles
B) Por selección sexual		
4) Selección intrasexual	Los diferentes morfos tienen ventajas que aumentan y disminuyen cíclicamente.	Pocos casos, la mayoría en polimorfismos de color.
5) Selección intersexual o emparejamiento selectivo	Los diferentes morfos tienen ventajas que aumentan y disminuyen cíclicamente según el morfo predominante del sexo opuesto.	Muchos casos, la mayoría en polimorfismo de color.
C) Por selección neutra	Ningún morfo tiene ventajas	Todos los casos que no apoyan mecanismos anteriores

La ventaja del heterocigoto (3), también llamado heterosis, es un mecanismo que explica el mantenimiento del polimorfismo (Kruger and Lindstrom 2001; Briggs et al. 2011; Boerner et al. 2013) en base a que los individuos heterocigóticos para un alelo tienen mayor fitness que los homocigóticos (Gray and McKinnon 2007) (Tabla 2). La heterosis proporcionaría una mayor eficacia biológica a sus portadores mediante tres posibilidades alternativas: 1) la sobredominancia, según la cual los individuos heterocigotos se encuentran fuera del rango fenotípico de sus progenitores de manera que mientras que el heterocigoto podría, por ejemplo,

transmitir ventajas y desventajas, los padres homocigotos podrían transmitir sólo una desventaja (Parsons and Bodmer 1961); 2) La aversión de genes deletéreos recesivos que contribuyen a la depresión endogámica, de manera que los individuos heterocigóticos expresarán menos alelos deletéreos, ya que, al estar combinados con un alelo distinto, en los casos en los que este alelo sea dominante sobre el deletéreo, este último dejará de expresarse (Fisher 1930; Charlesworth 2012). Y, 3) la mayor diversidad alélica de los individuos heterocigóticos que les ayudaría a la hora de afrontar distintos factores estresantes (Brown 1997; Hansson and Westerberg 2002). La heterosis explica el polimorfismo en la resistencia a enfermedades (Gemmell and Slate 2006), la fecundidad (Gemmell and Slate 2006), la resistencia a parásitos (e.g. Rödel et al. 2020), la visión del color (e.g. Riba-Hernández et al. 2004; De Araújo et al. 2006) o el metabolismo (Banaszek et al. 2009), entre otros. Aunque existe algún ejemplo de polimorfismos visibles explicados por este mecanismo (Krüger et al. 2001; Takahashi et al. 2015; Balfour et al. 2018; Kellenberger et al. 2019), la mayoría de estudios que analizan este mecanismo se centran en polimorfismos a nivel genético (e.g. Banaszek et al. 2009; Kekäläinen et al. 2009; Doyle et al. 2019; Kaňková et al. 2020) (Tabla 1).

Por su parte, la selección intrasexual (4) (Darwin 1871; Huxley 1938), que se da entre machos, podría también favorecer el mantenimiento del polimorfismo si la competencia entre individuos de un mismo morfo es mayor que la que se da entre individuos de morfo distinto (Dijkstra et al. 2008) (Tabla 2). En este escenario el polimorfismo podría persistir en el tiempo cuando existen más de dos morfos diferentes en el que cada uno es ventajoso respecto a uno de los otros, sólo en algunas condiciones. Este sistema en el que no existe un vencedor absoluto es el que se conoce en la teoría de juegos como el juego de piedra-papel-tijera. Cuando la interacción es doble, es decir juegan dos sujetos, siempre hay un claro vencedor. Sin embargo, a medida que se agregan más jugadores el juego se vuelve más complejo, con el éxito de diferentes estrategias que a menudo aumentan y disminuyen cíclicamente (e.g. Sinervo and Lively 1996; Fitze et al. 2014; San-Jose et al. 2014). Los pocos ejemplos de polimorfismo que han sido explicados en base a este mecanismo están basados en el color (Tabla 1) y están centrados en peces (e.g. Kingston et al. 2003) y reptiles (e.g. Pérez i de Lanuza et al. 2017).

La selección sexual también podría favorecer el mantenimiento del polimorfismo a través de un emparejamiento selectivo, es decir, por un mecanismo de selección intersexual (5) (Lank 2002; Rolán-alvarez et al. 2012) (Tabla 2). Este mecanismo tiene su base en la diferencia en inversión reproductiva que se da en los distintos sexos como consecuencia de los distintos costes de producir gametos masculinos y femeninos (Darwin 1871). Por lo general, las hembras, que son quienes suelen tener un coste mayor en la reproducción, son las encargadas de la elección de pareja. Para ello eligen machos con características que pueda conferir un mayor éxito a su descendencia, ya sea por los genes que transfieren a su descendencia o por los beneficios directos como por ejemplo el esfuerzo de los padres en el cuidado de la prole (Kokko et al. 2003). Existen numerosos estudios que apoyarían un papel de la selección intersexual en el mantenimiento del polimorfismo, y encontramos estudios mostrando tanto machos como hembras que seleccionan su pareja en base al morfo, con independencia del suyo propio (Gamble et al. 2003; Pierotti et al. 2008). La gran mayoría de estos estudios se refieren a emparejamientos discordantes en base al color (e.g. Lowther 1961; Sacchi et al. 2018) (Tabla 1).

Finalmente, algunos autores apuntan a que el polimorfismo también podría ser mantenido por una selección neutra (6) (Tabla 2), es decir, el carácter polimorfo no estaría sometido a selección sino que sería un simple correlato no funcional de variación genética al azar (Roulin 2004a).

EL CASO PARTICULAR DE LOS POLIMORFISMOS DE COLOR Y LOS FENOTIPOS COMPLEJOS COMO EXPLICACIÓN AL MANTENIMIENTO DEL POLIMORFISMO

A partir de la revisión realizada se constata que el polimorfismo ha sido estudiado de forma desigual en función de los rasgos, enfocándose el mayor número de estudios en el color (40% de los estudios, Tabla 1) y, en concreto, en coloraciones con base melánica. Este tipo de polimorfismo ha sido además más frecuentemente estudiado en las aves (21% de los estudios, Tabla 1).

Las melaninas son los pigmentos responsables de la mayor parte de las coloraciones no estructurales marrones, negras y grises de los vertebrados (Haase et al. 1992; Ito and Wakamatsu 2003). Estas melaninas son macromoléculas formadas por la polimerización

oxidativa de compuestos fenólicos o indólicos. La eumelanina y feomelanina son sus dos tipos principales y por lo general, las eumelaninas producen coloraciones negras y grises, mientras que las feomelaninas otorgan tonos rojizos y marrones (McGraw 2006).

Ambas tienen como precursor la tirosina que, por oxidación a L-3,4-dihidroxifenilalanina (L-DOPA, por sus siglas en inglés L-3,4- dihydroxyphenylalanine) o directamente a partir de L-DOPA, produce DOPA-melanina. Que esta DOPA-melanina sea feomelanina o eumelanina depende de si la dopaquinona, un metabolito extremadamente reactivo de la L-DOPA, reaccione con L-cisteína o no. Por lo tanto, la presencia de L-cisteína es un determinante clave para la producción de eumelanina o feomelanina (Ozeki et al. 1997).

Un gran número de estudios empíricos han mostrado que la variaciones en la coloración melánica se relacionan consistentemente con la variación en otros rasgos del fenotipo, pudiendo generar “fenotipos melánicos complejos”, que se integrarían por relaciones en el desarrollo, genéticas y funcionales (Pigliucci and Preston 2004). El estudio de las bases adaptativas de la coloración basada en melaninas desde una perspectiva integradora constituye un sistema idóneo para el estudio de la evolución de los fenotipos complejos y, por ende, para entender la variabilidad fenotípica. Puesto que los morfos de color presentan un alto componente genético y su expresión no es sensible, o lo es poco, al ambiente (Buckley 1987), los morfos podrían funcionar como marcadores genéticos del fenotipo. En estos casos los morfos melánicos podrían haber evolucionado en respuesta a la selección natural y/o social, pero podrían también representar una respuesta indirecta a la selección sobre otros rasgos del fenotipo genéticamente correlacionados con el color (Ducrest et al. 2008). Esto ocurriría porque la selección no actúa de forma aislada sobre los rasgos del fenotipo sino que lo hace sobre el conjunto de rasgos que integran el fenotipo (Lande and Arnold 1983). El estudio de fenotipos complejos (Pigliucci and Preston 2004), aporta por tanto un marco teórico novedoso para el estudio de los procesos evolutivos (Pigliucci 2003; Pigliucci and Preston 2004), en el que la selección natural podría favorecer la evolución de ciertos rasgos integrados, pero en otros casos limitarla (Merilä and Björklund 2004).

Se ha sugerido que las coloraciones basadas en melaninas podrían desempeñar un papel clave en los procesos de integración fenotípica (Ducrest et al. 2008; Fargallo et al. 2014; Kim and

Velando 2015; San-Jose et al. 2017). La biosíntesis de eumelaninas y feomelaninas se produce a través de rutas metabólicas en cuya expresión están implicados genes muy bien conservados dentro de los vertebrados (Ducrest et al. 2008). La melanogénesis se produce en el sistema de las melanocortinas, ubicado principalmente en el hipotálamo, donde las neuronas del núcleo arqueado producen proopiomelanocortina (POMC). El gen de las POMC codifica un precursor polipeptídico, llamado pre-proopiomelanocortina (pre-POMC) cuyo subproducto principal es la POMC. Tras sucesivos procesamientos por parte de células específicas y modificaciones de la prohormona POMC surgen las melanocortinas y algunas endorfinas (Pritchard and White 2007). Estas melanocortinas, que pueden ser al menos de 4 tipos distintos, se unen a uno de los 5 genes receptores de melanocortinas *MCR*. Algunos de estos genes se expresan en la piel, como es el caso del *MC1R* y su unión con las melanocortinas o bien con las proteínas de señalización Agouti (ASIP del inglés) determinando el tipo de melaninas producidas. Serán eumelaninas en el caso de la unión entre *MC1R* y melanocortinas, o feomelaninas si la unión se produce entre *MC1R* y el ASIP (Ducrest et al. 2008). Pero las melanocortinas y sus proteínas antagonistas, también se unen con otros genes *MCR*, modulando la actividad fisiológica y comportamental. Así pues, la unión o no de melanocortinas con el gen *MC2R* afecta a la respuesta fisiológica al estrés a través del eje hipotalámico-pituitario-adrenal y a la producción de hormonas esteroides. Por su parte el gen *MC3R* se sabe tiene implicaciones en el gasto energético, el consumo de alimento, actividad cardiovascular, renal y antiinflamatoria. Además del gasto energético y el consumo de alimentos, el *MC4R* afecta a la resistencia al estrés, la actividad sexual, el acicalamiento, el estiramiento y el bostezo, la regeneración nerviosa, sensibilidad al dolor, la actividad antipirrética (fiebre) y frente a la muerte celular (antiapoptótica). En lo que se refiere al gen *MC5R*, tiene efecto sobre la actividad de las glándulas exocrinas, la agresividad y la respuesta inmune (Ducrest et al. 2008). El hecho de que las melanocortinas necesiten la implicación del gen *POCM* en su biosíntesis y que estas melanocortinas afecten en la expresión de otros rasgos del fenotipo como los citados ocasiona que frecuentemente los rasgos melánicos co-varíen con rasgos comportamentales, morfológicos, fisiológicos o de historia vital (Galeotti et al. 2003; Jawor and Breitwisch 2003; Roulin 2004a), dando lugar a un efecto pleiotrópico de este gen o los genes encargados de su expresión y maduración (Ducrest et al. 2008).

El efecto pleiotrópico podría también manifestarse en estadios tempranos de la ontogenia (Wilkins et al. 2014). Durante el desarrollo embrionario, algunos tejidos del cerebro y las glándulas suprarrenales derivan de la cresta neural (Anderson 1997) y lo mismo sucede con los melanocitos (Singh and Nüsslein-Volhard 2015). Por tanto, se ha sugerido que variaciones en los genes implicados en el desarrollo de la cresta neural podrían, por pleiotropía, afectar tanto a rasgos de coloración melánica como a rasgos comportamentales o fisiológicos.

Alternativamente, la pleiotropía que explica los fenotipos melánicos podría basarse en efectos de las hormonas. Los niveles de algunas hormonas (p.e. la testosterona, la hormona del estrés, la hormona concentradora de melaninas o la hormona juvenil) juegan un papel clave en la regulación de distintas funciones metabólicas, y se han visto relacionadas con las coloraciones melánicas (e.g. Almasi et al. 2008, 2010, 2013). Por tanto, los genes que regulan la secreción hormonal, la afinidad hormonal por las proteínas transportadoras, las tasas de degradación y conversión y la interacción con los tejidos diana entre otros, podrían coordinar la co-expresión de rasgos fisiológicos, comportamentales y morfológicos (Ketterson et al. 2009). Estudios previos han analizado las relaciones entre la eumelanina y este tipo de hormonas, observándose de forma general que los individuos más oscuros presentan niveles menores de corticosterona (Rohwer and Wingfield 1981; Almasi et al. 2008, 2010) y el único trabajo que analiza la relación con las feomelaninas mostró niveles más elevados de corticosterona en sangre para aquellos individuos más feomelánicos (Saino et al. 2013). Con respecto a otras hormonas, también se ha sugerido la existencia de una correlación positiva entre las hormonas sexuales y las eumelaninas (Ducrest et al. 2008; Laucht et al. 2010; Muck and Goymann 2011; pero ver sin embargo Fargallo et al. 2007; Moreno et al. 2014; Béziers et al. 2017). Por lo que respecta a las feomelaninas, se ha observado un tamaño mayor en los ovarios de hembras menos feomelánicas (Roulin 2009), lo que podría estar vinculado a la producción de las hormonas sexuales femeninas. En el caso de la testosterona en machos, se ha correlacionado positivamente con la feomelanina en algunas especies (Safran et al. 2008; Eikenaar et al. 2011) y en otras se han observado patrones opuestos dependiendo de las zonas del plumaje analizadas (Haase et al. 1995).

Además de las relaciones entre hormonas y melaninas, las hormonas podrían jugar un papel como moduladoras de comportamientos (Carere et al. 2003; Kralj-Fišer et al. 2007; Schoech et

al. 2009; Garamszegi et al. 2012). Así, en mamíferos, se observó una mayor agresividad en los leones (*Panthera leo*) de melenas oscuras (West and Packer 2002), y esta misma relación positiva entre agresividad y coloración más melánica se ha descrito en aves (McGraw et al. 2003), aunque con patrones opuestos según el sexo (Boerner and Krüger 2009); también en reptiles (Mafli et al. 2011; Ibáñez et al. 2016; Bruinjé et al. 2019) y en algunas especies de peces (Horth 2003; Kim and Velando 2015 pero ver Höglund et al. 2000).

FENOTIPOS MELÁNICOS BASADOS EN FEOMELANINAS

Aunque numerosos estudios han mostrado la existencia de correlaciones entre las coloraciones melánicas y otros rasgos del fenotipo, la gran mayoría se han centrado en el papel de las eumelaninas, o bien en las melaninas en su conjunto, sin diferenciar entre los dos tipos de pigmentos melánicos (Ducrest et al. 2008, ver más arriba). Por ello, es difícil establecer el papel de las feomelaninas en la integración de fenotipos complejos, aun cuando la producción de feomelaninas puede comprometer la síntesis de las eumelaninas (Ducrest et al. 2008). De hecho, se ha sugerido que las relaciones entre ambos tipos de melaninas con otros rasgos fenotípicos debieran ser opuestas, o al menos diferentes (Hubbard et al. 2010; Roulin et al. 2011b; Jenkins et al. 2013; Galván and Solano 2016; ver si embargo Fargallo et al. 2014). Por tanto, es fundamental conocer si las feomelaninas promueven la correlación entre rasgos del fenotipo, más aún cuando este pigmento se considera un indicador de la calidad individual (hipótesis de condición- dependencia) (Roulin 2016; Arai et al. 2017; Galván 2018a).

Durante la melanogénesis, los niveles bajos de cisteína en los melanocitos permiten la síntesis de eumelaninas, mientras que cuando estos niveles son altos se sintetizan las feomelaninas (García-Borrón and Olivares Sánchez 2011; Riley et al. 2011). La cisteína tiene como su principal reservorio fisiológico al glutatión, un tripéptido que, además de ser considerado un antioxidante celular muy importante, está implicado en multitud de procesos fisiológicos (Wu et al. 2004). Es por ello que la deficiencia de glutatión en el organismo contribuye al estrés oxidativo, incrementando el envejecimiento celular y la patogénesis de muchas enfermedades. Como la síntesis de feomelaninas requiere de cisteína que consume glutatión (Pavel et al. 2011; Morgan et al. 2013), se ha sugerido que solo aquellos individuos en muy buena condición podrán permitirse el coste fisiológico que implica la elaboración de pigmentos feomelánicos (Galván et

al. 2015). De hecho, diversos estudios han observado correlaciones entre las coloraciones feomelánicas y distintos indicadores de condición (Roulin et al. 2011a; Galván et al. 2012; Grunst et al. 2014a, b; Emaresi et al. 2016; Arai et al. 2018; Galván 2018b; Hasegawa et al. 2019; Leclaire et al. 2019; Teerikorpi et al. 2019). En la mayoría de los casos se han observado concentraciones mayores de feomelanina en aquellos individuos de buena calidad y con un menor estrés oxidativo, si bien se dan algunas excepciones (e.g. Leclaire et al. 2019). Sin embargo, concentraciones elevadas de cisteína pueden resultar tóxicas, por lo que en periodos en los que el individuo está sometido a un nivel bajo de estrés resulta necesario eliminar el excedente (Galván 2017).

Conocer las implicaciones concretas de las feomelaninas en los fenotipos complejos es importante por múltiples razones: 1) como se apunta anteriormente, su producción podría comprometer la de eumelanina (Ducrest et al. 2008), lo que podría causar que ambos tipos melánicos mostrasen patrones distintos en su relación con otros rasgos del fenotipo (Hubbard et al. 2010; Roulin et al. 2011b; Jenkins et al. 2013; Galván and Solano 2016). 2) Además, las diferencias entre los dos pigmentos podrían venir dadas por las propias características físicas del pigmento (Slominski et al. 2004). Se considera que las eumelaninas protegen frente a la radiación ultravioleta evitando la aparición de melanomas, mientras que las feomelaninas se suponen fototóxicas (Huijser et al. 2011). Aunque no se conocen los mecanismos subyacentes, se ha observado en humanos una tasa mayor de apoptosis en las células adyacentes a feomelaninas, lo que genera una mayor sensibilidad de los individuos feomelánicos a padecer quemaduras solares o cáncer de piel (Takeuchi et al. 2004). 3) Además, existen diferencias en la energía absorbida procedente de la radiación solar entre ambas melaninas, siendo mayor para los pigmentos eumelánicos (Huijser et al. 2011), lo que podría tener implicaciones en la termorregulación. De hecho, bajo similares condiciones de radiación solar, los individuos con plumajes más oscuros de paloma bravía (*Columbia livia*) tienen temperaturas corporales más elevadas que aquellos más claros (Angelier 2020). 4) De igual forma, las coloraciones que confieren cada uno de los tipos melánicos pueden influir de distinta manera en la comunicación social o sexual, o bien en el camuflaje o cripsis, dado que las coloraciones a base de feomelaninas suelen ser más conspicuas, al menos desde la perspectiva del ojo humano y en determinados ambientes. 5) También se ha observado que las melaninas pueden conferir cierta

resistencia frente a la degradación de algunos tejidos (Bonser 1995), bien sea por parte de bacterias o por abrasión como sucede en las plumas y picos de las aves (Burtt and Ichida 1999; Schreiber et al. 2006), y, aunque no está claro que las distintas formas químicas de la melanina confieran una capacidad protectora distinta, se ha sugerido que las feomelaninas otorgarían una menor resistencia (Liu et al. 2005). 6) Por último, dado que la producción de feomelaninas tiene altos requerimientos de cisteína, las coloraciones feomelánicas tendrían un mayor potencial para evolucionar como señales honestas de calidad que las coloraciones eumelánicas. Todas estas diferencias entre los dos principales compuestos químicos melánicos, refuerzan la necesidad de ampliar el conocimiento en las relaciones de las melaninas con otros rasgos y en especial las feomelaninas.

OBJETIVOS

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OBJETIVOS

En este contexto, el objetivo global de esta tesis es estudiar la asociación entre distintos rasgos del fenotipo y la coloración feomelánica en una especie de ave nocturna con polimorfismo de color, el autillo europeo (*Otus scops*), con el objeto de contribuir al conocimiento sobre el origen y mantenimiento de los polimorfismos de color en la naturaleza. Para ello describiremos en la especie la variación natural en coloración del plumaje a lo largo del tiempo y en relación con la ontogenia, estudiando los mecanismos fisiológicos y moleculares de dicha variación, y la covariación entre esta coloración con rasgos comportamentales, fisiológicos y estrategias vitales. Finalmente, estudiaremos la dieta aportada a los pollos en el nido en relación a la coloración de sus padres con el objeto de evaluar la hipótesis de la segregación trófica para explicar el polimorfismo en la especie.

A continuación, se indican los sub-objetivos específicos de la tesis con los que se pretende alcanzar este objetivo general, indicándose además el capítulo donde se aborda cada uno de ellos:

Objetivo 1: Analizar la variación natural en la coloración del plumaje del autillo y su estabilidad temporal para poder establecer las bases del polimorfismo de color en la especie (Capítulo I).

Objetivo 2: Determinar los mecanismos próximos que determinan el color del plumaje en el autillo centrándonos en la composición y concentración de distintas melaninas y el polimorfismo en el gen *MC1R* (Capítulo II).

Objetivo 3: Evaluar el grado de covariación entre la coloración melánica, el comportamiento y la fisiología en el autillo (Capítulo III).

Objetivo 4: Estudiar la relación entre el grado de melanismo y el emparejamiento y entre el grado de melanismo y dos correlatos del fitness (éxito reproductor y supervivencia) en el autillo (Capítulo I).

Objetivo 5: Estudiar la dieta y las tasas de aprovisionamiento al nido en relación al color y en relación con el ambiente lumínico: un test de la hipótesis de la segregación trófica (Capítulo IV).

COHERENCIA Y JUSTIFICACIÓN UNITARIA DE LA TESIS

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El compendio de cuatro artículos que se presenta en esta tesis constituye una asociación conceptual lógica para abordar la complejidad del problema de las causas y consecuencias del polimorfismo de color en la naturaleza: 1) describir el fenómeno y su variabilidad, 2) estudiar los mecanismos en la base de dicha variación; 3) estudiar la covariación entre rasgos del fenotipo y coloraciones melánicas; 4) intentar integrar esas bases en una evaluación exhaustiva de una hipótesis clásica para explicar el mantenimiento del polimorfismo.

De estos artículos científicos, tres han sido publicados durante el desarrollo de la tesis. En el primero de ellos se describió el polimorfismo en el autillo europeo y se estudió la relación entre el emparejamiento y correlatos del fitness como éxito reproductor y supervivencia con las distintas coloraciones (Parejo et al. 2018). En el segundo de los trabajos se analizan los mecanismos fisiológicos y moleculares que determinan el color del plumaje en la especie, mostrándose que su coloración se basa fundamentalmente en feomelaninas y a genes o elementos reguladores diferentes del *MCR1* analizado (Avilés et al. 2020). Finalmente, en el tercer artículo se muestra la correlación entre rasgos comportamentales y fisiológicos con las coloraciones melánicas (Cruz-Miralles et al. 2020).

METODOLOGÍA

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METODOLOGÍA

ÁREA DE ESTUDIO

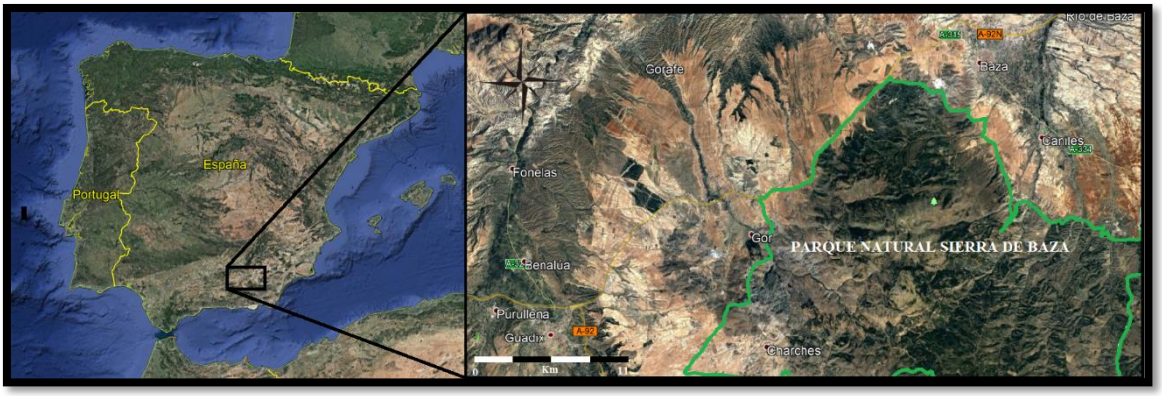

Figura 1: Área de estudio en el entorno de la Sierra de Baza.

Nuestro estudio se llevó a cabo en las inmediaciones del Parque Natural de la Sierra de Baza, en la provincia de Granada, al sureste de España (37°18'N, 3°11'W) (Figura 1) a unos 1000 m s. n. m. de altura promedio. La región presenta un clima mediterráneo típico, con precipitaciones anuales que rondan los 400 mm y caracterizado por veranos secos y calurosos, con temperaturas medias por encima de los 22 °C e inviernos húmedos y lluviosos, con temperaturas suaves. Esta zona tiene una vegetación variable, que incluye áreas de cultivo de cereal, encinares abiertos, plantaciones de almendros y olivos, así como zonas de ramblas y choperas (Figura 2). Aprovechando estas encinas dispersas, se instalaron progresivamente a lo largo de los años hasta 582 nidales artificiales de corcho, con unas dimensiones de 24 x 24 cm de lado en su base, una altura de 40 cm y un orificio de entrada de 6 cm de diámetro (Rodríguez et al. 2011). La red de nidales alberga una comunidad de aves cavernícolas que incluye autillos, mochuelos (*Athene noctua*), grajillas (*Corvus monedula*), carracas (*Coracias garrulus*), abubillas (*Upupa epops*), estorninos negros (*Sturnus unicolor*), carboneros (*Parus major)* y herrerillos *(Cyanistes caeruleus*). También las cajas nido son a veces ocupadas por roedores como el ratón de campo (*Apodemus sylvaticus*), ratón doméstico (*Mus musculus*) o lirón careto (*Eliomys quercinus*). Desde 2010 a 2018 se siguió la población de autillo europeo que se reprodujo en las cajas nido.

Figura 2: Paisajes agrarios del entorno de la Sierra de Baza.

MODELO DE ESTUDIO

Figura 3: Hembra de Autillo europeo capturada durante el seguimiento de la reproducción.

Para tratar de alcanzar los objetivos propuestos centramos nuestro estudio en el Autillo europeo (*Otus scops*), una especie que ha sido descrita como polimórfica según su coloración melánica (Del Hoyo et al. 1999; Sacchi et al. 1999; Galeotti et al. 2009).

El autillo europeo es una rapaz nocturna de tamaño pequeño (Figura 3) que se encuentra en latitudes medias-bajas en la región Paleártica (Cramp 1998). Aunque se reproduce en nuestras latitudes, la especie realiza una migración transahariana, desplazándose más al sur para su invernada, si bien se han detectado individuos residentes o poblaciones invernantes en el sur de Europa (BirdLife International 2019) (Figura 4). A nuestra área de estudio los individuos llegan

en abril, para comenzar la reproducción a lo largo del mes de mayo (Parejo et al. 2012). El macho es quien selecciona el territorio, para ello busca una cavidad en troncos de árboles, rocas o muros, incluso agujeros en taludes arenosos o en los tejados. También aceptan las cajas nido. Tras la aceptación de la oquedad por parte de la hembra, se asientan en ese territorio (König and Weick 2008). Durante el periodo de reproducción, la pareja muestra un comportamiento territorial (Galeotti and Sacchi 2001), defendiendo un área de unas 30 hectáreas alrededor del nido (Cramp 1998). Suelen ubicarse en áreas abiertas, como pastizales o tierras de cultivo (Cramp 1985; Panzeri et al. 2014). Realizan una sola puesta por periodo reproductor que oscila entre 2 y 6 huevos, que ponen a intervalos de 2 días. La incubación dura 24-25 días (Cramp 1998), es llevada a cabo por las hembras y comienza tras la puesta del segundo huevo (Del Hoyo et al. 1999). En las primeras semanas tras la eclosión, la hembra es la encargada de atender a la nidada. El macho se encarga de aportar presas a la hembra durante la incubación y también a su descendencia tras el nacimiento de estos. Desde el momento de la eclosión, la hembra va incorporándose poco a poco a la captura de presas para su prole. Los pollos permanecen en el nido entre 21 y 29 días (Cramp 1998) y tras abandonarlo se localizan en las ramas próximas del propio árbol o de algún arbusto cercano, donde escalan por medio de sus garras y pico. Cuando tienen alrededor de 33 días, los pollos son capaces de volar con autonomía, pero son cuidados y alimentados por los padres al menos durante un mes más (König and Weick 2008). La alimentación del autillo es básicamente insectívora, mostrando preferencias por individuos de la orden Orthoptera. Aunque también consumen otros invertebrados como polillas, escolopendras o fásmidos e incluso pequeños vertebrados como reptiles, paseriformes o roedores (Streit and Kalotás 1991; Bavoux et al. 1993; Marchesi and Sergio 2005; Latková et al. 2012). La especie ha sufrido reducciones significativas en sus poblaciones en toda Europa como consecuencia de las modificaciones en las prácticas agrícolas (Arlettaz 1990; Denac 2003). En España, donde se concentra la mayor población europea, se le considera la especie de búho más amenazada (Gragera 1996).

Figura 4: Mapa de distribución del Autillo europeo (BirdLife International 2019).

SEGUIMIENTO DE INDIVIDUOS

En cada temporada de cría, comenzando desde la última semana de abril, los nidales fueron revisados una vez a la semana hasta que se detectó el inicio de la puesta. Tras esa visita los nidos solo se visitaron una vez más al finalizar la puesta y otra más justo antes de la fecha estimada de eclosión para capturar a la hembra. Se trató de minimizar el número de visitas para reducir las molestias y evitar el abandono del nido. Tras la eclosión de los pollos, las visitas al nido fueron semanales, registrando en ellas los parámetros reproductivos como el número de pollos, su peso, longitud de pico, ala y tarso de estos como estimas de condición corporal, y su éxito para abandonar el nido. Además, todos los pollos fueron marcados con anillas metálicas para su identificación individual posterior.

La captura de los adultos se realizó a mano en el caso de las hembras, durante el día, cuando dormían en el nido y aun en el periodo de incubación. Los machos fueron capturados, después de la eclosión de los pollos, mediante trampas dentro de la caja nido cuando se disponían a cebar a las crías. Una vez capturados se les tomaban medidas del pico, tarso, ala y peso. Todos los adultos fueron anillados con anillas metálicas y se determinó el sexo mediante la observación de la placa incubatriz, la cual solo está presente en hembras. Además, todos los individuos adultos fueron fotografiados para la caracterización de su coloración. Se tomaron para ello dos fotografías estandarizadas del individuo, una frontal y otra de espaldas. Mediante un arnés de

velcro, los individuos se fijaron cuidadosamente a una caja de color neutro que garantizaba condiciones de luz estables (Figura 5). Junto a la cabeza del individuo se colocó una carta de color (X-Rite ColorChecker® Passport). Las fotografías se tomaron a una distancia de 50 cm del animal y para ello se utilizó una cámara digital (Canon EOS 1300D, Lens: EF-S 18-55 IS II) montada sobre un trípode. Las imágenes se captaron con flash, con una apertura del diafragma de 4.5, una velocidad de disparo de 1/200 y una sensibilidad del sensor para captar la luz (ISO) de 800.

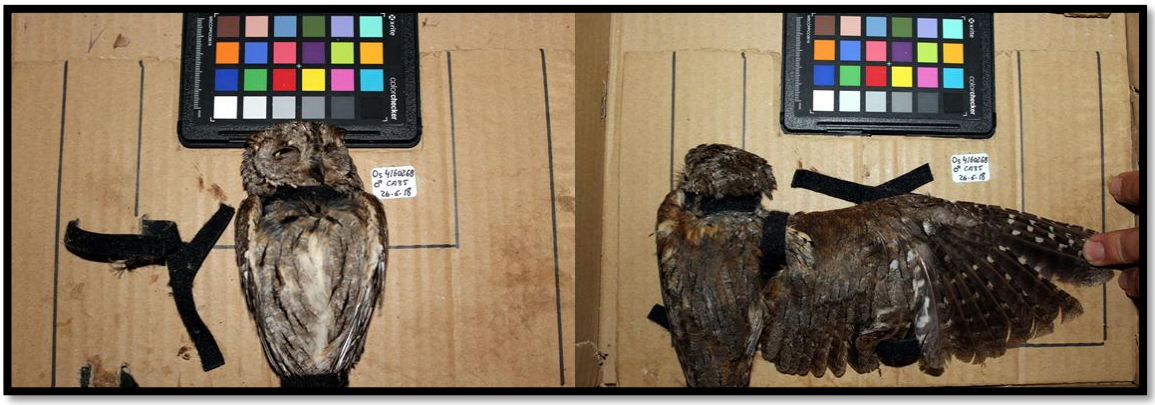

Figura 5: Macho de Autillo europeo sujetado mediante arnés para la toma de fotografías de la parte frontal y dorsal.

Adicionalmente, se recolectaron plumas de la cabeza, el pecho y la espalda para realizar medidas espectrofotométricas en condiciones de total oscuridad en el laboratorio. También se recolectaron plumas para medir la concentración de corticosterona acumulada en las mismas. Concretamente se extrajo la tercera cobertera primaria del ala izquierda de cada individuo. Además, se midió la frecuencia respiratoria, para lo que se estimó el número de movimientos del pecho durante 30 segundos, como una medida individual de respuesta al estrés por manejo (Fucikova et al. 2009). Para las hembras, además, se grabó en video su comportamiento en la caja-nido al ser molestada y también su comportamiento en mano, para poder clasificarlas con posterioridad como agresivas o no, en función del comportamiento exhibido.

ESTUDIO DEL COLOR, CLASIFICACIÓN DE LOS MORFOS

A partir de las fotografías se procedió a asignar una puntuación a cada individuo según su coloración. Para ello previamente todas las fotografías fueron estandarizadas usando el complemento Adobe® Photoshop Lightroom 6. Para otorgar la puntuación centramos nuestra atención en tres zonas del plumaje, la cabeza, el pecho y el dorso con las alas. Cada una de

estas partes fueron puntuadas con valores de 1 a 3 en función del color predominante, dando el valor de 1 cuando la tonalidad predominante del plumaje era gris y 3 cuando era marrón; se asignó un valor de 2 cuando ninguno de los dos colores destacaba sobre el otro. En aquellas partes del cuerpo en las que estas situaciones entre dos puntuaciones no quedaban claras, se asignaron puntuaciones intermedias de 1.5 o 2.5. Las puntuaciones otorgadas a las tres partes del cuerpo estuvieron fuertemente correlacionadas entre ellas (cabeza-pecho: $r_p = 0.65$, $p < 0.001$, $n = 224$; cabeza-dorso: $r_p = 0.50$, $p < 0.001$, $n = 224$; y pecho-dorso: $r_p = 0.40$, $p < 0.001$, $n = 224$), lo que indica que estas reflejan información similar respecto a la coloración del plumaje. Para conseguir un único valor de coloración por individuo, se sumaron los valores otorgados a cada una de las tres partes, oscilando las puntuaciones finales entre valores de 3 a 9. Para cerciorarnos de que el método de asignación de puntuaciones era fiable, 4 investigadores puntuaron individualmente a una muestra de 28 aves seleccionadas aleatoriamente, observándose una alta repetibilidad ($F_{27,28} = 11.054$; $p < 0.001$; $R^2 = 0.78$). Las puntuaciones otorgadas a todos los individuos objeto de este estudio fueron realizadas por dos investigadores, Juan Rodríguez-Ruiz y Ángel Cruz-Miralles. El valor definitivo que se asignó a cada ave fue resultado del valor promedio de ambas puntuaciones. Además, para verificar que nuestro método reflejaba la variación en la coloración del plumaje del autillo, se comparó con medidas espectrofotométricas tomadas de plumas de las distintas partes del cuerpo. Las medidas del espectrofotómetro que mostraron una coloración marrón rojiza mayor, fueron para aquellos individuos a los que se les había asignado una puntuación más alta en nuestra clasificación. Tanto las medidas del espectro como los análisis comparativos entre estas y las puntuaciones asignadas, se describen en detalle en el Capítulo I.

CAPÍTULO I: Determinants of color polymorphism in the Eurasian scops owl *Otus scops*.

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CAPÍTULO I: Determinants of color polymorphism in the Eurasian scops owl *Otus scops.*

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ABSTRACT

Understanding the evolutionary forces maintaining avian color polymorphisms is a major challenge in evolutionary ecology. Aiming to give new insights into the functional basis of color polymorphism, we studied plumage color variation and its associations with fitness proxies in an individually marked population of scops owls *Otus scops* during 8 yr. We found a repeatable method to assign individuals to three discrete morphs, using both photography and spectrophotometry. Individuals were either grey (33%), intermediate (37%) or brown (30%). Scops owl proved to be polymorphic as the three morphs were found in the two sexes and across ages. Frequency distribution of color within the population did not vary for the two sexes during the study period, and, within individuals, color was repeatable and not explained by plumage maturation. Females of the two extreme morphs seemed to mate at random while intermediate females seemed to mate assortatively. The color of females was not related to laying date, mean fledging mass or number of fledglings per nest. Finally, intermediate females survived slightly better than females of the other morphs. Hence, pairing seems to favor intermediate males, because all females include intermediate males among their mates, and survival seems to favor intermediate females. Despite this, the proportion of intermediate individuals did not increase during the study. This fact may allude to the importance, not analysed here, of larger scale temporal and/or spatial fluctuations in selection acting on different fitness-affecting factors, which may help to explain the maintenance of color polymorphism in the species.

INTRODUCTION

Species in which individuals of the same age and sex within a population display more than one of multiple existing color variants that are genetically inherited and whose expression is rather independent of the environment and body condition are considered to be polymorphic in color (Roulin 2004). This phenomenon is widespread in many animal taxa and specifically in vertebrates where it has been widely reported in lizards (Sinervo et al. 2001) and frogs (Medina et al. 2013), for instance. However, color polymorphism is relatively rare in birds, occurring only in 3.5% of the species (Roulin 2004), although it is surprisingly common in some bird orders as in Strigiformes (33.5% of species, Galeotti et al. 2003).

Color polymorphism has largely attracted the attention of evolutionary biologists (Huxley 1955, Mather 1955), and several maintenance mechanisms have been described for the different groups. Despite this, none of the mechanisms can be universally applied to all groups and species. Since morphs are mostly genetically determined (Mundy 2005, Hoekstra 2006), this question is critical to understand the maintenance of genetic diversity in nature. The most common mechanisms invoked to explain persistence of color polymorphisms are frequency-dependent selection, heterozygote advantage, genotype by environment interactions and local adaptation and sexual selection (Lank 2002, Roulin 2004, Briggs et al. 2011). In the end, the evolutionary stability of color polymorphisms implies a selective balance among alternative morphs in the long term (Losey et al. 1997, Bond and Kamil 1998, Roulin 2004).

Indeed, in birds, there are several examples showing that colour morphs may facilitate the exploitation of alternative conditions or develop different strategies. For instance, the white-throated sparrow *Zonotrichia albicollis* segregates spatially by morph color, with white males settling in high density areas exposed to high competition but providing high extra-pair copulations opportunities as compared to tan males (Formica et al. 2004). Also, the pale and dark morphs of the Eleonora's falcon *Falco eleonorae* adopt different breeding strategies, with pale individuals behaving highly colonially and dark ones being more territorial and more successful in most years (Gangoso et al. 2015). Polymorphic species belonging to the order Strigiformes seem to have wider spatial niches than monomorphic species (Galeotti and Rubolini 2004). Indeed, recent comparative evidence has shown that owls living under more variable luminal conditions, i.e., species with diurnal and crepuscular habits and those inhabiting in a mixture of open and closed habitats, were more likely to show color polymorphism (Passarotto et al. 2018), suggesting that different morphs may have an advantage in different environments. In one of these species, the tawny owl *Strix aluco*, annual morph frequencies vary over long time in relation to annual weather conditions (Galeotti and Cesaris 1996, Roulin et al. 2003). Viability selection against the brown morph was strong in cold years and diminished in milder winter conditions (Galeotti and Cesaris 1996, Karell et al. 2011), probably because of morph differences in thermoregulatory physiology (Mosher and Henny 1976) and/or in some other physiological property such as metabolism or immunity (Ducrest et al. 2008). In addition, different morphs may differ in other traits such as behavior, or reproductive strategies (Roulin 2004), which may lead to differential reproductive

success and/or survival. For example, alternative color morphs have shown differential defensive behavior in a number of raptor species such as the common buzzard *Buteo buteo* (Boerner and Krüger 2009), barn owl *Tyto alba* (Van den Brink et al. 2012), tawny owl (da Silva et al. 2013) and Eleonora's falcon (Gangoso et al. 2015). Defensive behavior towards predators and competitors might affect survival and reproduction (Boerner and Krüger 2009) because defense may determine vulnerability to predators and help to access and maintain the highest quality territories. Whenever defensive behavior triggered a trade-off between survival and reproductive success, dark and light individuals would achieve the same fitness (i.e. one morph may survive longer but produce smaller broods than the other morph), which could help to explain the maintenance of color polymorphism. Therefore, by the development of alternative strategies, the different morphs within a species might achieve the same fitness. Finally, polymorphism may be maintained by factors such as sexual selection (assortative mating, Lank 2002).

The Eurasian scops owl *Otus scops* is a Strigiform largely described as a color polymorphic species based on the occurrence of two (dark-reddish and grey) or three (including intermediates) main melanin-based morphs (Del Hoyo et al. 1999, Sacchi et al. 1999, Galeotti et al. 2009). However, none scops owl study has analysed morph distribution in relation to age and sex classes within populations. This raises the possibility that alluded polymorphism was rather a case of plumage coloration maturation (as in the collared pygmy owl *Glaucidium brodiei* (Lin et al. 2014)) or due to sexual dichromatism (as in the long-eared owl *Asio otus* and the burrowing owl *Athene cunicularia* (Johnsgard 1988)).

Here, we studied variation in morph frequency, and the functional basis behind, in a ringed breeding scops owl population. Temporal variation in morph frequency could occur due to within-individual changes in plumage coloration or because the composition of individuals changed in the population due to emigration and/or immigration. Aiming to assess the relative importance of these mechanisms, here, we analyse first whether morph frequency is maintained through time for the two sexes and later on, using individuals with repeated observations, we analyse the effect of age on coloration and whether individual coloration was repeatable through years. Furthermore, we examine the relationships between plumage coloration, pairing and fitness correlates (reproductive success and survival). Under the hypothesis stating that the Eurasian

scops owl is a polymorphic species we expected that: 1) all morphs occur in the same sex and age classes; and that 2) temporal variation in morph frequency, if it exists, was not due to individual plumage maturation. In addition, if the morphs are maintained by any of the proposed mechanisms linked to relative fitness of different morphs (Meunier et al. 2011), 3) color morphs should either get subtle different fitness advantages and/or the color-specific fitness should vary with time. Alternatively, if color polymorphism is the consequence of the pairing system, either by sexual selection or not, 4) pairing should be dissasortative (see however O'Donald 1983 and Krüger et al. 2001 for assortative mating maintaining polymorphism).

MATERIALS AND METHODS

STUDY SYSTEM

Our study was performed from 2010 to 2017 in the Hoya of Guadix-Baza, Granada, southeast Spain (37°18'N, 3°11'W) where a scops owl population breeding in nestboxes was monitored. The area is an extensive agricultural landscape with scattered holm oaks *Quercus ilex* where cork-made nestboxes (measurements: base of 24 × 24 cm, 40 cm height and opening of 6 cm in diameter) were installed to attract medium-sized hole-nesting birds (Rodríguez et al. 2011).

Scops owls are medium-sized (91 g) nocturnal and migratory birds (Cramp 1998) arriving throughout April into the study population from their winter quarters in Africa (Parejo et al. 2012). In our study area scops owls begin reproduction throughout May (Parejo et al. 2012), making one clutch per year of about 2–6 eggs that are laid each 1–3 d. Incubation starts from the laying of the second egg, takes 24–25 d, and is performed by the female (Del Hoyo et al. 1999). Nestling rearing takes 21–29 d on average (Cramp 1998).

Each year, nest-boxes were visited every seven days till the occupation by a scops owl pair from the beginning of the breeding season (end of April). After occupation, nests were only visited once more after the end of laying and just before the estimated hatching date to capture and ring the incubating female. After hatching, nests were visited weekly to record reproductive parameters.

Throughout the study period, we systematically trapped and photographed adults, which allows morph categorization. Females were trapped before egg hatching by hand while sleeping during the day. From 2012 onwards, males were also captured with nest-traps at night during the chick-rearing period. Birds were measured and banded with individually numbered metal rings to be

recognized in subsequent years. Sex of adults was determined by inspection of the brood patch that is only present in females. Most of the fledglings born in the study area that returned to breed (8 individuals recruited from 335 ringed juveniles through the study period) were at least 2-yr-old birds (87.5% of individuals), therefore we assigned a minimum age of 2 yr to every bird of unknown origin and age recruited in our population at the first time of capture, and calculated their relative age from that moment. For analytical purposes, as most breeding adults were new in the population (156 out of 234 captures), and hence assigned to the 2-yr-old category, the rest of individuals were either assigned to the 2 yr-old (native birds breeding for the first time) or to the older than 2 yr-old class (recaptured breeders being faithful to the study area).

Just before fledging, 20–21 d-old fledglings were ringed and weighed, and nests were re-visited after ten days, to verify fledging. Nestlings not found in the nests during that last visit were considered to have fledged.

COLOR SCORING

Two standardized photographs were taken for each captured individual: one head-on, in which we could observe head and breast plumage; and other to the back part in which we observed the back and wings. All pictures were taken at a distance of about 50 cm from the animal and always in shadow areas around the nests to homogenize light conditions. Pictures were then used to classify morphs by focusing on redness extension at three body parts, namely head, breast and wings–back (Supplementary material Appendix 1 Fig. A1a, b). Each body part was scored among 1 to 3 points depending if they were predominantly greyer or browner. In the head, score 1 was assigned when red-brown color was barely observed; score 2 was assigned when discontinuous red-brown spots could be observed; and score 3 was assigned to heads with continuous red-brown spots. Concerning the breast individuals scored as 1 had higher relative proportion of grey compared to red-brown in the breast and those scored 3 had higher relative proportion of red-brown compared to grey; score 2 was assigned to the even situation. Finally, in wings and back, we compared the red-brown secondary covert feathers, found in all our birds, with the rest of the back. Then, when red-brown feathers are dull and there is an evident contrast with grey of the rest of the back it was assigned a score of 1; we assigned score 2 if red-brown was vivid and was repeated in some parts of the back; and when bright red-brown was distributing through all the

back, we scored with 3. Half scores between two values were assigned when we found the intermediate situation between two consecutive scores. Scores of the three body parts highly correlated each other (head-breast: $r_p = 0.65$, $p < 0.001$, $n = 224$; head-back: $r_p = 0.50$, $p < 0.001$, $n = 24$; and breast-back: $r_p = 0.40$, $p < 0.001$, $n = 224$), suggesting that the different body parts reflected similar information concerning color. Scores of the three body parts were summed to get an individual score for every bird so that scores ranged from 3 to 9 points. Aiming to qualify reliability of our morph scoring method, photographs from 28 birds were randomly selected to be scored by 4 different researchers, obtaining a high repeatability ($F_{27,28} = 11.054$; $p < 0.001$; $R_2 = 0.78$). Hence, 219 birds (♂: 73, ♀: 146) were subsequently scored individually by AC and JR. A final score for each bird was obtained by averaging the two scores.

PLUMAGE COLOR VARIATION AND MORPHS

Also, we validated our morph classification by analyzing differences in spectrophotometric measures among the different morphs in a sample of individuals (n = 129 birds). Upon capture, we plucked three to five feathers from the same location of head, breast and back of each individual. For color measurements, feathers from each region and individual were carefully placed onto matte cardboard resembling the way they naturally lay on the bird (i.e. arranging the feathers in an overlapping fashion with the reverse side oriented up).

Spectral data was always recorded in total darkness with an Ocean Optics DH 2000 spectrophotometer. Plumage reflectance was quantified in the range 300–700 nm with a deuterium and a halogen light source using a bifurcated micron fibre optic probe at a 45° angle from the feather surface and illuminating an area of 1 mm^2. Using the spectra acquisition software package OOIBase, we sequentially recorded 10 spectra relative to a standard white reference (WS-2) and then averaged the spectra to reduce electrical noise from the collection array within the spectrometer. This process was repeated three times, the probe lifted and replaced on the feather sample between each scan. We then averaged the three spectra for each body region and individual (Supplementary material Appendix 1 Fig. A1c).

A principal components analysis (PCA) was performed on reflectance data (i.e. reflectance at the 215 possible 1.86 nm intervals between 300 and 700 nm) to reduce the number of correlated variables into a few orthogonal variables summarizing color variation (Cuthill et al. 1999, Avilés

et al. 2006). Invariably, the first principal component (PC1) obtained from reflectance spectra on natural objects describes achromatic variation, essentially brightness, and this often explains more than 90% of the spectral variation (Cuthill et al. 1999, Avilés et al. 2006). Principal components 2 and 3 (PC2 and PC3, respectively) represent variation in hue and saturation (i.e. chromatic variation).

STATISTICAL ANALYSES

We first investigated whether the frequency of color morphs varied with the sex and age of individuals by performing a generalized lineal mixed model (GLMM, GLIMMIX SAS procedure) with the morph score as the dependent variable, and the sex and the age (as two-years old individuals versus older individuals) as explanatory variables. For that purpose, we used all data from captured individuals.

ANNUAL VARIABILITY IN MORPH FREQUENCY AND CAUSES

We explored the annual variation in morph scores and morph class in relation to sex. For these analyses, we first used data from all the years from females (2010–2017) and then only used data from the years we captured individuals from the two sexes (2012–2017). First, we performed a general lineal model (GLM, GLM procedure in SAS) with the morph score as dependent variable, and the year as explanatory variable for the dataset on females and with the sex, the year and their interaction as explanatory variables for the subset of data on both sexes from 2012 to 2017. Also, we used the morph class assigned to each individual as a dependent multinomial variable and performed a generalized lineal mixed model (GLMM, GLIMMIX SAS procedure) with the year as the explanatory variable for the dataset on females and the sex, the year and their interaction as explanatory variables for the other subset of data on both sexes.

To investigate whether plumage coloration changes with age in adults, we used data from two-years-old or older individuals captured more than once. With this data we analysed first, by means of a lineal mixed model (LMM, MIXED procedure in SAS) and then by means of a GLMM, whether the age of the individual (as being two-years old individuals versus older ones) affected, respectively, the morph score (continuous variable) and the morph class (multinomial variable with three levels: grey, intermediate or brown) assigned to each individual. In the two analyses, the individual identity was introduced as a random factor to account for the fact that observations

from the same individual are not independent. In addition, for this subset of data, we analysed whether the colour score and the colour morph assigned to individuals captured more than once were repeatable.

PAIRING AND COLOR POLYMORPHISM

To test if scops owls mated assortatively regarding their color we first run a general lineal model (GLM) (GLM procedure in SAS) to analyse the relationship between the the color score of independent females and their corresponding males. Secondly, we tested whether the assigned color morph of paired individuals were related by means of a generalized lineal mixed model (GLMM, GLIMMIX SAS procedure) in which the female morph was introduced as a dependent multinomial variable. In these two analyses, the year was introduced as a factor to account for the yearly variation in the number of available individuals of each morph. We considered one breeding attempt per female, randomly selected among the available pairings when more than one mating event had been recorded, with males of known color.

Additionally, as mating may differ for different color morphs, we analyzed whether pairing was either assortative or not as a function of the individual morph. For this, we performed a logistic regression model (GENMOD procedure in SAS) in which the concordance of paired morphs (pairing with the same versus pairing with a different morph as a binomial variable) was the dependent variable and female and male morphs were the explanatory factors. Moreover, for the years with higher sample size (from 2014 until 2017), we rerun this same analysis but also including the year and the interaction with the other explanatory factors to take into account the potential yearly variation in availability of mates of each color morph.

FITNESS PROXIES AND COLOR POLYMORPHISM

For this purpose, only data from females were analysed because sample size for males was insufficient (73 observations from 45 different males in the 6 yr). We considered one breeding attempt per female, randomly selected among the available reproductive events to avoid pseudoreplication. As we only had one observation for many females (89) and only some of the females had two or more observations (25), we could not run an overall analysis by including individual as a random effect. As estimates of fitness, we used two fecundity components: 1) the average mass of fledglings until the day 21 of the nestling period, and 2) the fledging production

per female in the target year. These two variables are widely used estimates of fitness in birds (Lindström 1999, Chaine and Lyon 2008). We then tested whether fitness proxies varied for different color scores and morphs. We first modelled with GLMs the average fledgling mass until day 21 per nest (GLM SAS procedure with a Gaussian error distribution) and with Poisson regression analyses the no. of fledglings (GENMOD SAS procedure with a Poisson error distribution and a log link function). In these models, either the morph classification was introduced as a factor or the morph score as a continuous variable, laying date as a covariate accounting for individual quality and the year as a fixed effect to account for environmental effects. Also, in these models, we included the interaction year × morph classification or year × morph score to evaluate the possibility of changing selective pressures on morphs with time.

Additionally, we performed a capture–recapture analysis on the data for breeding females, categorized by color morph. Data from all individuals were analysed with the Cormack– Jolly–Seber (CJS) model implemented by the program MARK ver. 8.0 (White and Burnham 1999). This model estimates survival (ϕ) and capture (p) probabilities using a maximum-likelihood approach. We assumed that both probabilities of survival (ϕ) and capture (p) may be either constant (.), time dependent (t) (i.e. variable as a function of the year), color dependent (c) or both time and color dependent (t x c). Therefore, 16 possible models were constructed. Model fit was assessed by using quasi-Akaike's information criterion adjusted for small sample sizes (QAICc). The model with the lowest ΔQAICc values was selected as the best model but models with ΔQAICc < 2 was assumed to be equally parsimonious (Burnham and Anderson 2002). To make inferences of ϕ and p from the entire model set we used weighted model averaging since we had more than one model with ΔQAICc < 2.

Finally, we also performed a GLM to test for differences on laying date among morphs. In the model we included the morph classification, the year and its interaction as fixed factors. We considered one breeding attempt per female, randomly selected among the available attempts.

DATA DEPOSITION

Data available from the Dryad Digital Repository: < http:// dx.doi.org/10.5061/dryad.n88k968 > (Parejo et al. 2018).

RESULTS

PLUMAGE COLOR VARIATION AND MORPHS

PC1 was approximately flat, and it described achromatic variation, explaining 93.4% of the overall variation in plumage coloration. PC2 and PC3 were not spectrally flat (Fig. 1a) and together accounted for 92.0% of chromatic variance, which is the spectral variance remaining after discounting achromatic variance explained by PC1. PC2 had negative loadings at high wavelengths (625–700 nm) and, therefore, could be described as a redness gradient. PC3, however, had positive loadings approximately at the yellow (550–625 nm) wavelengths, and negative ones at the ultraviolet wavelengths (300–400 nm) and could be described as an ultraviolet– yellowness gradient. We averaged PC scores for the head, back and breast to obtain a global score for achromatic brightness, redness and ultraviolet-yellowness of each individual. These scores, thus, represent the global color appearance of scops owls.

Pearson correlation analyses revealed that degree of body redness (i.e. PC2 scores) ($r_p = -0.29$, $p = 0.001$, $n = 129$), but not body brightness (i.e. PC1 scores) ($r_p = 0.11$, $p = 0.22$, $n = 129$) or ultraviolet-yellowness (i.e. PC3 scores) ($r_p = -0.07$, $p = 0.403$, $n = 129$), was positively associated with morph scores based on photographs (Fig. 1b). Furthermore, body redness was higher in brown-red than intermediate and in intermediate than in grey visually classified individuals (one-way ANOVA: $F_{2,126} = 5.57$, $p = 0.004$, Fig. 1c). In addition, body redness was significantly related to head ($r_p = 0.24$, $p = 0.006$, $n = 129$) and back scores ($r_p = 0.21$, $p = 0.02$, $n = 129$), but not to scores of the breast ($r_p = 0.14$, $p = 0.10$, $n = 129$). This indicates that the determination of body color is mainly explained by head and back scores. A similar set of analyses based on calculations of standard descriptors of reflectance spectra (i.e. brightness, red chroma and hue) yielded qualitatively identical results (correlation red chroma versus morph scores based on photographs: $r_p = 0.24$, $p = 0.006$, $n = 129$). Hence, our morph scoring method reliably reflects variation in scops owl degree of body redness.

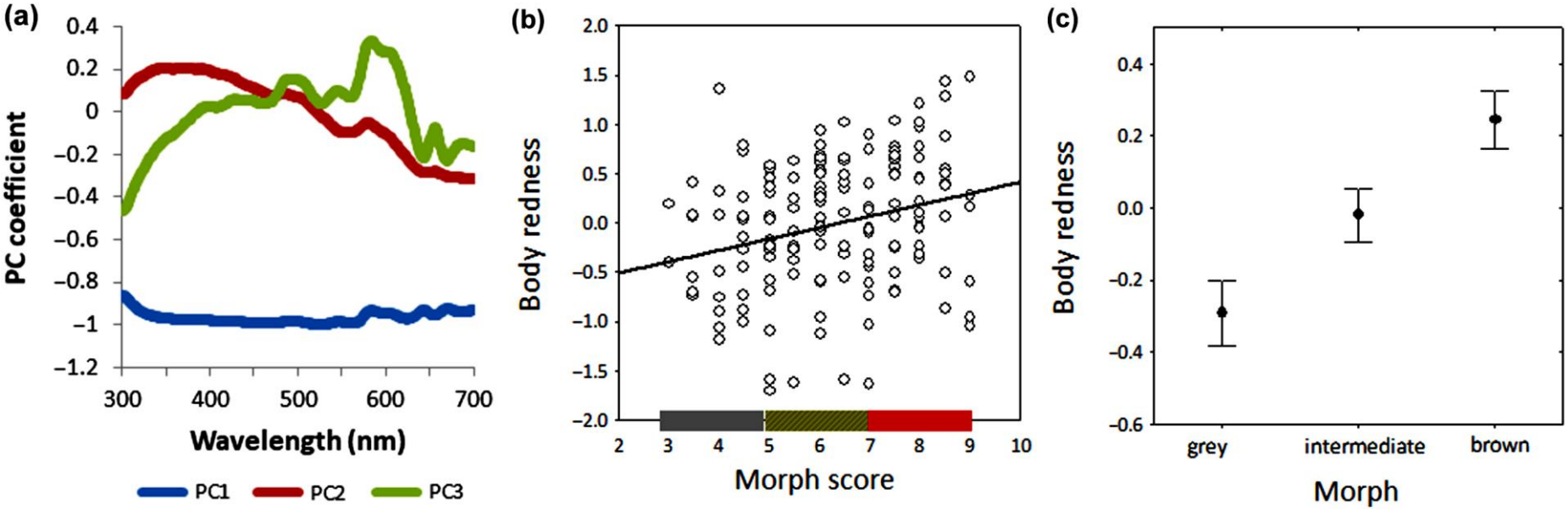

Figure 1. Color variation in relation to morph in adult scops owls. (a) Principal components in relation to wavelength derived from reflectance spectra of the head, breast and back of adult scop owls. PC1 indicates principal component 1, PC2 principal component 2, and PC3 principal component 3. PC1 describes achromatic variation and explains 93.4% of overall variation. PC2 (redness) and PC3 (ultraviolet-yellowness), explain, respectively, 47.8% and 43.9% of the chromatic variance. (b) Relationship between body redness (i.e. averaged (± SE)) PC2 scores for the head, breast and back for each individual) and morph score based on photographs. (c) Relationship between body redness (i.e. averaged PC2 scores for the head, breast and back for each individual) and morph categories based on photographs. n = 129 individuals (38, 52 and 39 classed as grey, intermediate and brown-red, respectively). For graphical purposes PC2 scores in panels (b) and (c) were multiplied by –1.

PLUMAGE COLOR POLYMORPHISM

We colour-scored 159 different birds (114 females and 45 males) from 2010 to 2017, producing 224 observations. From them, 41 individuals (25 females and 16 males) scored in more than one season produced 105 observations, the rest of them corresponding to individuals scored only once (89 females and 29 males).

Color varied throughout the entire range (from 3 to 9), the frequency of coloration being trimodal (Fig. 2a). In the basis of this distribution, individuals were categorized into three morphs: grey (scores lower than 5.5), intermediate (scores between 5.5 and 7, both included) and red-brown (scores higher than 7) (Supplementary material Appendix 1 Fig. A1a, b). In total, about 33% of the population was grey, 37% was intermediate and 30% reddish-brown.

We found the three described morphs in a similar frequency within the two sexes (sex effect: $F_{1,219} = 0.92$, $p = 0.34$) and the two defined age classes (2 yr-old individuals versus older individuals) (age effect: $F_{1,219} = 0.05$, $p = 0.82$) (Fig. 2b).

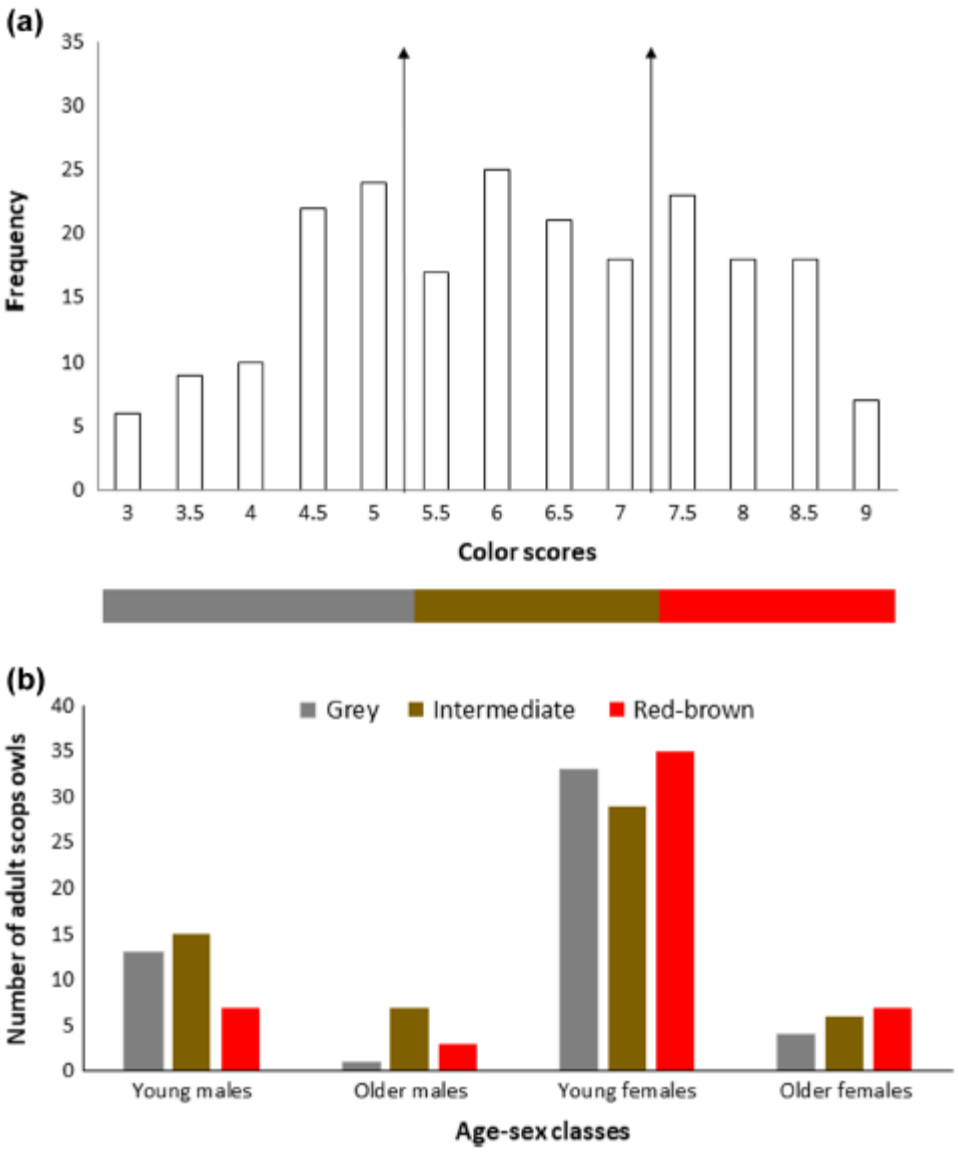

Figure 2. Plumage color polymorphism in the breeding population for the period between 2010 and 2017. (a) Frequency distribution of the color of individuals in order from more greyish (left) to more reddish (right). Color scoring is based on scoring of coloration on three different body parts (head, breast and back) of the plumage and ranges from 3 to 9 points. The frequency of coloration is trimodal so that individuals can be classified in three morphs: grey, intermediate and brown. The cut points (arrows) are established at the lowest intermediate points between two high points. (b) Number of adult scops owls assigned to each morph category within age and sex classes. Unknown individuals captured for the first time during breeding was considered as two-year old individuals and, hence, in the youngest age class. All the other individuals were included in the oldest age class. From each captured individual only one random observation through the study period was considered.

ANNUAL VARIABILITY IN SCOPS OWL COLORATION

Neither the average morph score ($F_{7,142} = 1.36$, $p = 0.23$), nor the frequency of color morphs ($F_{7,141} = 1.00$, $p = 0.43$) in the population varied with the year in females. We found very captured individuals from the two sexes: neither the average morph score (year effect: $F_{5,177} = 1.60$, $p = 0.16$; sex effect: $F_{1,177} = 1.03$, $p = 0.31$; and year × sex: $F_{5,172} = 1.21$, $p = 0.30$), nor the frequency

of color morphs (year effect: $F_{5,176} = 1.40$, $p = 0.23$; sex effect: $F_{1,176} = 0.72$, $p = 0.40$; and year × sex: $F_{5,171} = 0.57$, $p = 0.73$) in the population, varied with the year, the sex or their interaction.

For the sample of individuals captured more than once during the study period (n = 41 individuals), the assigned color score ($r = 0.55$, $F_{40,63} = 4.05$, $p < 0.001$) and color morph ($r = 0.43$, $F_{40,63} = 2.93$, $p < 0.001$) during each capture was repeatable (Lessells and Boag 1987), so that individuals tended to be similarly scored in different years. Nevertheless, some of these individuals (21 out of 41) were classified into a different morph in different years, which suggests that the use of discrete colour morphs is not perfect. Still, only 4 of these 21 changes in morph classification were between the two extreme morphs (from grey to brown or from brown to grey) and from them 2 individuals changed to brown and 2 to grey. In addition, the morph score ($F_{1,62} = 0.04$, $p = 0.84$) and morph classification ($F_{1,61} = 0.03$, $p = 0.86$) were not affected by age, suggesting that the color assigned to each individual is not a consequence of plumage maturation.

PAIRING

Neither color scores ($F_{1,39} = 2.83$, $p = 0.10$, n = 50) nor assigned color morphs ($F_{1,42} = 1.27$; $p = 0.27$; n = 50) of females and males breeding together were related, so that, in general, mating might be considered as a random process with respect to plumage coloration. Moreover, these relationships were not affected by the interaction with the year (color score × year: $F_{4,39} = 0.26$, $p = 0.90$, n = 50; color morph × year: $F_{4,38} = 0.81$; $p = 0.53$; n = 50).

However, when we analyzed assortative mating as a function of the individual morph, 36% of breeding attempts were assortative and 64% disassortative with respect to color morph (Fig. 3). Probability of assortative mating differed in relation to female morph ($\chi^2 = 9.11$, df = 2, $p = 0.01$), but not in relation to male morph ($\chi^2=1.71$, df = 2, $p = 0.42$). Females of the grey and brown morphs mated randomly, whereas females of the intermediate morph mainly mated with males of the same morph (Fig. 3). For years with enough sample size to perform analyses considering the year (4 yr), this pattern seemed to be consistent only in some of the years (effect female morph × year: $\chi^2 = 12.27$, df = 3, $p = 0.006$), so that probability of assortative mating only depended on female morph in 2 out of 4 analysed years.

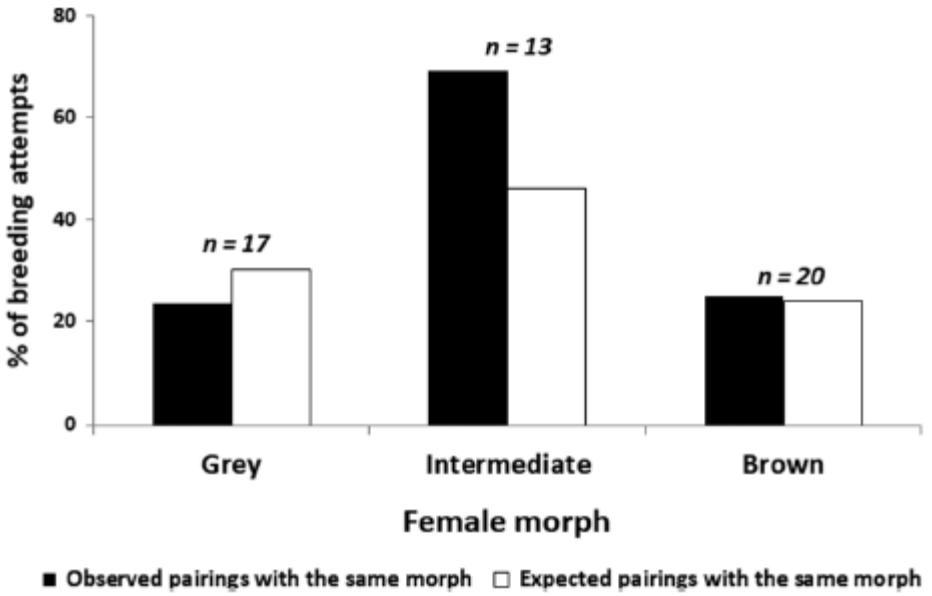

Figure 3. Percentage of assortative pairings in relation to female morph and expected values based on random pairing after considering the available males of the different morphs. Sample sizes are shown above bars and refer to the number of considered breeding attempts.

FECUNDITY SELECTION

Neither the number of fledglings nor the average fledgling mass at day 21 changed as a function of the female color score or morph (Table 1), this pattern being consistent across years (Table 1).

Table 1. Results of the models analysing the effects of the female color scores and morph on fitness proxies. 1) Poisson logistic regression models investigating the no. of fledglings per female; and 2) general lineal model investigating average fledgling mass until day 21 per female. In half of the models, we tested the effect of the female morph and in the others of the female color score. In all the models, laying date was introduced as a covariate, and year and the interaction between female color and year as fixed factors.

	No. of fledglings per female			Average fledging mass until day 21 per female		
Parameter	χ2	df	p	F	df	p
Morph score	0.69	1	0.41	0.00	1, 68	0.99
Year	4.59	7	0.71	0.79	7, 68	0.6
Morph score x year	6.86	7	0.44	0.63	7, 68	0.73
Laying date	0.00	1	0.99	1.82	1, 68	0.18
Morph class	0.80	2	0.67	0.06	2, 62	0.95
Year	10.02	7	0.19	4.96	7, 62	0.0002
Morph class x year	6.87	12	0.87	0.74	12, 62	0.71
Laying date	0.06	1	0.80	0.82	1, 62	0.37

VIABILITY SELECTION

Among the candidate models, two were chosen as the best fitting models because they differed in ΔQAIC by less than 2 points (Table 2). In these models, either female survival and capture probabilities were independent of time and color morph (ϕ(.), p(.)) or female survival probability depended on color morph (ϕ(c), p(.)) (Table 2). This second model is likely to be better because while differing by less than 2 points from the other best-fitting model, it showed the lowest quasi-deviance (Table 2). Nevertheless, we performed model averaging, which showed that intermediate females had the highest estimated survival (mean ± SE: 0.43 ± 0.07) compared to females assigned to the brown (mean ± SE: 0.39 ± 0.07) and grey morphs (mean ± SE: 0.37 ± 0.07).

Table 2. Results of the capture–recapture analyses for data on breeding female scops owls between 2010 and 2017. The analysis separate between survival (ϕ) probability and capture (p) probability that can be either constant (.), time dependent (t), color dependent (c) or both, time and color dependent (t x c). The best models (out of 16 possible) are shown in order of QAICc. Statistics given for each model include quasi-likelihood adjusted Akaike information criterion corrected for small sample sizes (QAICc), proportional support of the model (i.e. the QAICc weight), number of parameters, likelihood and quasi-deviance (QDeviance).

Model	QAICc	QAICc weight	No. param.	Likelihood	QDeviance
Φ(.) p(.)	152.0340	0.60047	2	1.0000	66.0945
Φ(c) p(.)	153.7254	0.25777	4	0.4293	63.4946
Φ(.) p(c)	156.2220	0.07398	4	0.1232	65.9911
Φ(c) p(c)	157.1614	0.04625	6	0.0770	62.4587

DISCUSSION

COLOR POLYMORPHISM

Individual scops owls were assigned to one of the three different morphs previously described for the species (Cramp 1998, Galeotti et al. 2009) by color scoring three different body parts of the birds (namely head, breast and wings-back) in standardized photographs. Coloration in this species, as in others considered to be polymorphic (e.g. Arctic skua *Stercorarias parasiticus*, O'Donald 1983, snow goose *Anser caerulencens*, Cooke and Cooch 1968, common buzzard *Buteo buteo*, Krüger et al. 2001), varies continuously and the three defined morphs aim to capture some of this variation. Indeed, the described method showed repeatability among observers, consistently classified individuals repeatedly captured and was validated by comparison of the morph assignment with spectrophotometric measurements of plucked feathers of a sample of the same individuals. Furthermore, we also showed that, intra-individually, variations in plumage coloration were not age-dependent, showing that the polymorphism was not an ontogenetic process. However, plumage maturation in other owl species mainly occurs between the ages of 1 and 2 yr (Dreiss and Roulin 2010), which are ages not considered here for scops owls. Still, the method seems to be a valid tool to classify individuals of this species.

The utilization of this method with birds from a wild population in southern Spain during 8 yr allowed us to show that the Eurasian scops owl is a polymorphic species. We found that the three morphs coexist within sex and age classes. Thereby, the polymorphism of the species, previously suggested but not proved in the literature (Cramp 1998, Galeotti et al. 2009), is demonstrated. The reported frequencies of the three morphs here only slightly differed from those reported in Galeotti et al. (2009), where grey birds constituted the most abundant morph. However, these variations seem to be of minor importance given the different methods used to estimate morphs. Furthermore, inter-population differences in relative frequencies of color morphs are common in polymorphic species and used to explain the maintenance of polymorphism (Galeotti and Cesaris 1996, Krüger et al. 2001, Roulin et al. 2011).

We found that the frequency of different morphs in the population did not significantly vary through the years. Therefore, colour polymorphism in this scops owl population seems to be stable throughout the study period, suggesting that some form of balancing selection is under the

maintenance of these frequencies during the study period. In other polymorphic birds as the tawny owl, micro-evolutionary changes in morph frequencies have been shown over short time periods (Roulin et al. 2003, Karell et al. 2011).

SELECTIVE ADVANTAGES PROVIDED BY POLYMORPHISM

We have found two pieces of evidence suggesting that polymorphism in the species might be not neutral.

First, mating seemed to be random for the two extreme morphs, grey and brown, and assortative for intermediate individuals. This may result in intermediate males being more frequently coupled than brown or grey males. Whenever color reflects different genotypes, this non-random mating may indicate selection for heterozygosity (Galeotti et al. 2003), perhaps because heterozygosity provides some fitness benefits. How plumage coloration is determined in the scops owl is unknown. Color polymorphism has usually a strong but simple genetic basis (Mundy 2005). In birds, melanistic plumage coloration has been suggested to be determined by Mendelian inheritance (Roulin 2004). More specifically, in owls, plumage color can be considered a continuous trait with relatively little non-additive genetic variation (Roulin and Dijkstra 2003, Brommer et al. 2005). Indeed, in the tawny owl, which shows similar color variation as scops owls, genes of the melanocortin system account for part of the interindividual variation in melanin-based coloration of nestlings (Emaresi et al. 2013). However, even more studies would be needed to understand mechanisms of determination of melanic coloration in other species. Non-random pairing may be explained by mechanisms others than mate choice as, for instance, by temporal or spatial variation in availability of males of different color for females. In our population, however, laying dates did not differ among females' morphs neither alone ($F_{2,90} = 0.19$, $p = 0.83$) nor in interaction with the year ($F_{13,90} = 0.78$, $p = 0.68$), allowing us to discard a role for temporal availability of morphs.

In agreement with the possible advantage achieved by intermediate males, intermediate females had slightly higher survival than grey or brown ones. The mechanism by which the intermediate scops owl morph may be favoured by viability selection compared to the other morphs may reside on selection acting directly on coloration. Indeed, predation might be more severe on grey and brown individuals, which would be less cryptic. However, due to the nocturnality of scops owls,

their predators are not likely to be visual predators, which would reduce the chances of this explanation to be correct. Alternatively, selection might be acting on other traits different to coloration, but related to it via pleiotropy or intragenic linkage. For example, pleiotropic effects between melanin pigmentation and personality, for which there is increasing evidence (Ducrest et al. 2008, Van den Brink et al. 2012, da Silva et al. 2013), could lead to differential predation pressure if intermediate individuals are, for instance, shyer than the other two morphs. Nevertheless, whichever the mechanism behind, as lifespan strongly affects lifetime reproductive success in many bird species (Brommer et al. 2005), a higher survival of intermediate females should involve a higher fitness. Despite this, we fail to find any relationship between fitness proxies and color-morph, so that females with different plumage coloration do not perform differently, at least in the short-term. However, the production of fledglings through the life of each individual, which was not measured here, is likely to differ among females of different morphs because intermediate females, due to their higher survival, have more opportunities to produce offspring than the other females.

In conclusion, our results show that scops owls are polymorphic in coloration, and evidence a small survival and mating advantage for intermediate over grey and brown morphs, which did not result into annual changes in morph frequencies in our population. In order to achieve a full understanding of how color polymorphism is maintained in the species, future studies should thus target on the study of differences among the three morphs in components of fitness here not considered as well as in morph inheritance patterns and genetics of the color polymorphism.

ACKNOWLEDGEMENTS

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MATERIAL SUPLEMENTARIO I

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MATERIAL SUPLEMENTARIO I

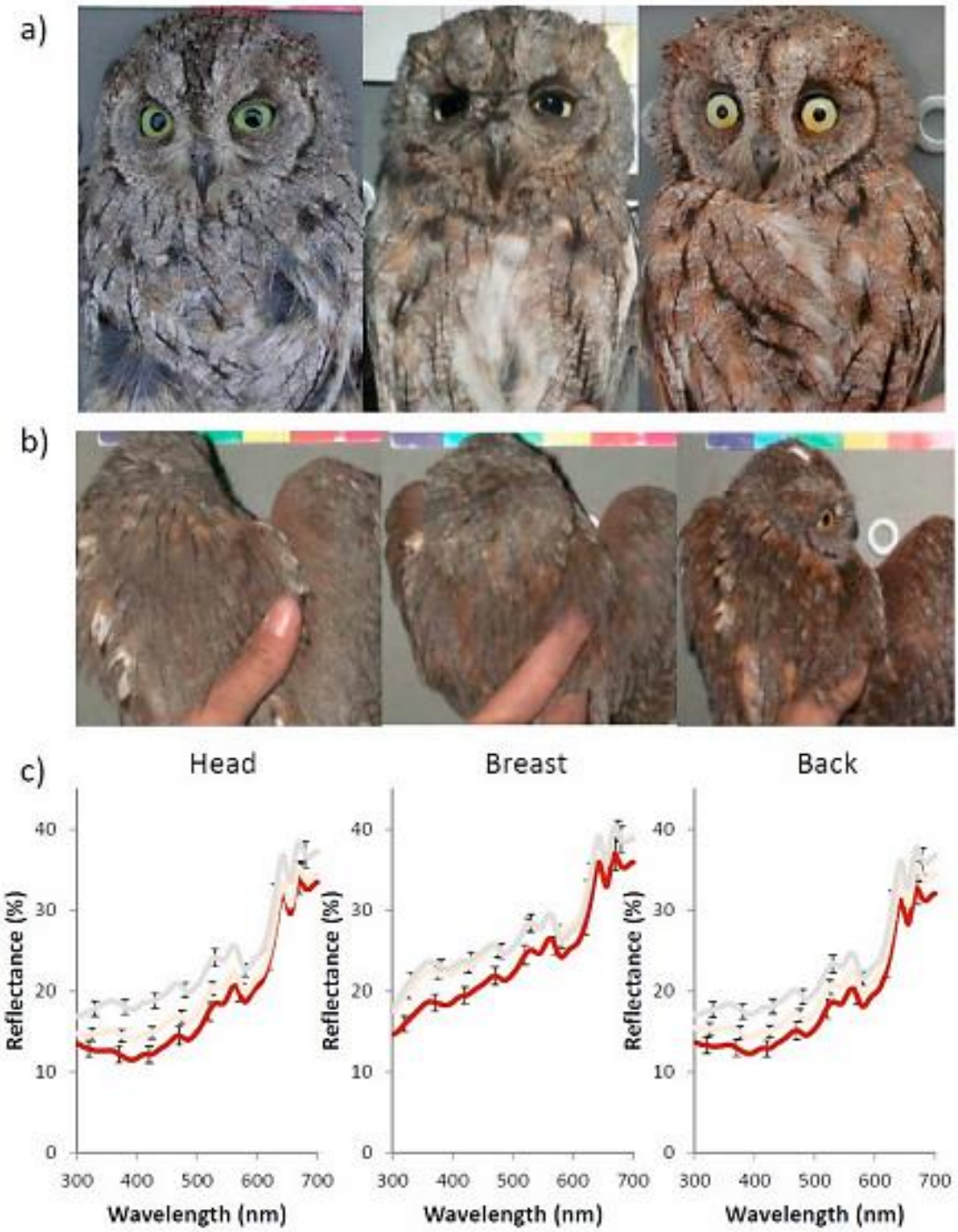

Figure A1 Color variation in scops owls. (a) Frontal and (b) dorsal photographs of representative adult scops owls of the grey (left), intermediate (center) and red-brown (right) morphs. (c) Average reflectance (± Standard error) spectra of the head, breast and back of representative adult scops owls of the grey (grey line, N=16 individuals with scores 8) morphs.

Table A1. Results of the models analysing the effects of the female color scores and morph on fitness proxies for the subset of nests for which we had data on females and males. 1) Poisson logistic regression models investigating the No. of fledglings per female; and 2) General lineal model investigating average fledgling mass until day 21 per female. The female morph was introduced as an explanatory fixed factor in half of the models, and in the others the female color score as a covariate. In all the models, laying date was introduced as a covariate, year and the interaction between female color and year as fixed factors and the male color either as a covariate (morph score) or as a factor (morph class).

	No. of fledglings per female			Average fledging mass until day 21 per female		
Parameter	χ^2	df	P	F	df	P
Morph score	0.51	1	0.57	0.12	1, 31	0.74
Year	1.23	4	0.87	0.73	4, 31	0.57
Morph score*year	1.85	4	0.76	0.31	4, 31	0.87
Laying date	0.09	1	0.76	1.00	1, 31	0.32
Male morph score	0.86	1	0.35	0.27	1, 31	0.61
Morph class	1.28	2	0.53	0.16	2, 26	0.85
Year	6.96	5	0.22	2.76	5, 26	0.04
Morph class*year	1.87	7	0.97	0.88	7, 26	0.53
Laying date	0.16	1	0.69	0.15	1, 26	0.70
Male morph score	2.99	2	0.22	0.36	2, 26	0.70

CAPÍTULO II: 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*)

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CAPÍTULO II: 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*)

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Ardeola, 2020, 67: 3-13.

ABSTRACT

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.

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 plumaje 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 color 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 color 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 color morph variants.

MATERIALS AND METHODS

FIELDWORK

The study was performed from 2010 to 2017 in the Hoya of guadix-Baza, Granada, southeastern Spain (37º18'N, 3º11'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 áreas 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 conversión 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 2.5m 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, Dubendorf, 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 homogeneity 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 Consejeria de Medio Ambiente y Ordenacion del Territorio of the Junta de Andalucia (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, 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).

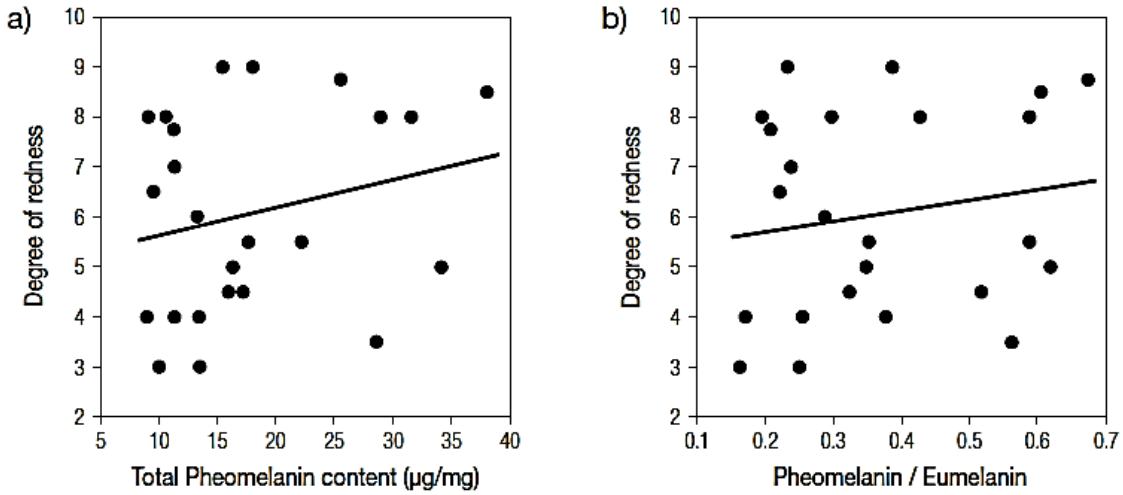

Figure 1. Relationships between degree of redness and a) pM content (i.e. BZ-PM + BT-PM) and b) PM / EM ratio in feathers of 25 Eurasian Scops-owls.

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 codón 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 abrasión 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 plumaje 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-José *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 Darkeyed Juncos *Junco hyemalis* (Abolins-Abols *et al.,* 2018).

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AUTHOR CONTRIBUTIONS

Jesús M. Avilés and Deseada Parejo conceived the study. Jesús M. Avilés, Ángel Cruz-Miralles and Deseada Parejo collected the data. Anne-Lyse Ducrest, Celine Simon and Alexandre Roulin performed the genetic analyses and Kazumasa Wakamatsu the pigments assessment of feathers. Jesús M. Avilés wrote a first draft of the manuscript and all authors contributed with comments.

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MATERIAL SUPLEMENTARIO II

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MATERIAL SUPLEMENTARIO II

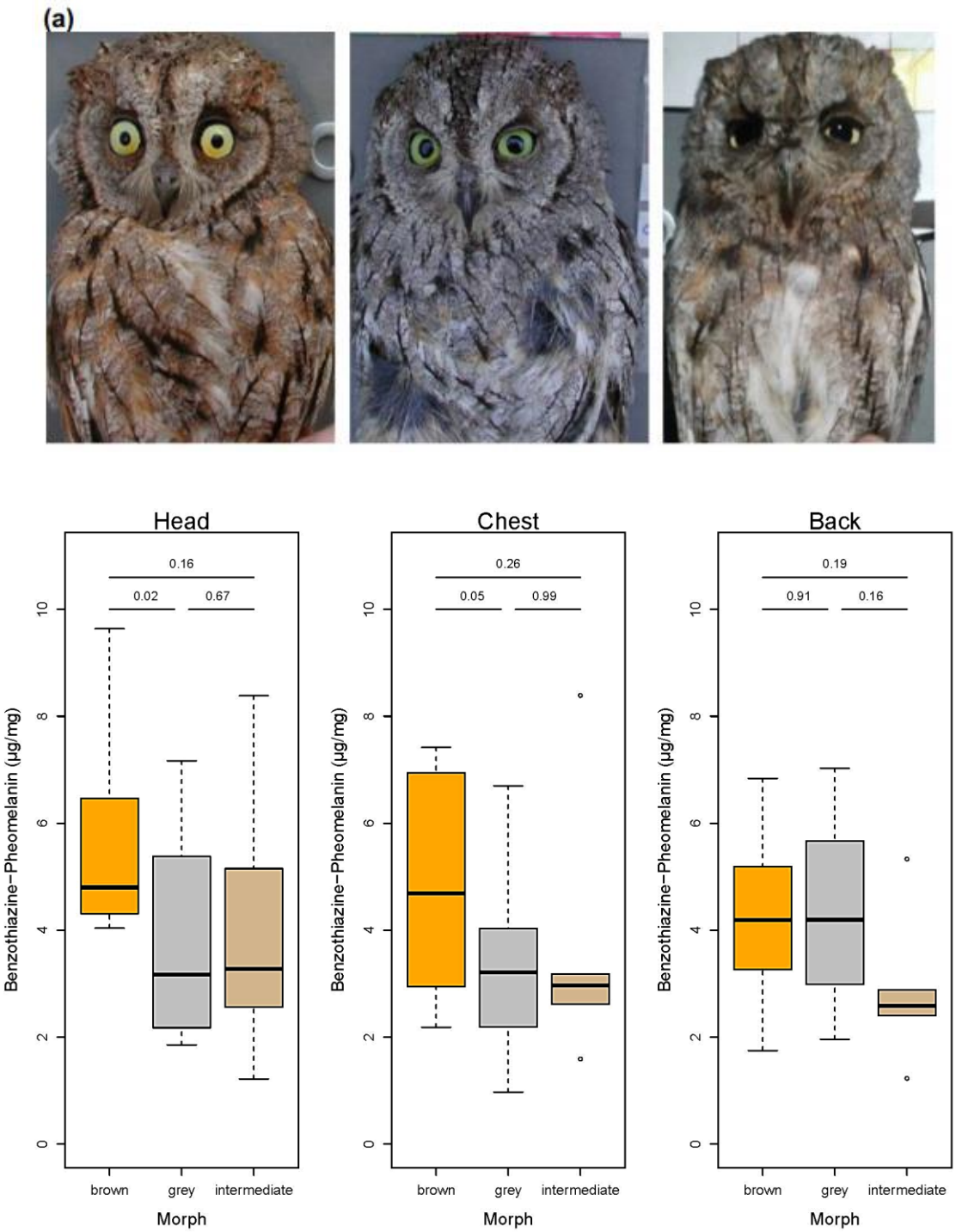

Figure A1. Colour variation and pheomelanin content in Eurasian Scops-owls. (a) Frontal photographs of representative adult Eurasian Scops-owls of the redbrown (left), grey (centre), and intermediate (right) morphs. (b) Boxplot showing benzothiazine-type pheomelanin (i.e. BT-PM) concentration in 10 red-brown, 10 grey and 5 intermediate individuals in feathers from the head, chest and back. Pairwise Scheffe differences are shown above horizontal lines designating pairs.

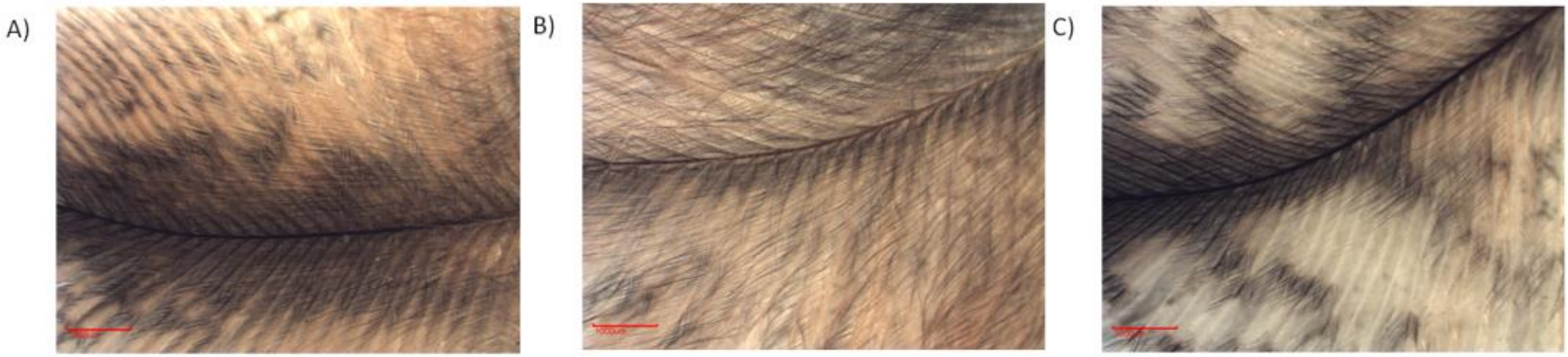

Figure A2. Light microscopy of Eurasian Scops-owl feathers [(A) head; (B) back; and (C) chest] showing different distribution of pheomelanin and eumelanin in barbules, barbs and rachis (50-70 x magnification).

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

Ring	Eumelanin (µg/mg)	Benzothiazole-Pheomelanin (µg/mg)	Benzothiazine-Pheomelanin (µg/mg)	Total Pheomelanin content (µg/mg)	Pheomelanin / Eumelanin	Total melanin content (µg/mg)
4130647	51.53	10.15	4.26	14.42	0.28	65.94
4130661	27.58	11.02	4.31	15.33	0.56	42.9
4130671	51.55	6.77	5.15	11.92	0.23	63.47
4130697	36.9	9.5	2.16	11.66	0.32	48.56
4150746	63.2	21.38	4.7	26.09	0.41	89.29
4160232	46.95	14.36	9.64	24	0.51	70.95
4160240	42	9.32	6.47	15.79	0.38	57.79
4160243	69.68	9.32	2.18	11.5	0.17	81.18
4160268	61.68	10.44	1.86	12.3	0.2	73.97
4160273	42.3	14.83	7.17	22	0.52	64.3
4160289	38.15	10.66	2.67	13.33	0.35	51.48
4160295	42.68	9.58	3.68	13.25	0.31	55.93
4160298	56	12.89	6.86	19.75	0.35	75.75
4160811	29.13	12.46	4.34	16.8	0.58	45.92
4160818	43.55	14.33	8.39	22.71	0.52	66.26
4160823	27.3	6.73	2.56	9.29	0.34	36.59
4160910	54.38	13.54	2.21	15.74	0.29	70.12
4160943	55.85	15.23	1.22	16.45	0.29	72.3
4160962	49.4	10.15	3.28	13.43	0.27	62.83
4165537	70.8	22.21	4.04	26.25	0.37	97.05
4165562	58.43	10.55	4.9	15.45	0.26	73.87
4178604	38.2	7.27	5.38	12.65	0.33	50.85
4178803	54.3	9.29	4.01	13.3	0.24	67.6
4178804	62.53	13.9	6.32	20.22	0.32	82.74
4178806	62.2	13	5.55	18.55	0.3	80.75
Average	**49.45**	**11.96**	**4.53**	**16.49**	**0.35**	**65.94**
Standard error	**2.49**	**0.76**	**0.42**	**95**	**0.02**	**2.96**

Table A 2. Colour morph score and *MC1R* sequence variation for 137 adult Eurasian Scops-owls. The only variable site leading to non-synonymous amino acid substitution was in position 70.

Ring	Sex	Color morph	Encoded Aa at site 70 on *MC1R*
4130647	Male	brown	Alanine
4130659	Female	brown	Alanine/Threonine
4130661	Female	brown	Alanine/Threonine
4130664	Female	brown	Alanine
4130685	Female	brown	Alanine/Threonine
4138734	Female	brown	Threonine
4138740	Female	brown	Alanine/Threonine

4138745	Female	brown	Alanine/Threonine
4138751	Female	brown	Alanine/Threonine
4138766	Female	brown	Alanine/Threonine
4138787	Female	brown	Alanine/Threonine
4150730	Female	brown	Threonine
4150746	Female	brown	Threonine
4150795	Female	brown	Alanine/Threonine
4160204	Male	brown	Threonine
4160205	Male	brown	Alanine
4160232	Male	brown	Alanine
4160240	Female	brown	Threonine
4160247	Female	brown	Alanine
4160258	Female	brown	Alanine
4160260	Female	brown	Threonine
4160282	Male	brown	Alanine/Threonine
4160298	Male	brown	Alanine
4160811	Male	brown	Alanine/Threonine
4160825	Female	brown	Alanine/Threonine
4160904	Female	brown	Alanine
4160911	Female	brown	Alanine
4160919	Female	brown	Alanine
4160928	Female	brown	Alanine
4161758	Female	brown	Threonine
4163159	Female	brown	Alanine/Threonine
4165519	Female	brown	Alanine
4165522	Female	brown	Alanine
4165523	Female	brown	Alanine/Threonine
4165525	Female	brown	Threonine
4165537	Male	brown	Alanine/Threonine
4165540	Male	brown	Threonine
4165561	Female	brown	Alanine
4165562	Female	brown	Alanine
4178804	Female	brown	Alanine
4121261	Female	grey	Alanine/Threonine
4130619	Male	grey	Alanine
4130657	Male	grey	Alanine/Threonine
4130660	Female	grey	Alanine/Threonine
4130662	Female	grey	Alanine
4130666	Female	grey	Alanine
4130675	Male	grey	Alanine/Threonine
4130697	Male	grey	Alanine/Threonine
4138741	Female	grey	Alanine/Threonine
4138754	Female	grey	Threonine
4138758	Female	grey	Alanine/Threonine
4138759	Female	grey	Threonine
4138762	Female	grey	Alanine

4150757	Male	grey	Threonine
4150766	Female	grey	Alanine/Threonine
4150797	Female	grey	Threonine
4160223	Male	grey	Alanine/Threonine
4160243	Female	grey	Alanine/Threonine
4160244	Female	grey	Threonine
4160246	Female	grey	Alanine
4160259	Male	grey	Alanine/Threonine
4160268	Male	grey	Threonine
4160273	Male	grey	Alanine
4160283	Male	grey	Alanine/Threonine
4160289	Female	grey	Alanine
4160290	Female	grey	Alanine
4160292	Female	grey	Alanine/Threonine
4160293	Female	grey	Alanine
4160295	Female	grey	Threonine
4160296	Female	grey	Alanine/Threonine
4160813	Male	grey	Threonine
4160822	Female	grey	Alanine
4160833	Female	grey	Alanine/Threonine
4160834	Female	grey	Threonine
4160836	Female	grey	Alanine/Threonine
4160838	Female	grey	Alanine
4160905	Female	grey	Alanine
4160910	Female	grey	Alanine/Threonine
4160924	Female	grey	Alanine
4160938	Male	grey	Alanine
4165510	Female	grey	Threonine
4165511	Female	grey	Alanine/Threonine
4165527	Female	grey	Alanine
4165528	Female	grey	Alanine
4165545	Female	grey	Alanine/Threonine
4178604	Female	grey	Threonine/Serine
4178606	Male	grey	Alanine/Threonine
4178615	Male	grey	Alanine
4178803	Female	grey	Alanine/Threonine
4178806	Male	grey	Threonine
4178811	Male	grey	Alanine/Threonine
4130618	Male	intermediate	Alanine
4130658	Female	intermediate	Threonine
4130671	Male	intermediate	Alanine
4130673	Female	intermediate	Alanine
4130674	Female	intermediate	Alanine/Threonine
4130679	Male	intermediate	Alanine/Threonine
4130686	Male	intermediate	Alanine
4138743	Female	intermediate	Alanine/Threonine

4138748	Female	intermediate	Alanine/Threonine
4138749	Female	intermediate	Threonine
4138750	Female	intermediate	Alanine
4138760	Female	intermediate	Alanine
4138761	Female	intermediate	Alanine/Threonine
4138765	Female	intermediate	Alanine
4138768	Female	intermediate	Alanine/Threonine
4138769	Female	intermediate	Alanine
4138770	Female	intermediate	Alanine/Threonine
4150739	Female	intermediate	Alanine
4158000	Female	intermediate	Alanine
4160245	Female	intermediate	Alanine/Threonine
4160248	Female	intermediate	Alanine/Threonine
4160249	Female	intermediate	Alanine
4160255	Male	intermediate	Alanine
4160277	Male	intermediate	Alanine
4160291	Female	intermediate	Alanine/Serine
4160294	Female	intermediate	Threonine
4160818	Male	intermediate	Alanine/Threonine
4160823	Female	intermediate	Alanine/Serine
4160832	Female	intermediate	Alanine/Threonine
4160862	Female	intermediate	Threonine
4160866	Male	intermediate	Alanine
4160902	Female	intermediate	Alanine
4160903	Female	intermediate	Alanine/Threonine
4160912	Female	intermediate	Alanine
4160943	Male	intermediate	Alanine
4160959	Male	intermediate	Alanine
4160962	Male	intermediate	Alanine
4165505	Male	intermediate	Threonine
4165552	Male	intermediate	Alanine/Threonine
4165595	Male	intermediate	Alanine
4178605	Male	intermediate	Alanine
4178802	Female	intermediate	Alanine
4178827	Male	intermediate	Threonine
4178828	Male	intermediate	Alanine/Serine
4178842	Male	intermediate	Alanine
4178845	Female	intermediate	Threonine/Serine

Table A3. *MC1R* sequence data for the 142 Eurasian Scops-owl individuals included in the study. Amino acid polymorphisms at SNP sites are reported in bold type. Numbers and codes correspond with the following nucleotide combinations (GG=1, AA=2, AG=3, GT=5K, AT=7W).

	SNP sites																											
RING code	**70/24**	69	86	87	111=37	120	166	207	259	278	282	306	319	322	366	411	444	477	514	522	525	531	574	627	682	711	788	876
4121261	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4130618	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4130619	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1	1
4130647	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4130657	3	1	1	1	3	1	1	1	1	3	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4130658	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1
4130659	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4130660	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1	3
4130661	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1
4130662	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1
4130664	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4130666	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4130671	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4130673	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3
4130674	3	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1
4130675	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4130679	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3
4130685	3	1	1	1	3	1	1	3	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1
4130686	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1
4130697	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4138734	2	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4138740	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1	3
4138741	3	1	1	1	3	1	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1
4138743	3	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

4138745	3	1	1	3	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4138748	3	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4138749	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1
4138750	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4138751	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	3	1	3
4138754	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	1	1
4138758	3	1	1	1	3	1	1	1	1	1	1	1	1	3	1	1	1	3	1	1	1	1	1	1	1	1	1	1
4138759	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1
4138760	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1
4138761	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3
4138762	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1
4138765	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4138766	3	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4138768	3	1	1	1	3	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3
4138769	1	1	1	1	3	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4138770	3	1	1	1	3	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4138787	3	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4150730	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	3	1	1	1	1	1	1	1
4150739	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4150746	2	1	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4150757	2	1	1	1	3	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

CAPÍTULO III: Phaeomelanin matters: Redness associates with inter-individual differences in behaviour and feather corticosterone in male scops owls *Otus scops*

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CAPÍTULO III: Phaeomelanin matters: Redness associates with inter-individual differences in behaviour and feather corticosterone in male scops owls *Otus scops*

Ángel Cruz Miralles, Jesús M. Avilés, Olivier Chastel, Mónica Expósito- Granados, Deseada Parejo.

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ABSTRACT

Individuals within populations often show consistent variation in behavioural and physiological traits which are frequently inter-correlated, potentially leading to phenotypic integration. Understanding the mechanisms behind such integration is a key task in evolutionary ecology, and melanin based colouration has been suggested to play a pivotal role. In birds, most of plumage colour variation is determined by two types of melanin, eumelanin and phaeomelanin, but the role of phaeomelanin in avian phenotype integration has been barely investigated. Here, we test for covariation between phaeomelanin-based colouration, behavioural traits (i.e. nest territoriality, aggressiveness, breath rate and parental behaviour) and corticosterone in feathers in the polymorphic scops owl *Otus scops*, a bird species in which more phaeomelanic individuals display reddish colourations. In males, we observed that reddish males took longer to return to their nests and showed higher levels of feather CORT than more greyish ones. Behaviour and feather CORT were not associated to plumage colour in females. The found associations between redness, behaviour and feather CORT in males, but not in females, might suggest the existence of a sex-specific integrated phaeomelanic phenotype in scops owls.

INTRODUCTION

Variation in behaviour and physiology can be correlated across individuals within animal populations (Gosling 2001; Sih and Bell 2008), giving rise to complex phenotypes (Pigliucci and Preston 2004). Phenotypic integration may reflect the effect of genetic, developmental or functional interactions between traits (Pigliucci and Preston 2004), and, because natural selection does not act on isolated traits, may have important consequences for the evolution of phenotypes (Lande and Arnold 1983). Melanin-based colouration of teguments has been suggested to play a key role in shaping phenotypic integration (Ducrest et al. 2008; Fargallo et al. 2014; Kim and Velando 2015; San-Jose et al. 2017).

Melanins are the pigments responsible for most non-structural brown, black and grey color in vertebrates (Haase et al. 1992; Kimball 2006), and variation is often associated with that in morphological, physiological, behavioural and life-history traits (reviewed in Jawor, Jodie and Breitwisch 2003; Roulin 2004; Ducrest et al. 2008; Hanley et al. 2013; Kim and Velando 2015). Birds constitute an ideal system for the study of phenotypic integration in relation to melanin-based colouration because plumage colour is frequently determined by eumelanin (responsible for grey-black colourations) and/or phaeomelanin (determining reddish-brown colour variation) (Delhey et al. 2010; Galván et al. 2011; Meunier et al. 2011). In most birds both eumelanin and phaeomelanin are found in the same feathers (McGraw et al. 2004, 2005; McGraw 2006). However, most of studies do not differentiate between eumelanin and phaeomelanin, or just refer to the role of eumelanin (e.g. Ducrest et al. 2008, but see Fargallo et al. 2014; Costanzo et al. 2018). This might be unfortunate because if the production of eumelanin compromises the synthesis of phaeomelanin (see Ducrest et al. 2008), covariation of the two pigments with other traits would be expected to differ (Hubbard et al. 2010; Roulin et al. 2011b; Jenkins et al. 2013; Galván and Solano 2016). Other studies, however, report results suggesting that the synthesis of one type of melanin would not reduce or inhibit the other (Fargallo et al. 2014), pointing to more complex mechanisms generating melanin plumage colour (Abolins-Abols et al. 2018). In this context, it seems critical to study the role of phaeomelanin-based colours to achieve a full understanding of the role of melanins in promoting trait covariation.

Covariation between phaeomelanic colouration and other traits may arise due to the cost of production of phaeomelanin (condition-dependence hypothesis). A number of studies provide support for the idea that phaeomelanic plumage colours may function as honest signals of quality (reviewed in Roulin 2016; Arai et al. 2017; Galván 2018). The synthesis of phaeomelanin depends on the amount of available cysteine and glutathione. Glutathione may play a critical role in anti-oxidative defence, nutrient metabolism or in regulating immune function (Kosower and Kosower 1978). Hence, there could be a physiological trade-off between for example anti-oxidative defence and the expression of phaeomelanin, so that only the fittest individuals would be able to display the reddest phenotypes without compromising physiology (Galván and Jorge 2015). Based on these production costs, phaeomelanic colourations would have a higher potential to evolve as honest signals of quality than eumelanic ones (Galván and Solano 2016). Alternatively, covariation between phaeomelanic colouration and other traits could be due to pleiotropy, either mediated by genes involved in phaeomelanogenesis or in hormone synthesis (pleiotropy hypotheses) (San-Jose and Roulin 2018). The melanocortin system comprises a set of five membrane receptors (*MCRs*) which regulate several functions such as melanogenesis, sexual behaviour, aggressiveness, and stress response, depending on the binding of melanocortins and the agouti signalling protein (ASIP) (Ducrest et al. 2008). The binding of the melanocortins to the receptor *MC1R* (coded by melanocortin-1 receptor gene) promotes eumelanin synthesis, whereas the binding of ASIP would promote the formation of phaeomelanin (Robbins et al. 1993; Prota 2012). As melanocortins also bind to other *MCRs* than *MC1R* that regulate behaviour (e.g. *MC5R*, aggressiveness) and physiology (e.g. *MC2R*, hypothalamus-pituitary-adrenal (HPA hereafter) stress response), this may result in trait covariation (Ducrest et al. 2008). Alternatively, changes in hormone secretion, hormonal affinity for carrier proteins, rates of degradation and conversion, and interaction with target tissues could potentially coordinate the co-expression of behavioural, physiological and morphological traits (hormonal pleiotropy hypothesis *sensu* (Ketterson et al. 2009). In particular, corticosterone (CORT hereafter), a glucocorticoid widely investigated in birds that affects the response to stress through the activation of HPA axis, might play such a modulator role. Indeed, several studies have shown that CORT associates with behaviour (Carere et al. 2003; Kralj-Fišer et al. 2007; Schoech et al. 2009; Garamszegi et al. 2012) and melanin-based colouration (Almasi et al. 2008, 2010, 2013). However, only a handful

of studies have studied covariation between phaeomelanin colourations and other phenotypic traits (Piault et al. 2009; Van den Brink et al. 2012b; da Silva et al. 2013; Emaresi et al. 2014).

Here we study covariation between phaeomelanic plumage colour, behaviours (territoriality, aggressiveness during researcher visits and parental care) and likely correlates of stress response (breath rate and feather CORT) in male and female Eurasian scops owls (*Otus scops*) (scops owl hereafter). Breath rate is considered a reliable proxy of handling stress (Fucikova et al. 2009), whereas the amount of corticosterone (CORT) deposited in growing feathers provides a long-term, integrated measure of HPA activity in birds (Bortolotti et al. 2008). In the scops owl, feathers contain eumelanin and phaeomelanin, but most of redness variation is due to phaeomelanin (Avilés et al. 2020). Moreover, color does not change with age in this species (Parejo et al. 2018), making this an ideal system to study covariation between phaeomelanic colours and other phenotypic traits. Based on the assumption that the synthesis of phaeomelanin blocks the synthesis of eumelanin (Roulin et al. 2011b; Van den Brink et al. 2012a), and, knowing that more eumelanic individuals usually display more proactive behaviours and are less stress-sensitive than less eumelanic ones (reviewed in Ducrest et al. 2008), we predict:1) that reddish individuals should exhibit more reactive behaviours (i.e. be less territorial and aggressive and show lower nest attentiveness when threatened) than greyish ones; and 2) that reddish individuals would have higher breath rate and levels of corticosterone in feathers than greyish ones. Finally, because behaviours could be subjected to sex-specific selection (Ketterson et al. 2009), and the hormonal pathways is likely to differ between males and females due to sexual hormones (Sapolsky et al. 2000; Korte et al. 2005), we predict 3) sex-specific differences in the relationships between colour, behaviour and feather CORT.

MATERIALS AND METHODS

STUDY SYSTEM

The study was performed from 2012 to 2018 in the surroundings of the Natural park of Baza, Granada, southeast of Spain (37˚18'N, 3˚11'W). The area is an extensive agricultural landscape with scattered holm oaks (*Quercus ilex*) where cork-made nest-boxes have been set up to favour the reproduction of hole-nesting birds (see details in Rodríguez et al. 2011).

The scops owl is a medium-sized nocturnal owl arriving from Africa into the study area in April (Cramp 1998; Parejo et al. 2012) and starting its reproduction throughout May (Parejo et al. 2012). Scops owls produce one clutch per year of about 4 eggs on average that are laid every second days. Females start incubating after laying the second egg, and incubation takes 24–25 days (Del Hoyo et al. 1999). Nestling rearing takes approximately 21–29 days (Cramp 1998).

SAMPLING PROCEDURE

Starting in the fourth week of April, nest-boxes are visited once a week until egg-laying is detected. After detection of a breeding attempt, nests are visited once more after the end of laying, and only once again just before the estimated hatching date to avoid nest desertion. After hatching, nests are visited weekly to record reproductive parameters.

Adult females were captured by hand while they were sleeping in the nest-boxes during incubation, whereas males were trapped with nest-traps while delivering food to owlets (Parejo et al. 2018). Upon capture individuals were metal ringed, sexed based on inspection of the brood patch (only present in females), and photographed for colour assignment (see below). We also collected feathers for assessment of corticosterone and measured female aggressiveness and breath rate.

COLOUR CHARACTERIZATION

Each captured individual was photographed twice: once head-on, so that head and breast plumage could be observed; and the other back on, targeting on back and wings. Photographs were taken using a digital camera (Canon EOS 1300D, Lens: EF-S 18–55 IS II) mounted on a tripod at a fixed distance of 50 cm and with a flash (aperture: 4.5, shutter speed: 1/200, ISO: 800). Owls were gently fixed with a harness inside a neutral-coloured box with the head placed next to a colour chart (X-Rite ColorChecker® Passport). Photos were standardized using the Adobe® Photoshop Lightroom 6 plugin and used to measure redness extension at the head, breast and wings–back. Each body part was scored among 1 to 3 points depending if they were predominantly greyish or reddish (see S1 Table in Parejo et al. 2018). Previous results have shown that scores of the three body parts are highly correlated within individuals, and, that scores assigned by different observers on the same individual are highly repeatable (Parejo et al. 2018), hence scores of the three body parts were summed to get an individual score for every bird

(ranging from 3 to 9). Pigment analyses have revealed that although eumelanin is the most abundant pigment in scops owl feathers, redness variation is related to phaeomelanin: the higher the score the larger the amount of phaeomelanin in head and breast feathers (Avilés et al. 2020). Hence, colour scores were used to characterize phaeomelanic redness colouration.

BEHAVIOURAL TRAITS

MALE TERRITORIALITY

Territoriality was measured in 35 males from 2014 to 2018 by recording behavioural responses to a simulated territorial intrusion. All trials were conducted when clutches were completed, between nightfall (mean initial time: 22:38 ± 35 minutes) and 01:00 a.m., when owls were expected to be more active. Territorial intrusions were simulated by broadcasting calls of a male scops owl with a MP3 player (takeMS “Deseo”) connected to a speaker (MOLGAR 3” 20W 4 ohm) placed under the closest tree to the target nest (at an approximate distance of 25 meters). Broadcasted records consisted of an initial 2-minutes silent track, as an acclimation period, followed with a 2-minutes track of male territorial calls followed by another 10 minutes of silence track, and a final territorial call track of 2 minutes of the same male. To avoid recognition by familiarity we extracted tracks from 3 unknown males to our population from xeno-canto (https://www.xeno-canto.org/). Territorial tracks were edited using version 2.0.3 of Audacity (R) software. MP3 compression files are widely and successfully used to imitate songs in behavioural studies of birds (e.g. Szymkowiak et al. 2016), so we do not expect to find effects of the MP3 format on the behavioural response in this species. Male territorial behaviour was measured using two different variables: 1) Latency of response, as the time in seconds from broadcasting to the first male hooting response; 2) Duration of response, as the time lasted in seconds from the first to the last male hooting response.

We captured nine breeding males that were deployed with radio transmitters tags (PIP Ag392 de Biotrack Ltd., Wareham, UK) the night before the simulated territorial intrusion in 2016. This allowed us to confirm whether males responding to the playback were the territory´ owners. Tags weighted between 1.10 and 1.90 g and were attached with cyanoacrylate glue onto the feathers of the back. Individuals were located by means of receivers Yaesu FT-290R II antennas (frequency range of 150 MHz). All the individuals hooting back to the simulated intrusion carried

the transmitter, suggesting that they were the territory´ owners. Deployed males were re-captured the night after the simulated territorial intrusion to remove the tag without any apparent harmful effect, and, none of the nests owned by these males were abandoned after tag deployment.

FEMALE AGGRESSIVENESS

Female aggressiveness was measured in 45 females based on video recordings (video camera Sony DCR-SR32) made at the nest-boxes during the day, when females usually sleep. Female behaviour inside the nest-box was filmed during 20 seconds after gently opening the roof, while slowly approaching the camera to the female, and 10 seconds more while holding it in hand after its capture. Based on films females were classed as either aggressive, when they displayed any of the following behaviours: clicked the beak, hissed, swelled their body, laid on their back with claws raised, grabbed with bill or claws and/ or tried to get away through that 30 seconds; or, as non-aggressive females, those feigning death in the nest and in the hand and not exhibiting any of the above behaviours.

PARENTAL CARE

We measured parental provisioning in most scops owl nests from 2012 to 2018 (98 nests) at the beginning of the chick-rearing period (3 days after the hatching of the last egg). Parental activity was recorded at night for at least 60 minutes using infrared cameras (KPC- S500, black and white CCD camera, Esentia Systems Inc.). Upon capture, females were marked with a white Tippex spot on the head that allowed their identification in recordings. In subsequent visits to the nests and in video recordings we did not find any apparent effect of these marks on females.

From recordings, we determined: 1) latency of entering the nest-box in minutes after setting the microcamera, and, 2) adults' feeding rates as the number of prey delivered at the nest per hour.

STRESS RESPONSE

BREATH RATE

Breath rate, estimated as the number of breast movements during 30 seconds, was measured from 2015 to 2018 in 51 females and 35 males as a measure of individual response to handling stress (Fucikova et al. 2009). Birds were held loosely on its back on the hand, fixing it by keeping its head between thumb and index finger and gently laying the middle finger of the other hand on the breast (Fucikova et al. 2009).

FEATHER CORTICOSTERONE

Upon capture, we collected the third covert feather of the left wing of 27 males and 43 females from 2012 to 2015 to determine CORT in feathers. Feathers were kept in hermetic plastic bags until analyses, that were performed in two batches (autumn 2014 (for samples collected from 2012 to 2014) and autumn 2015 (for samples collected in 2015)).

CORT levels in feathers were estimated by ME at the Centre d'Etudes Biologiques of Chize´, France using the method described by Bortolotti et al. (Bortolotti et al. 2008), based on a methanol-based extraction technique. Radioimmunoassay was used to measure the CORT extracts (Lormée et al. 2003), with a highly cross-reactive rabbit anti-mouse antibody from Sigma (C8784). The detection limit of the method was 0.28 ng/mL (lowest measure was 1.23 ng/mL). Although CORT in feathers was calculated in ng/mL, values were transformed to ng/mm for which feathers length (without calamus) were previously measured with a calliper to the nearest 0.1 mm.

STATISTICAL ANALYSIS

Analyses were performed using SAS 9.3 software (SAS Institute Inc., Cary, NC).

In a first step we estimated repeatability of behaviours for the subset of individuals with repeated samples in different years (male territoriality n = 15; breath rate, males = 15 and females = 11) by performing a linear mixed model with the trait measure as the dependent variable and the individual ID as the random intercept. This allowed us to obtain among-individual variance and within-individual variance that are used to estimate repeatability following Lessells and Boag (Lessells and Boag 1987). A behaviour was considered repeatable if among-individuals variance was significantly higher than within-individuals variance, which is a reasonably assumption given low repeatability of behavioural traits (see Bell et al. 2009). Non-repeatable behavioural traits were not considered in subsequent analyses. We did not calculate repeatability in female aggressiveness because this feature was measured at different time in different years for a given female, potentially conditioning the test. Also, we disregarded repeatability in parental care because the number of nestlings raised by a single individual and mate identity varied among years.

Second, we run general linear models to investigate the relationships between colour scores and latency of response of males to territorial intruders, latency to enter the nest-box, feeding rates

and breath rate, as dependent variables, respectively. The study year (as a categorical variable with seven levels) was also included as a fixed term. In birds, early breeders in the season are generally better quality individuals than late breeders. Hence, date of measure was introduced in the models as a further covariate to account for variation in individual quality through the season potentially affecting colouration and behaviour. Finally, brood size was included as a further covariate to control for its possible effect on parental investment.

In addition, we run generalized linear models for analysing females' aggressiveness as a binomial dependent variable (aggressive vs non-aggressive) in relation to colour. In these models, we replicated the model structure performed above for continuous traits, but including as a covariate the hour of the day (as time in minutes until sunset) at which the response was measured to account for the fact that females were captured at different hours during the day.

Finally, we run two general linear models for analysing the relationship between CORT in feathers as dependent variable and colour scores in females and males separately. In these two models the study year was included as a fixed factor. Standard model validation graphs (Zuur et al. 2009) revealed that model assumptions of homogeneity of variance and normality of residuals were fulfilled after corticosterone in feathers, latency to return to nests and feeding rates were log-transformed.

ETHICAL STATEMENT

Animal data collection complies with the current laws of Spain and the fieldwork was authorized by Consejería de Medio Ambiente y Ordenación del Territorio de la Junta de Andalucía (projects CGL2011-27561/BOS, CGL2014-56769-P and CGL2017-83503-P; license code: P06-RNM-01862). The study protocol was reviewed and approved by the ethical committee of the CSIC. Spanish law does not require ethical approval for this specific study from an International Animal Care and Use Committee (IACUC).

RESULTS

Latency of response of males to territorial intrusions was marginally not repeatable ($r = 0.45$; $F_{7, 9} = 2.75$, $P = 0.08$), but we decided to analyse it anyway. Duration of response was not repeatable ($r = 0.23$; $F_{11, 12} = 1.59$, $P = 0.22$), and, hence, disregarded in subsequent analyses. By contrast, breath rate was repeatable ($r = 0.31$; $F_{27, 43} = 2.16$, $P = 0.01$).

PLUMAGE COLOURATION AND BEHAVIOURAL TRAITS

Latency of response to the playback of males and female aggressiveness were not explained by colouration (Tables 1 and 2).

Concerning parental behaviours, latency to enter the nest-box was related to colour in males, but not in females (Table 3). Individuals with a more reddish plumage take longer to enter the nest-box after disturbance (Fig 1).

Feeding rates of female and male scops owls were not associated with plumage color (Table 3).

Table 1. Results of the statistical models analysing male territoriality in scops owls as latency of response against an intruder in relation to plumage colouration (N = 35 individuals).

		Colour score				
Explanatory term		***β***	**SE**	***F***	**df**	***P***
Intercept		-0.66	0.52	1.27		0.210
Male colour		0.08	0.09	0.78	1, 27	0.384
Year*	2014	0.35	0.44	0.25	4, 27	0.908
	2015	0.41	0.44			
	2016	0.20	0.42			
	2017	0.27	0.36			
	2018	0.00	0.00			
Date		-0.01	0.16	0.00	1, 27	0.961
Brood size		0.00	0.13	0.00	1, 27	0.980

*The reference category for the year effect was 2018.

Table 2. Results of the statistical models analysing female aggressiveness in relation to plumage colouration (N = 45 individuals).

		Colour score				
Explanatory term		***β***	**SE**	χ^2	**df**	***P***
Intercept		-0.53	1.36	0.15	1	0.690
Female colour		-0.09	0.22	0.18	1	0.670
Hour		0.58	0.41	2.20	1	0.138
Year*	2014	2.66	1.32	7.11	4	0.130
	2015	0.87	1.21			
	2016	0.17	1.10			
	2017	1.89	1.11			
	2018	0.00	0.00			
Date		1.03	0.59	3.47	1	0.063
Brood size		0.09	0.32	0.08	1	0.775

*The reference category for the year effect was 2018.

PLUMAGE COLOURATION AND STRESS RESPONSE

Breath rate was not related with plumage colouration neither in male nor in females (Table 4).

However, levels of feather CORT in males, but not in females, was related to plumage colouration (Table 4). Reddish males had higher levels of feather CORT than greyish ones (Fig 2).

Table 3. Results of statistical models analysing parental care in relation to plumage colouration.

			Males (N = 63)					Females (N = 39)				
Dependent variable	**Explanatory term**		***β***	**SE**	***F***	**df**	***P***	***β***	**SE**	***F***	**df**	***P***
Latency	Intercept		1.20	0.49	**2.46**		**0.020**	1.83	0.65	**2.82**		**0.008**
	Colour		0.17	0.08	**4.23**	**1, 54**	**0.045**	0.00	0.09	0.00	1, 31	0.968
	Year*	2013	0.41	0.68	0.38	5, 54	0.545	-	-	-	-	-
		2014	0.31	0.42				0.60	0.48	0.95	4, 31	0.449
		2015	0.42	0.42				0.53	0.50			
		2016	0.28	0.37				0.77	0.44			
		2017	0.04	0.35				0.67	0.45			
		2018	0.00	0.00				0.00	0.00			
	Date		0.25	0.15	2.87	1, 54	0.051	0.38	0.18	**4.65**	**1, 31**	**0.039**
	Brood size		-0.24	0.12	3.98	1, 54	0.096	-0.08	0.14	0.29	1, 31	0.594
			Males (N = 69)					**Females (N = 69)**				
Dependent variable	**Explanatory term**		***β***	**SE**	***F***	**df**	***P***	***β***	**SE**	***F***	**df**	***P***
Feeding rate	Intercept		2.15	0.37	**5.78**		**<0.0001**	1.36	0.34	**4**		**0.0002**
	Colour		-0.03	0.06	0.16	1, 58	0.692	-0.04	0.05	0.81	1, 59	0.373
	Year*	2012	-	-	-	-	-	-0.14	0.67	0.93	6, 59	0.482
		2013	-0.57	0.45	0.84	5, 58	0.526	-0.66	0.48			
		2014	-0.03	0.33				-0.41	0.27			
		2015	-0.45	0.32				-0.21	0.28			
		2016	-0.34	0.28				-0.19	0.26			
		2017	-0.23	0.27				0.06	0.23			
		2018	0.00	0.00				0.00	0.00			
	Date		-0.19	0.11	2.81	1, 58	0.099	0.11	0.10	1.24	1, 59	0.270
	Brood size		0.17	0.10	3.06	1, 58	0.086	0.11	0.08	1.87	1, 59	0.177

*The reference category for the year effect was 2018.

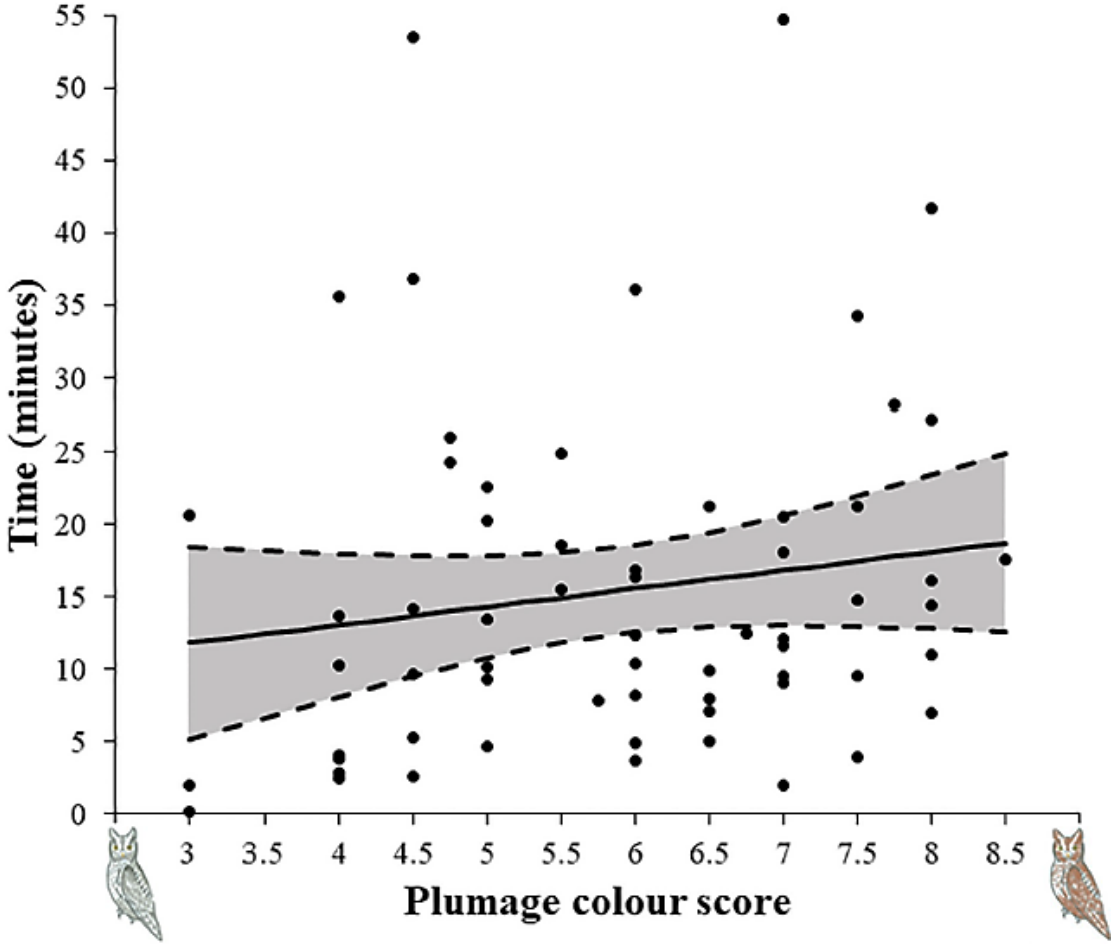

Figure 1. Latency to enter the nest-box of male scops owls (mean with 95% confidence interval) after setting the microcamera in relation to plumage colouration scores (N = 63).

Table 4. Results of GLMs to analyse breath rate and CORT in feathers in relation to plumage colouration in males and females scops owls.

			Males (N = 35)					Females (N = 52)				
Dependent variable	**Explanatory term**		***β***	**SE**	***F***	**df**	***P***	***β***	**SE**	***F***	**df**	***P***
Breath rate	Intercept		0.49	0.69	0.70		0.489	0.17	0.60	0.28		0.777
	Colour		-0.02	0.12	0.02	1, 27	0.894	-0.01	0.10	0.02	1, 43	0.895
	Hour		-0.32	0.27	1.48	1, 27	0.234	-0.39	0.16	5.95	**1, 43**	**0.019**
	Year*	2015	-0.56	0.57	1.56	3, 27	0.222	0.22	0.49	0.20	3, 43	0.895
		2016	-1.21	0.67				0.00	0.45			
		2017	-0.61	0.41				-0.16	0.41			
		2018	0.00	0.00				0.00	0.00			
	Date		0.22	0.20	1.25	1, 27	0.274	-0.09	0.21	0.20	1, 43	0.660
	Brood size		-0.21	0.17	1.46	1, 27	0.238	-0.21	0.13	2.60	1, 43	0.114
			Males (N = 28)					**Females (N = 42)**				
Dependent variable	**Explanatory term**		***β***	**SE**	***F***	**df**	***P***	***β***	**SE**	***F***	**df**	***P***
CORT in feathers	Intercept		0.89	0.09	**9.77**		**<0.0001**	1.14	0.05	**23.62**		**<0.0001**
	Colour		0.03	0.01	4.13	1, 22	0.055	-0.01	0.01	2.27	1, 36	0.140
	Year*	2012	-0.07	0.10	0.34	3, 22	0.794	-0.06	0.03	1.70	3, 36	0.184
		2013	0.00	0.05				-0.04	0.03			
		2014	0.02	0.04				-0.06	0.03			
		2015	0.00	0.00				0.00	0.00			

*The reference categories for the year effect were 2018 for the models on breath rate, and 2015 for the models on CORT in feathers, respectively.

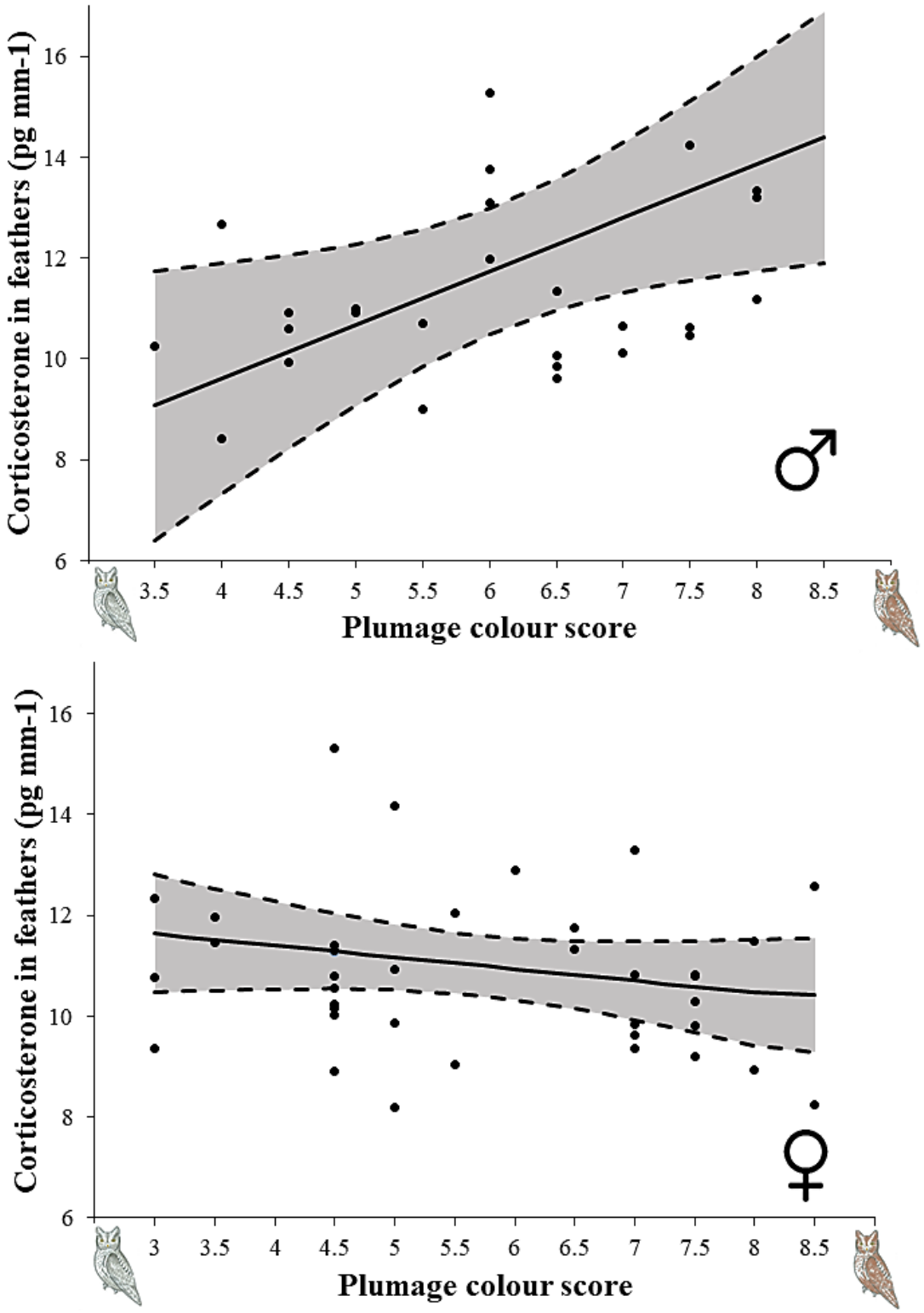

Figure 2. Levels of CORT in feathers (mean with 95% confidence interval) in relation to plumage colouration in male (A) and female (B) scops owls.

DISCUSION

Our results tentatively support the existence of a phaeomelanic syndrome encompassing a suite of correlated behavioural and physiological traits in male scops owls. First, feather CORT differed with redness so that the reddish males had higher CORT values than grey ones. On the other hand, reddish males required more time to resume feeding than greyish ones after being disturbed in their nests. Colour variation, however, did not associate with behaviour and feather CORT in females. Below we discuss the most likely explanations for the causes and sense of patterns based on current knowledge about the role of phaeomelanin and corticosterone in determining phenotypic variation.

One possibility to explain the association between male colouration and feeding activity would be provided by the melanocortin hypothesis (Ducrest et al. 2008). Membrane receptors (*MCRs*) involvedin melanogenesis could control sexual behaviour, aggressiveness, and stress response (Ducrest et al. 2008). However, we recently have found that variation in the coding sequence of the *MC1R* does not explain variation in redness in this species (Avilés et al. 2020). Nonetheless, more than 150 genes involved in colour expression in animals have been identified, and many of them could be involved in phaeomelanins synthesis (Hubbard et al. 2010). Hence, we can only discard a pleiotropic effect of *MC1R* but not of other genes potentially involved in melanogenesis such as *MIFT*, *ASIP*, *TYR*, *SLC45A2* and *TYRP1* (e.g. Chang et al. 2006; Gunnarsson et al. 2007; Minvielle et al. 2010; Lehtonen et al. 2012).

Alternatively, the found association between colour and behaviour in males could be mediated by corticosterone (San-Jose and Roulin 2018). We found that reddish males have higher levels of CORT in feathers than more greyish ones, suggesting that individuals differing in phaeomelanic colour may have different sensitivity to stress during the moult period. However, breathing rate (i.e. a proxy of handling stress) was unrelated with phaeomelanic colour in males, a pattern that was also found in rock pigeons (*Columba livia*) in relation to eumelanic colouration (Angelier et al. 2018). Previous studies have found that breath rate might also be related to personality and risk taking (Fucikova et al. 2009), and, therefore, we cannot exclude the possibility that breathing rate was reflecting differences in personality rather than in stress response. Alternatively, given that feather CORT is likely to reflect the accumulated stress during the time of feather growth

(Bortolotti et al. 2008), the pattern could arise because males differing in colouration have faced different stressors during moult time (i.e. moulting at different time or places or following a different moult pattern (e.g. Karell et al. 2013). A particularly fruitful field of future research that may help to disentangle these possibilities would be to study how individuals differing in phaeomelanic colour use space and time and forage outside the breeding season by deploying GPS devices in combination with the study of feather CORT.

The relationships between phaeomelanic colours and other functional traits seemed to differ between sexes, a pattern that has already been reported for eumelanic colourations (e.g. common buzzard *Buteo buteo*; (Boerner and Krüger 2009); marsh harrier *Circus aeruginosus*; (Sternalski and Bretagnolle 2010); masked boby *Sula dactylatra* (Fargallo et al. 2014). Sexual differences in covariation could arrive from variation in the relative role of eumelanin versus phaeomelanin influencing trait expression in the two sexes. However, pigment analyses have revealed no sexual differences in the relative importance of the two melanin pigments in scops owls (Avilés et al. 2020). In females, however, feather CORT was not associated with colouration. Differences between females and males may be due to the role of sexual hormones. Indeed, levels of testosterone have been reported to negatively correlate with CORT (Sapolsky et al. 2000; Korte et al. 2005), whereas oestrogens enhance glucocorticoids responses (Figueiredo et al. 2007). Also, sexes could show differential sensitivity to hormones in the brain mediated by the abundance of androgen receptors, aromatase or oestrogens (Rosvall et al. 2012; Burns et al. 2013). Alternatively, given that patterns found in males are based on feather CORT, which likely reflects stress during feather development, it could be argued that males and females are not under the same stressors when they moult feathers because they moult at different places, or that they do not moult at the same time.

Our results would support expectations from a key role of mechanisms involved in phaeomelanin synthesis in determining traits associations in male scops owls. Whatever the physiological mechanism behind, the expression of eumelanin and phaeomelanin colours are expected to be inversely related to other functional traits (Hubbard et al. 2010; Roulin et al. 2011b; Galván and Solano 2016). So far, covariation between eumelanic colours and behaviour has been widely investigated in birds (reviewed in Ducrest et al. 2008), but only recently a few studies have

considered covariation between phaeomelanic colours and behaviour, showing contradictory results (Van den Brink et al. 2012a, b; da Silva et al. 2013; Costanzo et al. 2018). First, Van den Brink and co-workers did not detect any relationship between behaviour and the reddish phaeomelanic colouration in both the Eurasian kestrel *Falco tinnunculus* (Van den Brink et al. 2012b) and the barn owl *Tyto alba* (Van den Brink et al. 2012a). However, redness was positively associated with antipredator behaviour in tawny owls *Strix aluco* (da Silva et al. 2013) and barn swallows *Hirundo rustica* (Costanzo et al. 2018). As expected, our results show that reddish scops owl males show more reactive behaviour than more grey ones. Differences among studies might be due to the different relative role of eumelanin versus phaeomelanin in determining colouration and behaviour in different species, a possibility that merits further investigation.

Concerning corticosterone levels, a number of studies have previously investigated their association with eumelanin-based traits in birds and shown that in general darker eumelanic individuals have lower stress-induced CORT levels (Almasi et al. 2008, 2010), see however (Fargallo et al. 2014). The association of phaeomelanin and the stress response, however, remains elusive. Some studies do not find a significant association between phaeomelanin colourations and circulating basal or stressinduced CORT (North America barn swallows *Hirundo rustica erythrogaster*, (Jenkins et al. 2013) or feather CORT (yellow warblers *Setophaga petechia*, (Grunst et al. 2015) levels. However, another study in barn swallows shows that darker phaeomelanic males had higher baseline and stress-induced levels of circulating CORT (Saino et al. 2013). In agreement with expectation from a contrary role of eumelanin and phaeomelanin, we found that reddish scops owl males showed higher levels of feather CORT which may suggest that reddish individuals would be less prepared to cope with stress during moulting. Nevertheless, as above stressed, it is also possible that variation in phaeomelanic colour determined first the behaviour and/or moulting pattern (Karell et al. 2013), and, as a by-product, the stress response.

LIMITATIONS OF THE STUDY

Our study has some weaknesses worth mentioning that may affect the strength of our conclusions. First, due to logistic issues during data collection, we did not study multiple trait covariation, but independent pair-wise covariation. This limits our potential to conclude about complete phenotype integration in scops owls, and it remains to be studied if the found patterns

in relation to colour morphs form part of a higher level of integration. Second, given huge differences in reproductive behaviours between sexes, we could not measure the same behaviours in males and females. Future studies aiming to investigate sex-specific phenotypic integration should ideally target non-reproductive periods, which are less likely affected by sex.

CONCLUSION

The found relationships between phaeomelanin-based colour, behaviour and feather CORT in males might suggest the existence of an integrated phaeomelanic phenotype in scops owls. This is one of the first studies showing a role of phaeomelanin underlying the covariation between melanic colouration and other phenotypic traits, urging for more investigation into the genetic basis linking behaviour and stress-related hormones with the production of this pigment. Finally, although we have found sex-specific covariation among functional traits, our work identifies practical difficulties to study phenotype integration in the reproductive period, where selective pressures for the functional association among behaviour, endocrine profile, and colouration might differ between sexes.

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MATERIAL SUPLEMENTARIO III

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MATERIAL SUPLEMENTARIO III

Table 1A. Number of individuals, grouped by year and sex, in which the different behavioural traits and feather CORT were measured.

	Years															
	2012		**2013**		**2014**		**2015**		**2016**		**2017**		**2018**		**Total**	
	Females	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**
Territoriality	-	-	-	-	-	6	-	6	-	6	-	11	-	6	-	35
Aggressiveness	-	-	-	-	6	-	7	-	9	-	12	-	11	-	45	-
Latency	-	-	-	2	6	9	9	11	7	10	9	16	8	15	39	63
Feeding rate	1	-	2	3	11	9	12	12	11	11	17	17	15	15	69	67
Breath rate	-	-	-	-	-	-	11	6	12	6	15	16	13	7	51	35
Corticosterone in feathers	8	1	13	5	12	10	104	11	-	-	-	-	-	-	43	27

CAPÍTULO IV: Trophic segregation based on moonlight in the colour polymorphic Scops owl *Otus scops*

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CAPÍTULO IV: Trophic segregation based on moonlight in the colour polymorphic Scops owl *Otus scops*

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(Manuscript in preparation)

ABSTRACT

The moon is a major source of luminosity that affects the behaviour of nocturnal animals, and that might promote colour polymorphism *via* disruptive selection if the different morphs were differently adapted to luminosity variation along the moon cycle. Here we test the trophic segregation hypothesis based on moonlight in the Eurasian scops owls (*Otus scops*) by simultaneously studying owlet diet, parental feeding behaviour and prey activity under different moon light conditions. This hypothesis predicts that individuals differing in coloration would differ in the diet they provide to their owlets and in feeding rates depending on moon light. Insects constituted 89.9% of delivered biomass to owlets, being Orthoptera (69.7% of prey), particularly grasshoppers, the most abundant prey, mainly provided by males. Brownish males fed less grasshoppers than greyish ones in full moonlight conditions, but no so in new moonlight conditions. However, male feeding efficiency was unrelated to moon light. The number of grasshoppers fed by females was influenced by their coloration, but unrelated with moon light: the browner the females was, the lower the number of grasshoppers it provided to owlets. Locusts were more active at new moon than at full or waning moon. Our results provide support for the existence of a colour-specific trophic segregation based on moon light variation in scops owls, pointing toward a key role of moon light variability in the maintenance of polymorphism in this nocturnal species.

INTRODUCTION

Colouration determines how animals interact with the environment (Cuthill et al., 2017). The reflective properties of furs or plumages, the environmental light, the colour background and the visual system of conspecifics, prey or predators, influence the way colours are perceived (Endler, 1990; Endler and Mappes, 2017), playing a fundamental role in camouflage and crypsis (Troscianko et al., 2016), in intraspecific communication (Endler, 1990) and mate choice (Bortolotti et al., 2008). Colour polymorphism, so named when in a population individuals of the same age and the same sex display one of several coloration variants that are genetically inherited (Roulin, 2004b), constitutes a paradigm for the study of evolutionary processes in which colouration is relevant. However, the mechanisms leading to the origin and maintenance of this phenomenon remains unclear (Huxley, 1955; Dearn and Davies, 1983; Tate et al., 2016). The paradox comes because natural selection theoretically predicts that only the best adapted individuals (those with the fittest coloration) should thrive. Therefore, polymorphism could be only maintained because the different colour variants achieve equal fitness over time (Ford, 1945). So, alternative variants fall into a selective trade-off, the different variants enjoying some advantages but also incurring some costs (Fisher, 1930). Colour polymorphism is widespread in many animal taxa and specifically in vertebrates (e.g. Sinervo et al., 2001; Medina et al., 2013). In birds, colour polymorphism occurs in 3.5% of the species (Galeotti et al., 2003; Roulin, 2004b; Rueffler et al., 2006), but its occurrence reaches up to 33.5% in some orders as in Strigiformes (Galeotti et al., 2003). It has been suggested that the high occurrence of colour polymorphism among raptors is due to a key role of predator-prey interactions (Paulson, 1973; Rohwer and Paulson, 1987; Roulin and Wink, 2004). The apostatic selection is a likely mechanism explaining this phenomenon, where prey mainly avoid the attacks of more common recognised colour predators favouring the spread of the least frequent (new invaders) colour variants (Allen, 1988; Bond and Kamil, 1998, 2002). Another hypothesis potentially explaining colour polymorphism is the existence of colour-specific trophic segregation (Furness, 1987; Skulason and Smith, 1995; Bolnick et al., 2003). Accordingly, different colour morphs could coexist if they exploited different prey in a population (Skulason and Smith, 1995), being in this scenario colour morphs under disruptive selection. Supporting this mechanism, Roulin (2004a) found that reddish-brown barn owls (*Tyto alba*) mainly

prey on common voles (*Microtus arvalis*), while the lighter morph mainly preyed on wood mice (*Apodemus spp*.).

Environmental luminosity may determine prey and predator perception (Endler and Théry, 1996; Théry, 2006) and hence influence the evolution of colour polymorphism. Indeed, there is comparative evidence showing that in birds, colour polymorphism might be maintained by the selective advantage of morphs under different light conditions of habitats *via* disruptive selection (Galeotti et al., 2003; Passarotto et al., 2018). Variation in luminosity within habitats may also influence the persistence of polymorphism if the different color morphs were differently adapted to luminosity variation. For instance, in the black sparrowhawk (*Accipiter melanoleucus)*, dark morphs showed a decrease in foraging activity and success with increasing light-levels, whereas white morphs showed higher foraging success in the same conditions (Tate et al., 2016; Tate and Amar, 2017).

Another potential source of variation in luminosity within environments is moonlight. It is known that changes in luminosity due to the moon cycle drive different behaviours along the animal kingdom, as for example the vertical migrations of zooplankton (Last et al., 2016) or how large herbivores as wildebeests (*Connochaetes taurinus*) and buffaloes (*Syncerus caffer*) adjust their behaviours in dark nights to prevent lions´ attacks (Palmer et al., 2017). Hence, moonlight might theoretically lead to the maintenance of colour polymorphism whether the different morphs were differently adapted to variation in luminosity along the moon cycle. Indeed, a recent study has shown that the reddest barn owls are less successful at hunting and providing food to their owlets during moonlit nights, and that, under full-moon conditions, white barn owl plumage would facilitate prey catchability (San-José et al., 2019). It remains unclear, however, whether this is a general mechanism at work in nocturnal polymorphic birds, or rather an exception in barn owls.

In this study we aim to unravel the effect of moon-light in the maintenance of colour polymorphism in the Eurasian scops owls (*Otus scops*) (scops owl hereafter) by simultaneously studying owlet diet, parental feeding efficiency and prey activity under different moon light conditions. Specifically, we test the trophic segregation hypothesis based on moonlight. This hypothesis states that variation in moonlight may allow the persistence of polymorphism because the different color morphs are differently adapted to feed based on moonlight variation. It, hence, predicts i)

that individuals differing in coloration would provide their owlets with different prey depending on moon light. Moreover, it is expected ii) that the feeding rates of colour variants differed with moonlight. Finally, we will analyse in an *ex-situ* study the activity of locusts (the main scops owl´s prey) in relation to moonlight aiming to understand whether locust activity was behind trophic segregation.

MATERIALS AND METHODS

STUDY AREA

Our study was performed in the surroundings of sierra de Baza, Granada, southeast Spain (37°18'N, 3°11'W) where a monitored population of scops owls breed in nest-boxes. The area is an extensive agricultural landscape with scattered holm oaks *Quercus ilex* where cork-made nest-boxes (measurements: base of 24 × 24 cm, 40 cm height and opening of 6 cm in diameter) were installed to attract medium-sized hole-nesting birds (Rodríguez et al. 2011).

STUDY SPECIES

The scops owl is a medium-sized nocturnal and trans-Saharan migrant owl arriving into the study area in April and starting its reproduction throughout May (Parejo et al., 2012). The species makes one clutch per year of about 2-6 eggs that are laid every 1-3 days. Females start incubating after laying the second egg, and incubation takes 24-25 days (Del Hoyo et al., 1999). Nestling rearing takes 21-29 days in average (Cramp, 1998).

Scops owl plumage coloration varies continuously from grey to brown in relation to the amount of phaeomelanin (Avilés 2020), and previous studies have reported the occurrence of at least three (dark-reddish, intermediate and grey) melanin-based morphs (Del Hoyo et al., 1999; Galeotti et al., 2009; Parejo et al., 2018).

SAMPLING PROCEDURE

Starting in the last week of April all nest-boxes are regularly visited once a week until egg-laying is detected. Once a breeding attempt is confirmed, nests are visited only twice before hatching to minimize the risk of nest desertion (see Parejo and Avilés, 2020): one at the end of laying and then around the estimated hatching date. After hatching, nests are visited weekly to record breeding and nestling biometric parameters. Nestlings estimated older than 23 days in a given visit not found in a subsequent visit were considered to have fledged.

Females were captured by hand while sleeping at the nests during incubation and males were trapped with nest-traps while they were delivering food to offspring (Parejo et al., 2018). This capture methodology has a negligible effect on nest desertion in this species (Parejo et al., 2018). All individuals were metal ringed and sexed based on inspection of the brood patch (only present in females). Moreover, upon capture, all adults were photographed for colour scoring (see below). Females were marked with a white Tippex spot on the head that allowed their identification in video recordings. In subsequent visits to the nests and in video recordings we did not find any apparent effect of these marks on females.

PLUMAGE COLOUR SCORING

We systematically took two standardized photos for each captured individual: one head-on, in which we could observe the head and breast plumage; and other to the back part in which we observed the back and wings. Photographs were taken using a digital camera (Canon EOS 1300D, Lens: EF-S 18-55 IS II) mounted on a tripod at a constant distance of 50 cm and with a flash (aperture: 4.5, shutter speed: 1/200, ISO: 800). Owls were gently fixed with a harness inside a neutral-coloured box that ensured stable light conditions and with the head placed next to a colour chart (X-Rite ColorChecker® Passport). Photos were standardized using the Adobe® Photoshop Lightroom 6 plugin and used to determine coloration by focusing on redness extension at the head, breast and wings–back. Each body part was scored among 1 to 3 depending on if they were predominantly greyish or reddish (Parejo et al., 2018). We have previously shown that scores of the three body parts are highly correlated within individuals and that scores assigned by different observers on the same individual are highly repeatable (Parejo et al., 2018). Therefore, we summed scores of the three body parts of each bird to get an individual score (ranging from 3 to 9) that were used to characterize variation in plumage colouration.

NESTLINGS DIET AND PARENTAL FEEDING BEHAVIOUR

We studied owlet diet based on parental provisioning video recordings in 70 scops owl nests from 2015 to 2018. We recorded prey provisioning at day 8th after the hatching of the first egg during at least 60 minutes at night (recording began 37 min after the sunset on average) using infrared cameras (KPC- S500, black and white CCD camera, Esentia Systems Inc.) settled on the roof of the nest-box.

Based on visualization of video recordings prey delivered by both parents were identified. Diet composition was estimated based on the frequency of each prey taxon identified relative to the total number of identified prey, and by the percentage of biomass that each taxon represents in relation to the total biomass consumed. Average dry weights of different identified prey used for estimating consumed biomass were extracted from previous studies carried out in the Mediterranean region (see weights and source references in Table 1). Moreover, aiming to characterize nest provisioning in relation to plumage coloration and moonlight, we extracted from the video recordings the number of feeds delivered by each parent in a nest together with the length of the video recording.

MOONLIGHT VARIATION

Moonlight data for the study area were extracted from The United States Naval Observatory (https://aa.usno.navy.mil/data/docs/RS_OneYear.php). Moonlight was estimated as the visible percentage of the moon during each night, but this percentage was modified in those days when moonrise and moonset occurred during light sun period. In such cases the moon was not visible in the night and hence we assigned a moonlight value of zero. We can discard a potential effect of cloudiness on moonlight because in case of intense cloudiness, we postponed the recordings to protect the video-recording equipment against storms.

LOCUSTS´ ACTIVITY

We performed an *ex-situ* study using *Locusta migratora* to study prey activity in relation to moonlight in the knowledge that *Acrididae* are the main prey delivered to scops owls owlets in the study area (see results). *Locusta migratora* is an *Acrididae* widely distributed through Africa, Europe and Asia, and it is abundant in the Iberian Peninsula and in particular in Granada province, where the study was performed (Presa et al., 2007). The species can be found in many natural habitats, but it is also abundant in anthropic habitats as cultivated fields, pasturelands or irrigated fields (Latchininsky et al., 2011). Adult locusts (3-4 cms) used in the experiment were bought in a local pet shop and kept in captivity for 48 hours until they were video filmed. Every locust was just exposed to a moon phase and then put it out of the experiment to minimize the risk of habituation. After removal, all locusts were frozen and used for a food supplementation experiment.

We monitored locusts' activity in 21 different PVC boxes placed in the field in triads and separated from other triads by a minimum of 500 m. Boxes were 29x60x40 cm, and the floor was covered with 3 cm of soil and in a side provided with a grass patch of 25x30 cm, which served as a food source (Figure 1). In addition, we placed in every box a bird water disperser in which we placed cotton at their exit to avoid drowning (Figure 1). We opted to locate the boxes in triads to account for possible differences in environmental conditions within the study area potentially affecting locust activity.

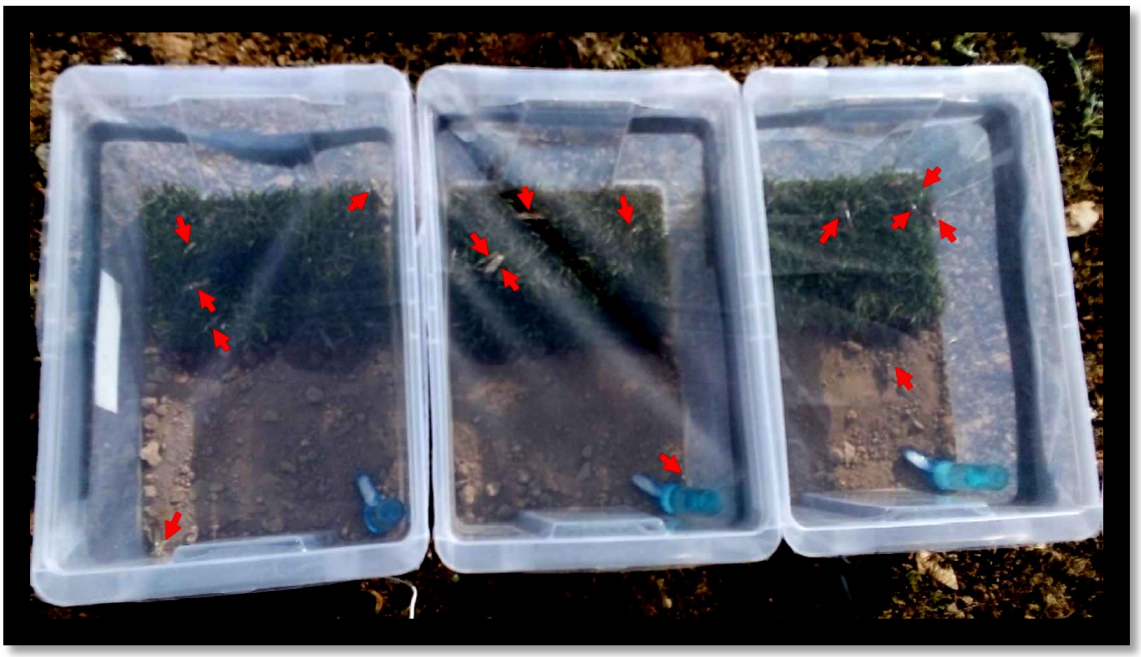

Figure 1. Photograph showing a triad of boxes used to record locust activity. The red arrows indicate the position of the five locust in each box. Seven triads separated by 500 meters were simultaneously placed in field to record locust behaviour during the night in three moon phases (waning moon, new moon and full moon, see methods for details).

In each box, we recorded locusts' activity in three different nights through the breeding time of scops owls in 2019 and coinciding with three periods of the moon cycle: full moon, waning moon and new moon. Each experimental day, we introduced 5 locusts in each box around 7 p.m. and their activity was registered continuously until dawn the following day by means of video cameras that recorded three boxes at once. Video cameras (model Sricam SP009) were installed at a height of 1.5 m on a tripod in a corner of each triad. Boxes were covered with a tulle tissue attached with an elastic rubber that allows visualization of locusts but prevents them from escaping.

To measure locust activity in relation to moon cycle we visualized the recordings and registered the activity in each box at each minute from 30 minutes after sunset to 30 minutes before sunrise. Activity was coded as 1 (when any of the five locusts was moving) or 0 (if none locust did it). We opted to use this codification because in many occasions most of locusts did not move. Daily sunset and sunrise time were extracted from United States Naval Observatory.

Animal data collection complies with the current laws of Spain and the fieldwork was authorized by Consejería de Medio Ambiente y Ordenación del Territorio de la 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.

STATISTICAL ANALYSES

Analyses were performed using SAS v.9.4 statistical software (SAS Institute, Cary, NC, USA).

After describing the owlet diet, we run two Generalized Linear Mixed Models to investigate factors affecting the number of the most abundant prey (grasshoppers, see Table 1) delivered per nest by males and females separately during a night record (distribution: Poisson; link function: log; GLIMMIX procedure in SAS). The models include colour score of the adult and the moonlight (quantified as the visible percentage of the Moon) as covariates, and the interaction between these. The models also include the year of study as a random intercept to account for yearly environmental variation.

We analyse factors influencing number of feedings per nest by fitting two General Lineal Models (GLM procedure in SAS), in which the dependent variable was the number of preys delivered into the nest either by both parents or only by males. In these models, male and female coloration and moonlight were entered as covariates. Moreover, we entered the interaction between male coloration and moonlight. Given that females did not feed during the video-recordings in a number of nests, we disregarded including the interaction between female coloration and moonlight. Aiming to account for environmental variation, in a first step we included study year as a random intercept, but due to the non-convergence of models, we opted to include study year as a fixed explanatory term. Finally, we entered nest brood size and recording duration as two further covariates to control for possible nest differences in feeding requirements and length of video-recordings possibly affecting the delivered number of feedings, respectively.

Finally, to test for the effect of moonlight variation on the activity of locusts we ran a Generalized Linear Mixed Model, in which activity of locusts per box in each minute (coded as active if any of the five locusts was moving, or non-active if none did it) was the response variable (distribution: Binomial; link function: log; GLIMMIX procedure in SAS). The moon cycle as the illuminated portion of the moon's visible face (i.e. 0, 0.5 and 1) was fitted as fixed factors in the model. Box ID was entered as a random intercept to account for non-independence of activity measures within a box. In addition, we included Box ID nested within the Triad ID as a second random term to control for non-random variation among boxes in a given triad potentially affecting locust behaviour.

Standard model validation graphs (Zuur et al., 2009) revealed that model assumptions of homogeneity of variance and normality of residuals were fulfilled. P values smaller than 0.05 were considered significant.

RESULTS

DIET OF SCOPS OWL

2755 feeding events were registered from which 960 prey (34.86%) could be identified, belonging to 16 taxa (from eight classes and twelve orders) (Table 1). Insects constituted 89.9% of delivered biomass to owlets, being *Orthoptera* (69.7% of prey), particularly grasshoppers of the family *Acrididae*, the most abundant prey (Table 1). *Lepidoptera* were the second most delivered prey (11.9%), followed by *Scolopendromorpha*, *Phasmida* and *Araneae* (Table 1). The rest of insect taxa constituted less than 2% of the diet. Vertebrates were scarcely represented, being reptiles (0.6% of preys) the most delivered prey (Table 1).

Males delivered 74.8% of prey in the 2734 feeding events in which we could assign the sex of the adult.

Table 1. Prey delivered in the nests by male and female Scops owls. Total columns also include prey that were not assigned to any sex. Data by prey class are shown in bold and were calculated from the sum of minor prey taxa.

	Males			Females			Total		
Prey taxa	**n**	**Frecuency (%)**	**Biomass (%)**	**n**	**Frecuency (%)**	**Biomass (%)**	**n**	**Frecuency (%)**	**Biomass (%)**
Class Clitellata	**2**	**0.3**	**0.5**	**0**	**0.0**	**0.0**	**2**	**0.2**	**0.4**
Order Crassiclitellata	2	0.3	0.5	0	0.0	0.0	2	0.2	0.4
Fam. Lumbricidae[1]	2	0.3	0.5	0	0.0	0.0	2	0.2	0.4
Class Arachnida	**24**	**3.3**	**0.3**	**15**	**6.4**	**0.5**	**39**	**4.1**	**0.3**
Order Araneae[2]	24	3.3	0.3	15	6.4	0.5	39	4.1	0.3
Class Chilopoda	**35**	**4.9**	**4.0**	**27**	**11.5**	**9.3**	**63**	**6.6**	**5.4**
Order Scolopendromorpha[3]	35	4.9	4.0	27	11.5	9.3	63	6.6	5.4
Class Diplopoda	**3**	**0.4**	**0.1**	**0**	**0.0**	**0.0**	**3**	**0.3**	**0.1**
Order Julida[4]	3	0.4	0.1	0	0.0	0.0	3	0.3	0.1
Class Insecta	**647**	**90.0**	**91.6**	**190**	**81.2**	**84.5**	**834**	**86.9**	**89.9**
Order Odonata[5]	2	0.3	0.2	0	0.0	0.0	2	0.2	0.1
Order. Phasmida[6]	48	6.7	5.1	9	3.8	2.9	57	5.9	4.5
Order Orthoptera	503	70.0	85.4	160	68.4	81.2	669	69.7	84.4
Fam. Acrididae[7]	497	69.1	85.4	157	67.1	81.1	660	68.8	84.3
Fam. Tettigoniidae[8]	5	0.7	0.1	3	1.3	0.1	8	0.8	0.1
Fam. Gryllidae[9]	1	0.1	0.0	0	0.0	0.0	1	0.1	0.0
Order Mantodea[10]	1	0.1	0.2	0	0.0	0.0	1	0.1	0.1
Order Lepidoptera	93	12.9	0.7	21	9.0	0.5	114	11.9	0.7
Imago[11]	90	12.5	0.7	21	9.0	0.5	111	11.6	0.6
Larvae[12]	3	0.4	0.1	0	0.0	0.0	3	0.3	0.1
Class Sauropsida	**6**	**0.8**	**1.7**	**0**	**0.0**	**0.0**	**6**	**0.6**	**1.2**
Order Squamata	6	0.8	1.7	0	0.0	0.0	6	0.6	1.2
Fam. Gekkonidae[13]	1	0.1	0.5	0	0.0	0.0	1	0.1	0.3
Fam. Lacertidae[14]	3	0.4	0.5	0	0.0	0.0	3	0.3	0.4
Fam. Amphisbaenidae[15]	2	0.3	0.7	0	0.0	0.0	2	0.2	0.5
Class Aves	**0**	**0.0**	**0.0**	**1**	**0.4**	**2.9**	**1**	**0.1**	**0.7**
Order Passeriformes[16]	0	0.0	0.0	1	0.4	2.9	1	0.1	0.7
Class Mammalia	**2**	**0.3**	**1.8**	**1**	**0.4**	**2.8**	**3**	**0.3**	**2.0**
Order. Rodentia[17]	2	0.3	1.8	1	0.4	2.8	3	0.3	2.0

Mass in grams of the different taxa: 1: 4.73g (Wroot 1985); 2: 0.19g (Avilés and Parejo 1997); 3: 2g (Franco and Andrada 1977); 4: 0.5g (Franco and Andrada 1977); 5: 1.59 g (as overage of *Coenagrion puella*, *Aeshna cyanea*, *Aeshna mixta* and *Sympetrum striolatum* (Clarke et al. 1996; Torralba-Burrial 2015)); 6: 1.85g (comparing with *Obrimus asperrimus* (Frantsevich and Cruse 1997; Moya 2015)); 7: 3g; (Franco and Andrada 1977); 8: 0.19g (Marchesi and Sergio 2005); 9: 0.6g (Franco and Andrada 1977); 10: 3g (Franco and Andrada 1977); 11: 0.13 g (Marchesi and Sergio 2005); 12: 0.4 g (Naef-daenzer and Keller 1999); 13: 8.03g (as overage of data provided by Zuffi et al., 2011); 14: 3g (Franco and Andrada 1977); 15: 6g (Franco and Andrada 1977); 16: 17g (Parejo and Avilés 2001) and 17: 16g (Franco and Andrada 1977)

FACTORS INFLUENCING GRASSHOPPER PROVISIONING

The number of grasshoppers fed by males was significantly influenced by the interaction between the moonlight and the colour (Table 2). Specifically, brownish males fed less grasshoppers than greyish ones when moonlight was high, but no so when moonlight was low (Figure 2).

Table 2. Results of the generalized linear mixed model analysing variation in the number of Acrididae prey delivered by male and female scops owls in relation to plumage colour and moonlight. The model also includes the study year as a random intercept. Significant effects are shown in bold.

MALES Dependent variable	Explanatory term	β	SE	Statistic *F/Z*	df	*P*
Number of acrididae	**Moonlight*Colour**	**-0.005**	**0.002**	***F*=7.49**	**1,56**	**0.008**
	Colour	0.06	0.05	*F*=1.66	1,56	0.203
	Moonlight	**0.02**	**0.01**	***F*=4.94**	**1,56**	**0.030**
	Year	0.61	0.51	*Z*=1.19		0.116
FEMALES Dependent variable	**Explanatory term**	**β**	**SE**	**Statistic *F/Z***	**df**	***P***
Number of acrididae	Moonlight*Colour	0.002	0.002	*F*=0.98	1,60	0.326
	Colour	**-0.300**	**0.07**	***F*=16.26**	**1,60**	**0.0002**
	Moonlight	-0.01	0.01	*F*=1.14	1,60	0.289
	Year	0.89	0.79	*Z*=1.12		0.130

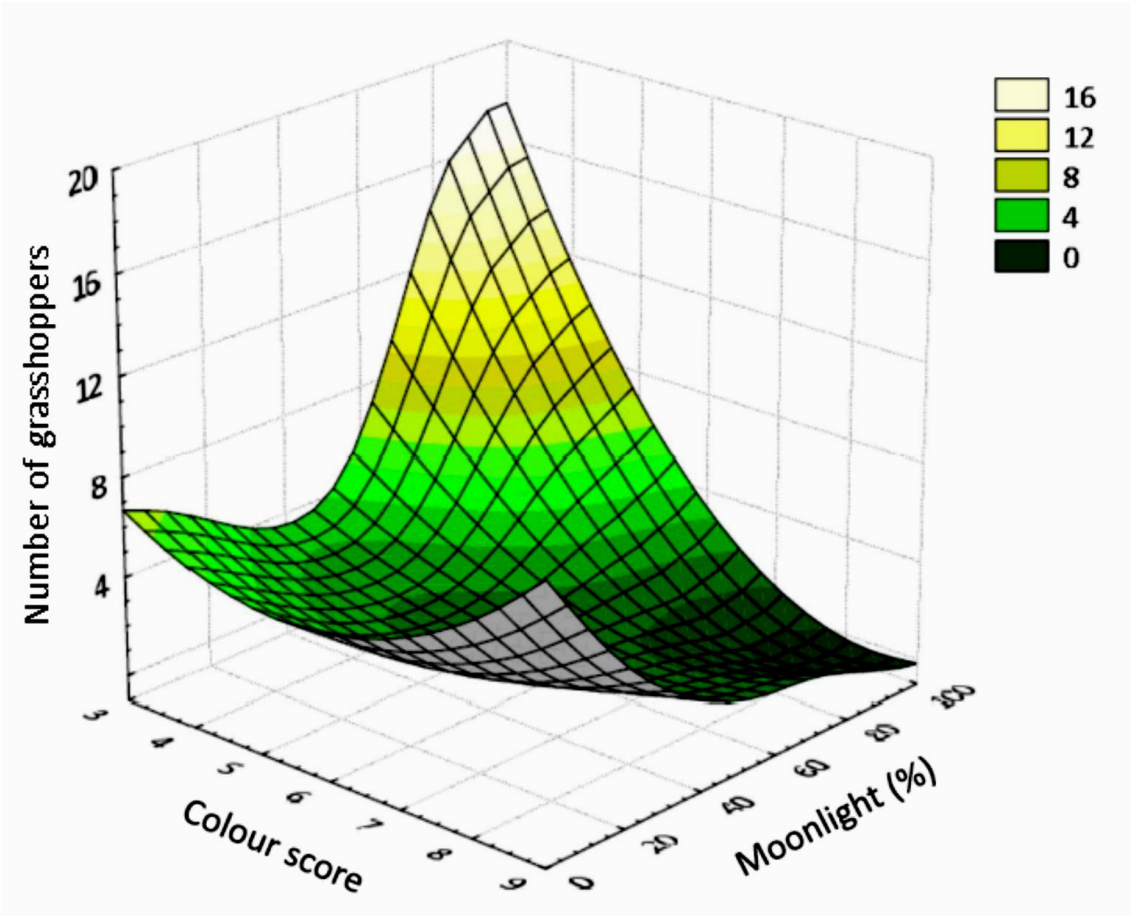

Figure 2. Relationship between the total number of grasshoppers delivered by male parents in relation to their colour scores (higher is browner) and the visible percentage of the moon disc.

The number of grasshoppers fed by females, however, was only influenced by their coloration, but not by moonlight (Table 2). The browner the females were, the lower the number of grasshoppers it fed in a nest (Figure 3).

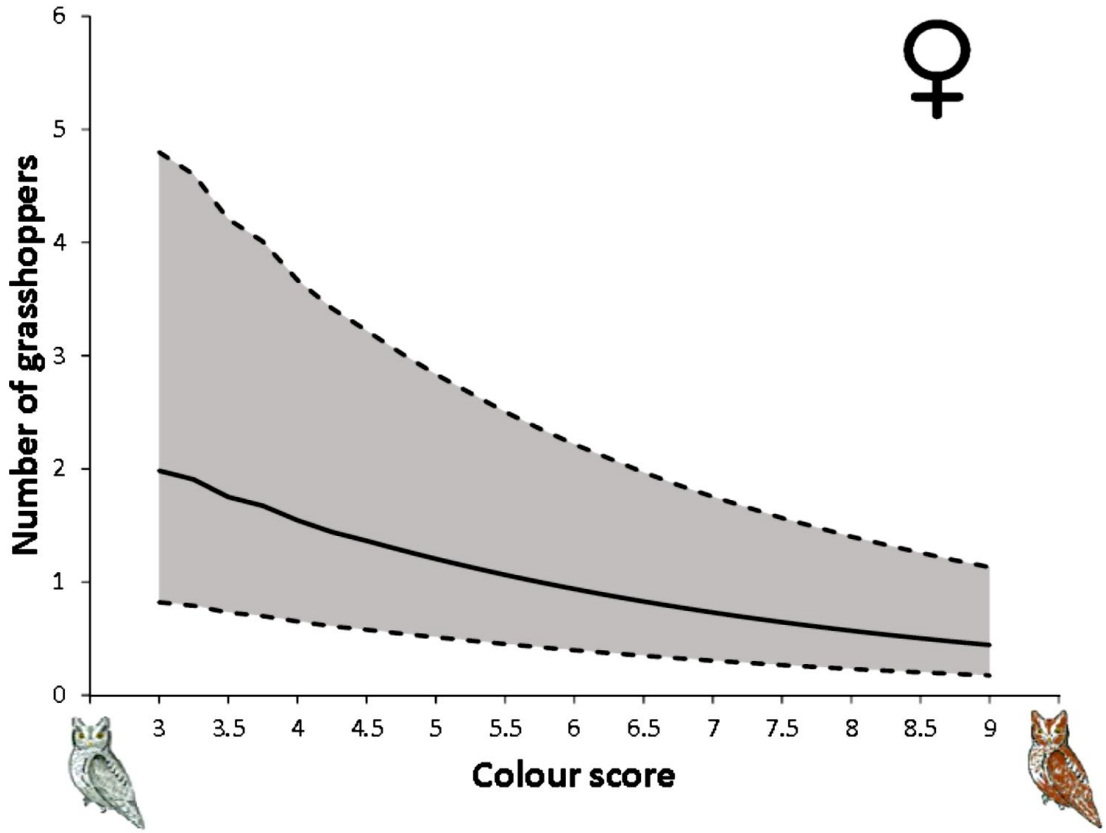

Figure 3. Estimated number of grasshopers delivered per nest (with 95% intervals) by female scops owls in relation to their colour scores (higher is browner).

NOCTURNAL FEEDING BEHAVIOUR

The number of feedings was not influenced by moonlight and/or plumage coloration neither if prey were fed by both parents or only by males when we accounted for the significant effect of recording length (Table 3).

Table 3. Results of the general linear model analysing variation in feeding rate per nest in relation to scops owl adult colour and moonlight. The model also includes the study year as a fixed term and brood size and recording duration as two further covariates. Significant effects are shown in bold.

			Both sexes					Males				
Dependent variable	Explanatory term		β	SE	Statistic *F*	df	*P*	β	SE	Statistic *F*	df	*P*
Feeding rate	Moonlight*Male colour		0.06	0.14	0.16	1, 31	0.690	0.12	0.14	0.71	1, 31	0.407
	Male colour		-0.07	0.13	0.31	1, 31	0.581	-0.07	0.13	0.33	1, 31	0.567
	Female colour		-0.19	0.12	2.73	1, 31	0.109	-0.19	0.12	2.58	1, 31	0.118
	Moonlight		-0.15	0.83	0.03	1, 31	0.862	-0.49	0.83	0.35	1, 31	0.558
	Brood size		0.07	0.18	0.15	1, 31	0.699	-0.01	0.18	0.00	1, 31	0.951
	Year	2015	0.07	0.54	0.15	3, 31	0.928	0.27	0.54	0.20	3, 31	0.897
		2016	-0.27	0.56				-0.20	0.56			
		2017	-0.18	0.45				-0.01	0.45			
		2018	0.00	0.00				0.00	0.00			
	Recording duration		**0.55**	**0.23**	**5.95**	**1, 31**	**0.021**	**0.57**	**0.23**	**6.26**	**1, 31**	**0.018**

LOCUSTS´ ACTIVITY

Locust activity at night changed with the moon phase (Table 4), so that they were more active at new moon than at full or waning moon (Figure 4).

Table 4. Results of the statistical model analysing the activity of locusts in relation to moon cycle. The model also includes Box ID and Box ID nested within the Triad ID as two random intercepts (see methods). Significant terms are shown in bold.

Dependent variable	Explanatory variable		β	SE	Statistic *F/Z*	df	*P*
Activity of locusts	**Moon cycle**	New moon	0.93	0.21	**20.09**	**2, 1188**	**<0.001**
		Waning moon	-0.48	0.27			
		Full moon	0.00	0.00			
	Box ID (Triad)		0.22	0.14	*Z* = 1.55		0.06
	Box ID		0.09	0.13	*Z* = 0.64		0.26

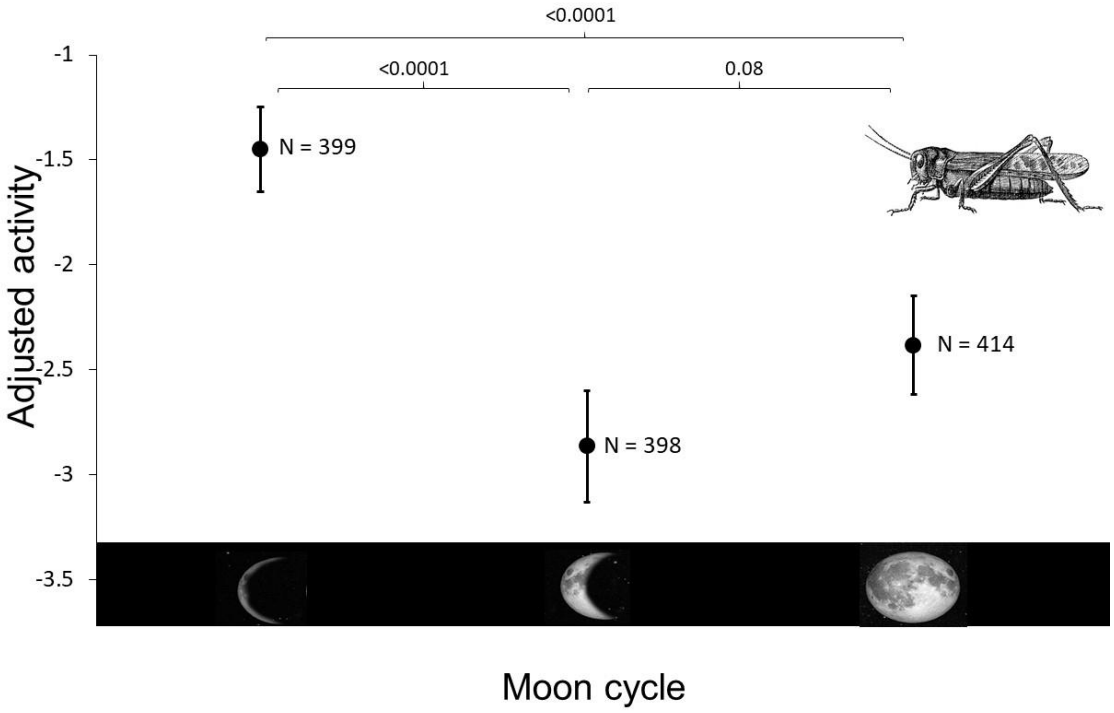

Figure 4. Adjusted activity of locusts (mean ± SE) in relation to moon phases. Results of pairwise comparisons among moon phases are shown above whiskers.

DISCUSSION

Scops Owls feed their owlets mainly with insects in our population as has been reported for other populations from north to south of its distributional range (e.g. 96.8% of prey in Russia (Cramp, 1985); 97.9% of prey in Slovakia (Sotnar et al., 2008); 97.2% in Hungary (Streit and Kalotás, 1991); in Austria varied between 89.6% in Bungenland and 77.6% in Wien (Keller and Parrag,

1996; Muraoka, 2009); 94.6% in Romania (Latková et al., 2012); 100% and 92.2% in two populations of Switzerland (Henninger and Banderet, 1990; Arlettaz et al., 1991); 89.3% in France (Bavoux et al., 1993); 98.0%, 99.3%, 98.0% and 98.3% in four different locations of Italy (Sorace, 1991; Perani et al., 1997; Marchesi and Sergio, 2005; Panzeri et al., 2014) and 94.3% in Spain (Herrera and Hiraldo, 1976)). Among the insects, the order *Orthoptera*, and particularly those included in the family *Acrididae*, constitute the main prey fed to owlets. *Lepidoptera* was the second most abundant prey, confirming the importance of this food source reported in many other populations (Cramp, 1985; Henninger and Banderet, 1990; Arlettaz et al., 1991; Sorace, 1991; Streit and Kalotás, 1991; Bavoux et al., 1993; Marchesi and Sergio, 2005; Muraoka, 2009). A peculiarity of our results is the absence of *Coleoptera* in the owlet diet, as this prey has been frequently reported as abundant in many other locations (Streit and Kalotás, 1991; Keller and Parrag, 1996; Perani et al., 1997; Latková et al., 2012; Panzeri et al., 2014). Biparental care during offspring food provisioning occurs in about 90% of bird species (Bennett and Owens, 2002), but in most of these the two sexes do not contribute equally (e.g. Aho et al., 1997; Lewis et al., 2002). Our study provides novel insights into the role of the sexes in scops owl reproduction. We found that males are the sex responsible to bring most of food for nestlings during the first part of the nestling period, but that females also feed during that time.

THE TROPHIC SEGREGATION HYPOTHESIS

Our results provide support for the existence of a colour-specific trophic segregation in scops owl related to moonlight. Indeed, brownish males fed less grasshoppers (the main prey of owlets in the population (this study)) than greyish ones when the visible percentage of the moon was large, but fed similarly to greyish individuals when it was small. Interestingly, males do not modify their feeding rates depending on their coloration or moonlight, which suggest that trophic segregation of colour morphs in scops owls may arise due to differences in the capture of grasshoppers depending on moonlight.

There are several at least two alternative ways in which the moon, by determining luminosity at night, may condition the presence of grasshoppers in the diet of owlets: 1) Moonlight may modify detectability of adult scops owls by grasshoppers prey. In this scenario, prey detection would be the selective force behind trophic segregation, and brown adult owl phenotypes should be more

conspicuous than grey ones at high moon light. A growing body of evidence is accumulating about the remarkable visual abilities (including colour discrimination) of nocturnal insects in dim light (Warrant, 2017). Also, locusts show nocturnal activity as shown in our *ex-situ* study. Moreover, a recent study shows differences in foraging efficiency of morphs in the black sparrowhawk linked to light variability, suggesting a key role of predator detection by prey on the maintenance of colour polymorphism (Tate et al., 2016). Hence, it might be possible that grasshoppers could recognize brown scops owls as a predator and escape. Against this possibility, a recent study based on visual modelling has calculated the degree of background matching with the vegetation of brown and grey scops owls in our population during day light conditions, and, found that grey individuals should be those showing a poorer background matching (Parejo et al. submitted). However, visual model calculations became unreliable under nocturnal conditions, which impedes us to rely on this to approach to compare detectability of brown and grey phenotypes in the context of moonlight variation. Alternatively, 2) moonlight may modify the activity of grasshoppers prey and hence the hunting ability of scops owls. In our *ex-situ* study, we have found that locusts were less active at high moonlight, which may suggest that the lower presence of grasshoppers in the owlet diet of brown males might be due to a low ability of these individuals to hunt immobile prey. A lower activity may probably render a lower visual and acoustic detectability of grasshoppers. Therefore, although the mechanism behind a lower hunting efficiency of brown scops owls on static grasshoppers is unknown, it may relate to a differential visual or auditory capacity of the different morphs. Supporting colour-based differences in hunting efficiency, we also found that brown females, although providing comparatively far less feeds than males, fed less grasshoppers than greyish ones within our study area. However, we must acknowledge that our inference about prey activity was based on our *ex-situ* study where all the replica were done in the same moonlight conditions and in the same dates, raising the possibility that other correlated variables with moon phases (as temperature or date) were under the found activity pattern.

CONCLUSIONS

Niche segregation is an evolutionary mechanism that allows avoiding or minimizing competition among co-occurring species (Macarthur and Levins, 1967; Corrêa et al., 2009; Maire et al., 2012), that usually takes place along resources, habitats and/or temporality (Schoener, 1974). Niche segregation may also favour the maintenance of polymorphism within a population. Our results

stress the importance of moonlight in determining colour-specific trophic segregation in nocturnal scops owls. These findings add to recent evidence suggesting a key role of moonlight on the evolution of coloration in nocturnal species (San-Jose et al., 2019), and, innovatively suggest that the moon, by determining luminosity at night, may favour the coexistence of polymorphism in nocturnal animals.

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DISCUSIÓN INTEGRADORA

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Conocer los mecanismos a través de los cuales la variación fenotípica se mantiene en las poblaciones naturales es uno de los desafíos más importantes a los que se enfrenta la ecología evolutiva. Esta tesis ahonda en el conocimiento de estos mecanismos a través del estudio del polimorfismo de color en una población de autillo europeo (**Capítulo I**), especie para la que se han analizado los mecanismos próximos (i.e. pigmentos y genes) responsables de dicha coloración (**Capítulo II**). Dado que la selección natural actúa sobre conjuntos de rasgos del fenotipo, se analizó la covariación entre la coloración y rasgos de comportamiento y fisiológicos, observándose una asociación entre rasgos del fenotipo diferente para los dos sexos (**Capítulo III**). Esta tesis, además, sugiere que podrían ser varios los mecanismos implicados en el mantenimiento del polimorfismo de color en el autillo. Por un lado, el análisis de los patrones de emparejamiento y las tasas de supervivencia sugieren que los individuos con coloraciones intermedias se verían favorecidos (**Capítulo I**), mientras que, a la vez, se encontró evidencia de segregación trófica en relación con la coloración del plumaje, lo que podría favorecer el aumento de frecuencia de las formas más extremas de color en la población (**Capítulo IV**).

Para conocer los mecanismos que determinan la evolución y persistencia de las variantes de color en la naturaleza, el primer requisito es conocer cómo se distribuyen estas variantes en cada especie y en el tiempo. Por ello, se clasificó a los individuos reproductores dentro de una población de autillo en base al grado de “rojismo” de su plumaje, durante varios años (**Capítulo I**). Ello permitió observar una variación continua en la coloración del plumaje presente dentro de todas las clases de edad y en los dos sexos, lo que confirma por primera vez la existencia de un polimorfismo de color en la especie.

Tras describir el polimorfismo de color y su variación temporal, el siguiente paso lógico es conocer las bases fisiológicas y moleculares que originan dicha variabilidad. Por un lado, se trató de establecer el tipo de pigmento melánico que proporcionaba las tonalidades observadas. Para ello, se analizaron plumas de la cabeza, pecho y espalda de individuos adultos reproductores mediante técnicas de cromatografía líquida de alta resolución, encontrándose que, aunque las eumelaninas aparecían en las plumas en concentraciones hasta tres veces superiores a las feomelaninas, es la variación en este último pigmento la que se relaciona con los cambios en la

coloración del plumaje (**Capítulo II**). Es llamativo que sea la feomelanina el pigmento que confiere la variabilidad del color en el autillo, dado que tradicionalmente el estudio de la coloración melánica se ha basado en la eumelanina. Se ha mostrado que la coloración feomelánica en algunas especies es un indicador de la calidad individual debido a que su síntesis precisaría de cisteína, compuesto cuyo mayor reservorio fisiológico lo constituye el glutatión, que es a su vez un importante antioxidante celular. En el autillo parece descartable esa función por varias razones. Por una parte, en aquellos individuos presentes en la población durante varias temporadas de cría se observó que la coloración feomelánica era repetible (**Capítulo I**). Es decir, los individuos mostraron similar coloración, aunque las condiciones ambientales cambiaron, apuntando a que la coloración sería independiente de la condición individual. En el mismo sentido, la fecha de puesta y estimadores de fitness tradicionales como el peso promedio de los pollos al vuelo o el número de volantones, que deberían diferir en función de la calidad de los progenitores, no se relacionaron con el color de las hembras de autillo (**Capítulo I**).

Por otro lado, se exploraron las bases moleculares de la variación en la coloración melánica del autillo. Se optó por analizar el papel del gen receptor *MC1R* porque se encuentra implicado en la síntesis de melaninas en un gran número de especies de vertebrados (Ducrest et al. 2008). Mediante el genotipado de este gen, se comprobó que las mutaciones encontradas no guardaban relación con las variaciones de color del plumaje, con lo que se puede descartar la implicación directa del gen *MC1R* en la variación de color (**Capítulo II**). No obstante, cabe señalar que existe una pequeña región de 27 pares de bases que no fue secuenciada en este estudio. Nuestros resultados no descartan la posibilidad de que otros genes distintos del gen *MC1R* implicados en el sistema de las melanocortinas y no considerados en esta tesis pudieran modular la expresión del color en el autillo.

Con el objetivo de explorar la existencia de un posible fenotipo melánico complejo en la especie, en un siguiente estadio analizamos la covariación entre rasgos fisiológicos y comportamentales con la coloración del plumaje. Los resultados pusieron de manifiesto que los machos con coloración más feomelánica exhibieron comportamientos más precavidos, necesitando más tiempo para retornar al nido tras una molestia en el mismo (**Capítulo III**). Estos resultados concuerdan con lo observado en la mayoría de estudios en los que los individuos más

eumelánicos muestran comportamientos más agresivos e intrépidos (Ducrest et al. 2008). Dado que la producción de feomelanina podría limitar la producción de eumelanina, y viceversa, estos resultados apoyarían la idea de un papel opuesto en la covariación de rasgos entre los dos tipos de pigmentos melánicos. Adicionalmente, se encontró que los machos con coloraciones más feomelánicas tenían mayores niveles de corticosterona en las plumas (**Capítulo III**). Los niveles de corticosterona en plumas reflejan cómo los individuos afrontan las situaciones de estrés durante el periodo de desarrollo de la pluma, relacionándose de manera general unos altos niveles de corticosterona con una mayor supervivencia. Sin embargo, no se observó un incremento de la frecuencia de machos marrones (i.e. más feomelánicos) en la población, como sería esperable si esta posibilidad fuese cierta. Este resultado podría explicarse por los costes fisiológicos asociados al mantenimiento de elevados niveles de corticosterona, de manera que los individuos feomelánicos se verían sometidos a un mayor estrés oxidativo (Spiers et al. 2015) y una peor inmunología (Berger et al. 2005).

Estos resultados sugerirían una mayor susceptibilidad de los individuos marrones (i.e. más feomelánicos) ante las situaciones estresantes, lo que también se aprecia en una mayor percepción del riesgo (**Capítulo III**). En otras especies polimórficas de búhos se ha documentado que los patrones de muda difieren según la coloración (Karell et al. 2013), lo que potencialmente podría influir en los niveles de glucocorticoides depositados en las plumas. Por ello sería conveniente investigar en el futuro los patrones de muda en la especie, tratando de descifrar los agentes estresantes que podrían condicionar a cada sexo y morfo durante dicho periodo.

Los resultados de este capítulo (**Capítulo III**) muestran además una marcada diferencia en la covariación entre rasgos del fenotipo según el sexo, no apreciándose signos de covariación en las hembras. La existencia de covariación diferencial entre sexos ha sido reportada en rapaces y otras aves, lo que podría deberse al posible papel de las hormonas sexuales. Es bien conocido el vínculo entre algunas hormonas sexuales y la agresividad, y también se han descrito correlaciones entre hormonas sexuales y corticosterona (Sapolsky et al. 2000; Korte et al. 2005; Figueiredo et al. 2007). Alternativamente, la diferente sensibilidad neuronal de machos y hembras para percibir las concentraciones de hormonas sexuales podrían afectar a la cantidad de glucocorticoides y los comportamientos exhibidos. Como mecanismo alternativo, podría

suceder que las hembras modulasen su comportamiento en función del de su pareja, como así se ha observado en otras rapaces. Sin embargo, análisis previos de los distintos comportamientos en nuestra población y especie que consideraron el morfo de la pareja, no encontraron que este fuese importante. Se ha sugerido también que las presiones selectivas podrían ser distintas en machos y hembras (e.g. Jormalainen et al. 1995) y de ahí que las correlaciones entre rasgos sean diferentes. Es fácil pensar que en aquellas especies donde el macho sea el encargado de proveer el alimento durante el desarrollo de los pollos, como sucede en el autillo, estos queden más expuestos a depredadores, mientras que las hembras queden a resguardo en el nido. Sin embargo, nuestros datos a largo plazo no mostraron diferencias en la frecuencia de aparición de los distintos morfos ni en machos ni en hembras (**Capítulo I**).

Para tratar de dilucidar los posibles mecanismos que promueven el mantenimiento del polimorfismo en la población se analizó el patrón de apareamiento y la eficacia biológica en relación a la coloración (**Capítulo I**), así como el posible efecto de la selección disruptiva a través del estudio de la segregación trófica dentro de la población (**Capítulo IV**). Se observó que los individuos con plumajes situados en los extremos del gradiente de coloración se emparejan de manera aleatoria, pero que los individuos con coloración intermedia prefieren otros de similares características. Este patrón de emparejamiento aumentaría la probabilidad de reproducción de los individuos intermedios, ya que son preferidos por los morfos intermedios y también son elegidos por los morfos extremos. Por otro lado, se evidenció que la supervivencia de las hembras intermedias era mayor que la de las hembras con coloración gris o marrón. La mayor supervivencia de las hembras intermedias, unida al patrón de emparejamiento encontrado, debería propiciar un aumento en la frecuencia de los individuos intermedios, que, sin embargo, no se constató. Esto hace pensar que otros mecanismos compensatorios no considerados en esta tesis podrían igualar la eficacia biológica de las distintas variantes de color.

Un posible mecanismo que podría compensar la ventaja de los morfos intermedios a través del emparejamiento y la supervivencia sería la selección disruptiva. Para indagar esta posibilidad se analizó la dieta aportada al nido por parte de los padres en relación a la coloración del plumaje (**Capítulo IV**), observándose evidencia de segregación trófica en base a la coloración del plumaje y las condiciones lumínicas durante la noche (**Capítulo IV**). Además, el análisis de las cebas

aportadas al nido permite describir, por primera vez y de forma detallada, la dieta del autillo europeo en el país que alberga la población más grande de esta especie dentro del continente europeo. Al igual que en otras poblaciones, los insectos constituyen la mayor parte de la dieta, siendo los ortópteros el orden predominante y el macho el sexo que aporta la mayor parte del alimento durante los primeros días de vida de los pollos. Se observa también que los machos más marrones ceban con menos ortópteros en las noches con mayor iluminación. En las hembras se constata que, con independencia de las condiciones lumínicas, las más marrones capturan menos ortópteros. En el caso de los machos la segregación trófica dependiente del ciclo lunar y la coloración, podría promover a través de la selección disruptiva una especialización diferencial en el tipo de presa entre los extremos del continuo de coloración, mediante la cual se favorecería la coexistencia de las variantes de color en la población. Los motivos por los cuales se pueden dar diferencias tróficas entre los morfos son dos principalmente. Por un lado, los individuos más feomelánicos podrían ser más conspicuos y más aún con mejores condiciones lumínicas, lo que podría permitir su detección por los ortópteros, repeliendo así su ataque. Esta opción parece poco probable porque análisis con modelos visuales que simulan la percepción de los morfos desde la visión de una presa mostraron que en realidad son los individuos más grises los que destacan más en la vegetación del área de estudio (Parejo et al. submitted). La otra posibilidad que explicaría las diferencias en la dieta se refiere a cambios comportamentales en las presas en función del ciclo lunar. El estudio del comportamiento de las presas en relación al ciclo lunar que llevamos a cabo durante la tesis mostró una menor movilidad de los saltamontes durante las noches de luna llena (**Capítulo IV**), lo que podría dificultar su percepción en base a pistas de movimiento por los autillos. Así pues, los individuos feomelánicos tendrían una menor pericia visual o auditiva para detectar a estas presas cuando reducen su actividad. Aunque serán necesarios estudios futuros que verifiquen esas diferencias sensoriales, se han descrito relaciones entre las melaninas y la pérdida de capacidad auditiva (Murillo-Cuesta et al. 2010) incluso se sugiere que los distintos tipos melánicos puedan tener funciones distintas en este cometido (Barrenäs and Holgers 2000; Bartels et al. 2001). En cualquier caso, la segregación trófica observada en función de la fase lunar pone de relevancia la importancia de la iluminación nocturna en los procesos evolutivos que dirigen los patrones de coloración en las especies nocturnas.

Los resultados de esta tesis sugieren que varios mecanismos podrían promover, de forma simultánea, el mantenimiento del polimorfismo en el autillo europeo. Mientras la selección disruptiva que opera por mediación de la segregación trófica favorecería un incremento de la frecuencia de las variantes de coloración extremas (Tabla 2), la selección intersexual de los machos, junto a las diferencias en supervivencia de las hembras, debida posiblemente a heterosis (Tabla 2), favorecerían a los individuos de coloración intermedias. La acción conjunta de estos mecanismos podría por tanto originar la gradación continua en coloración del plumaje encontrada.

CONCLUSIONES

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CONCLUSIONES

1. El autillo europeo es una especie polimórfica con tres morfos que se encuentran en los dos sexos y en todas las edades, si bien está coloración varía de manera continua desde las formas grises a las marrones-rojizas.
2. La distribución de frecuencias de las variantes de color en la población no cambia durante los ocho años de estudio, sugiriendo la existencia de mecanismos promotores de selección estabilizadora.
3. El grado de rojismo del plumaje se asocia con la cantidad de feomelanina en las plumas, aunque la cantidad de eumelanina en estas es tres veces superior que la de feomelanina. Se detectó una sustitución sinónima y una sustitución no sinónima en el *MC1R* que no se asocian con la variación en la coloración del plumaje. Por tanto, 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*.
4. Los machos marrones-rojizos tardaron más tiempo en retornar al nido tras una molestia y muestran niveles más altos de corticosterona en plumas que los grises. En las hembras, el comportamiento y los niveles de corticosterona en plumas no se asocian con el color del plumaje. Las asociaciones encontradas entre el color, comportamiento y corticosterona en las plumas de los machos, pero no en las hembras, podría sugerir la existencia de un fenotipo feomelánico integrado dependiente del sexo en el autillo europeo.
5. Las hembras con coloraciones extremas no mostraron preferencias con respecto al color de los machos con los que se emparejan, mientras que las hembras intermedias prefieren machos de coloración intermedia y muestran una mayor supervivencia. Por tanto, el emparejamiento parece favorecer a los machos intermedios, porque todas las hembras incluyen machos intermedios entre sus parejas; y la supervivencia parece favorecer a las hembras intermedias. A pesar de esto, la proporción de individuos intermedios no aumentó durante el estudio. Este hecho puede deberse a fluctuaciones temporales y/o espaciales a mayor escala en la selección sobre el color.
6. Los insectos constituyeron el 89,9% de la biomasa aportada a los pollos durante el desarrollo temprano, siendo los ortópteros (69,7% de las presas), y en particular los saltamontes, la presa más abundante que traen al nido, principalmente los machos.

7. Los machos marrones-rojizos cebaron menos saltamontes que los grises en condiciones de luna llena (i.e. alta luminosidad). Sin embargo, la eficiencia de alimentación de los machos no estaba relacionada con la luz de la luna. El número de saltamontes aportado por las hembras depende de su coloración, pero es independiente de la luz de la luna: las hembras marrones-rojizas ceban menos saltamontes que las grises. En el autillo, se evidencia la existencia de una segregación trófica dependiente del color, basada en la variación de la luz de la luna.
8. Los resultados de esta tesis sugieren de manera global que el polimorfismo de color que se da en el autillo es complejo y explicable por varios mecanismos de selección natural y sexual que podrían funcionar simultáneamente. Además, estos mecanimos funcionarían de manera diferente en los dos sexos sugiriendo un posible papel de las hormonas sexuales. La observación de una variación continua en la coloración en el autillo podría derivarse del efecto conjunto de la selección disruptiva que llevaría a favorecer las formas más marrones y grises del continuo, mientras que la mayor supervivencia y probabilidad de emparejamiento favorecería a los individuos intermedios.

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