THE ROLE OF ECOLOGY IN THE EVOLUTION OF COLORATION IN OWLS

PhD Thesis

Arianna Passarotto

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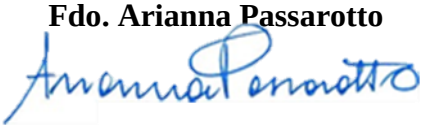
The role of ecology in the evolution of coloration in owls

Memoria presentada por la Licenciada en Ciencias Naturales, Arianna Passarotto, para optar al título de Doctor por la Universidad de Sevilla

Fdo. Arianna Passarotto



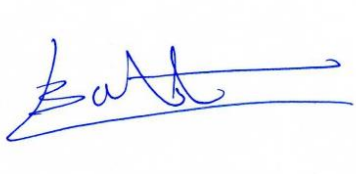
Dr. Jesús Miguel Avilés Regodón, Científico Titular de la Estación Experimental de Zonas Áridas (EEZA-CSIC), como director, y Javier Balbontín Arenas, Profesor Titular de la Universidad de Sevilla, como tutor

CERTIFICAN:

Que los trabajos de investigación desarrollados en la Memoria de Tesis Doctoral "*The role of ecology in the evolution of coloration in owls*", son aptos para ser presentados por la Licenciada Arianna Passarotto ante el Tribunal que en su día se designe, para aspirar al grado de Doctor por la Universidad de Sevilla.

Y para que así conste, y en cumplimiento de las disposiciones legales vigentes, firman el presente documento en Sevilla, a 23 de junio de 2020.

Director:



Fdo. Jesús Miguel Avilés Regodón

Fdo. Javier Balbontín Arenas (Tutor)

There is a driving force more powerful than steam,

electricity and nuclear power: the will.

Albert Einstein

Dedicated to my family

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ABSTRACT

Birds show an extraordinary variability in their colour patterns shaped by natural and sexual selection to fulfil several functions like communication, camouflage and/or protection. The factors promoting colour variation can be diverse and act differently on different traits and levels of organization. Therefore, to fully understand how ecology influences the evolution of so outstanding colour diversity, it is necessary to consider holistic approaches combining multiple levels of study, including different traits, but also different species with a wide range of ecological conditions.

In this thesis, I adopted such a multiple-scale approach combining i) ecogeographical analyses at a world scale, ii) comparative analyses to control for the possible effects of common ancestry, and iii) field data of two species collected at the population level, to assess the relative importance of a number of ecological and evolutionary factors in determining colour variation in owls. More specifically, I aimed to investigate i) large scale geographic variability of melanin-based colour patterns in relation to environmental gradients in the frame of classic ecogeographical rules; ii) interspecific variability in colour polymorphism in relation key ecological drivers; iii) the evolution of interspecific iris colour in relation to activity rhythm; and iv) to assess the potential of iris coloration as a quality indicator in different communication contexts in two owl species.

Ecogeographical analyses at global scale revealed that owls display darker phenotypes near the equator. In particular, owls inhabiting regions with high temperature and living in more densely vegetated areas were darker, excluding a role of thermoregulation in promoting large-scale plumage variation in owls. On the other hand, it was found that owl species inhabiting areas with a denser tree cover were more likely eumelanic, which would agree with a potential role of eumelanin in camouflage. Finally, the proportion of pheomelanin color was higher in species inhabiting warmer and wetter areas, suggesting that several alternative selective forces may have simultaneously contributed to shape large-scale plumage colour variation within the clade.

In a second step, I examined in a comparative framework the relative importance of several ecological drivers in promoting the evolution of colour polymorphism in owls in the frame of three mutually non-exclusive evolutionary scenarios: the apostatic selection hypothesis, the niche divergence hypothesis and the no selection hypothesis. In agreement with the niche divergence hypothesis, I found that species 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 colour polymorphic. Correlated evolution analyses revealed that a change in the luminal niche might be a fundamental requisite for the evolution of colour polymorphism. Moreover, polymorphism was more frequent among owl species occupying lower trophic levels, which could be explained by a particularly strong selection for crypsis on small predator owls. Results, thus, provide support for the idea that colour polymorphism in owls is an adaptive character likely maintained by the selective advantage of colour morphs under different environmental conditions via disruptive selection mechanisms.

Afterwards, I used phylogenetic comparative models to test the camouflage hypothesis for eye colour, a remarkably coloured feature whose functional basis remains poorly understood. I found that the proportion of dark-eyed owl species is higher among strictly nocturnal owls than among diurnal ones. Ancestral state reconstruction revealed that the ancestor of the family *Strigidae* was more likely bright-irided whereas the ancestor of the family *Tytonida*e was more likely dark-irided. These results show robust support for the coevolution of iris coloration and nocturnality in the owls, and suggest that shifting to a nocturnal niche would be a prerequisite leading to the evolution of dark eyes in owls. The specific evolutionary pathway by which iris coloration and activity rhythm coevolved, however, remains to be investigated further as I have found only partial support for the idea that dark irises in owls might be an adaptive feature evolved due to the selective advantage of concealment from undesired visual receptors.

Finally, given that iris colour is a remarkably striking feature in the wholly cryptic pattern of many owls, which may suggest it may potentially play a signalling function, I studied variation and potential signaling of iris yellowness as an indicator of quality in parent-offspring communication and other social contexts in Little Owl (*Athene noctua*) and Eurasian Scops-Owl (*Otus scops*). Yellowness did not differ between the sexes; however, adults of the two species had more intensely yellow irises than owlets. Most of variation in iris yellowness of owlets occurred between rather than within nests and seemed to be linked to parental qualities in Little Owls, but was unrelated with condition among Eurasian Scops-Owl owlets. In adults, however, I found that iris yellowness of females was positively associated with nest success (an index of female fitness) in Little Owls, but not in Eurasian Scops-Owls. This study suggests that iris color variation is unlikely to play a role in parent-offspring communication in these two owl species, but that iris yellowness in female Little Owls may potentially play a signalling role in social contexts.

Summing up, the results of my thesis show that environmental variation may act in several ways promoting the evolution of colour variation in owls at different spatial scales, and more specifically, that plumage and eye colour can potentially serve several, previously overlooked, adaptive functions in this clade.

RESUMEN

Las aves muestran una extraordinaria variabilidad en sus patrones de color modelados por la selección natural y sexual para cumplir diferentes funciones como la comunicación, el camuflaje y/o de protección. Los factores ambientales que promueven dicha variación pueden ser muy diversos y actuar de forma diferente sobre distintos rasgos y niveles de organización. Por esta razón, para comprender de manera integral como la ecología influye en la evolución de tan excepcional diversidad de formas coloreadas, es necesario considerar aproximaciones holísticas que combinen múltiples niveles de estudio, incluyendo diferentes rasgos y diferentes especies que ocupen un amplio rango de condiciones ecológicas.

En esta tesis doctoral, he utilizado una aproximación multidisciplinar en la que se combinan i) análisis eco-geográficos a escala mundial, ii) análisis comparativos para controlar por el posible efecto de un ancestro común, y iii) datos de campo de dos especies recogidos en una población, para evaluar la importancia relativa de una serie de factores ecológicos y evolutivos a la hora de determinar la variación de rasgos coloreados en los estrígidos. Más concretamente, he estudiado i) la variación geográfica a gran escala de la coloración con base melánica en relación a gradientes ambientales en el marco de reglas eco-geográficas clásicas, ii) la variabilidad interespecífica en el polimorfismo de color en relación a factores ecológicos clave, iii) la evolución de la variación interespecífica de la coloración del iris en relación al ritmo de actividad y iv) he analizado el potencial de la coloración del iris como indicador de la calidad en diferentes contextos de comunicación en dos especies de búhos.

Los análisis eco-geográficos a escala global mostraron que los búhos presentan fenotipos más oscuros cerca del ecuador. En particular, las especies de búhos que viven en regiones con temperatura más altas y vegetación más densa son más oscuras, lo cual excluye un posible papel de la termorregulación a la hora promover la variación del plumaje a una escala global. Por otro lado, se

observó que las especies de búhos que habitan en áreas con una cobertura arbórea mayor eran más eumelánicas, lo cual está de acuerdo con un potencial papel de la coloración basada en eumelanina en el camuflaje. Finalmente, la importancia de la coloración basada en feomelanina era mayor en especies que viven en zonas más cálidas y húmedas, sugiriendo en conjunto que distintas fuerzas selectivas podrían haber simultáneamente contribuido a modelar la variación global en el color del plumaje en este clado.

En segundo lugar, se examinó en un contexto comparativo la importancia relativa de diferentes factores ecológicos a la hora de promover la evolución del polimorfismo de color en los búhos en el marco de tres escenarios evolutivos mutuamente no exclusivos: la hipótesis de la selección apostática, la hipótesis de la divergencia de nicho y la hipótesis de no selección. De acuerdo con la hipótesis de la divergencia de nicho, se encontró que las especies que viven bajo condiciones lumínicas variables, i.e. especies con hábitos crepusculares y diurnos, y aquellas que ocupan una mezcla de hábitats abiertos y cerrados, eran con más probabilidad polimórficas. Los análisis de coevolución mostraron que un cambio en el nicho lumínico podría ser un prerrequisito fundamental para la evolución del polimorfismo de color en los búhos. Además, el polimorfismo resultó más frecuente entre las especies que ocupan los niveles tróficos más bajos, lo cual podría venir explicado por una selección más fuerte para la cripsis en las especies depredadoras de pequeño tamaño. Los resultados, por tanto, proporcionan apoyo a la idea de que el polimorfismo de color en los búhos es un rasgo con valor adaptativo probablemente mantenido por una ventaja selectiva de los morfos bajo diferentes condiciones ambientales a través de mecanismos de selección disruptiva.

A continuación, se utilizaron modelos filogenéticos comparativos para testar la hipótesis del camuflaje para el color del iris, un rasgo coloreado cuya base funcional no está aun suficientemente clara. Se encontró que la proporción de especies con ojos oscuros es más alta entre las especies estrictamente nocturnas que en las diurnas. La reconstrucción ancestral mostró que el ancestro de la familia *Strigidae* tenía con más probabilidad ojos claros, mientras que el ancestro de la familia

Tytonidae tendría probablemente ojos oscuros. Estos resultados proporcionan evidencia en favor de una coevolución de la coloración del iris y la nocturnalidad en los búhos, y sugieren que el cambio a un nicho nocturno sería un prerrequisito que ha llevado a la evolución de los ojos oscuros en los búhos. Sin embargo, la ruta evolutiva mediante la cual la coloración del iris y el ritmo de actividad han evolucionado en concierto necesitaría ser más investigada, dado que se encontró solo un apoyo parcial a la idea que los ojos oscuros podrían haber evolucionado por una ventaja selectiva de ocultación frente a receptores visuales no deseados.

Finalmente, puesto que el color del iris es un rasgo muy llamativo en el diseño generalmente críptico de los búhos, sugiriendo que podría jugar un posible papel señalizador, evalué la variación y el potencial para señalizar calidad del color amarillo del iris en la comunicación paterno-filial y entre conspecíficos en el mochuelo *Athene noctua* y en el autillo *Otus scops*. La coloración del iris no varió entre sexos; sin embargo, los adultos de ambas especies presentaron iris más intensamente coloreados que sus pollos. La mayor parte de la variación en la coloración del iris de los pollos se dio entre los nidos, y no dentro de ellos, y pareció estar relacionada con la calidad de los padres sólo en los mochuelos, mientras en los pollos de autillos no se encontró ninguna relación con la condición. En los adultos, se encontró que la intensidad del color amarillo de las hembras se asociaba positivamente con el éxito del nido (un índice del fitness) en el mochuelo, pero no en el autillo. Este estudio sugiere que es muy improbable que la variación de color del iris esté involucrada en la comunicación paterno-filial en estas dos especies, pero que, en las hembras de mochuelo, el color del iris podría potencialmente desempeñar un papel en la señalización en contextos sociales.

En conclusión, los resultados de esta tesis muestran que la variación ambiental podría actuar de manera diferentes promoviendo la evolución de la variación de color en los búhos a diferentes escalas espaciales y, más específicamente, que el color del plumaje y de los ojos podría potencialmente servir para diferentes funciones adaptativas previamente poco consideradas en este clado.

GENERAL BACKGROUND

INTRODUCTION

1.1 Colourful birds

Birds present a huge diversity in their phenotypic traits, including an outstanding variation in coloration (del Hoyo et al. 1999). Thanks to methodological advances in objective colour quantification made in the last decades, it is now possible to measure, for example, the variability in feathers and tegument coloration of bills and foots, which is critical to understand its functions (Cuthill et al. 2017). The importance of understanding why colour varies dwells in the fact that coloration plays a fundamental role in key biological functions, such as communication and camouflage, and it is determined by pigments and/or structures involved in many other important physiological processes (Hill and McGraw 2006, Hill and McGraw 2006). In addition, colour plays a relevant role in visual discrimination at multiples levels, helping to avoid possible misidentifications of relatives, partners, predators or parasites that could lead to a decrease in fitness (Savalli 1995).

The colour we perceive in birds comes from either the refraction of light caused by the microscopic structure of feathers or integuments (i.e. structural coloration) (Prum 2006), or from pigments that are included in these and that determine a chemical coloration (Hill and McGraw 2006), or is the result of the joint action of pigments and structural coloration. Pigments are coloured compounds that can be found both in plants and animals, and whose chemical origin is very heterogeneous. Their importance, from a functional point of view, is that they are actively involved in physiological processes, acting, for example, as powerful antioxidants (McGraw 2005), or playing a role in the functioning of the immune system (Pérez-Rodríguez et al. 2010, Sepp et al. 2011). Furthermore, they can exert a protective action against the negative effects of ultraviolet radiation (Kirschfeld 1982), and are important in reinforcing tissue structure (Bonser 1995). Five types of pigments have been identified in birds: carotenoids, melanins, pteridines (or pterins), porphyrins, and psittacofulvins (McGraw 2005). With the exception of psittacofulvins, which are exclusive of

Psittacifomes, the rest of pigments are present in all bird families and their combination gives rise to a wide pallet of colour shades (McGraw 2005).

Because coloration of plumage or any other body parts depends on the availability of pigments and/or dietary nutrients, pigmentary colorations are commonly considered as honest signals of individual quality (Hill 1999). Structural coloration requires a good condition through the moult period, and thus, also the expression of structural coloration may serve as a signal of individual quality in several multiple social contexts (Bennett 1996, McGraw et al. 2002, Hill and McGraw 2006). Therefore, irrespective of their pigmentary or structural origin, avian colorations might transmit information about adult and young individuals' quality, playing a key role as communication signals (e.g. Hill 1991, Torres and Velando 2010, Soler and Avilés 2010).

Colour patterns, therefore, have been always considered to play a main role in communication among diurnal birds. On the other hand, crepuscular and nocturnal birds have traditionally been expected to rely mostly on acoustic communication, but this old belief has been recently challenged. Several studies have provided evidence that nocturnal birds seem to decipher achromatic cues, i.e. black and white colours, probably thanks to their higher contrast that makes them more visible under scant luminal conditions (reviewed in Penteriani and Delgado 2017). The importance of achromatic patterns is widely documented in diurnal birds, and studies on different species have shown that they may play an informative role in several social context (e.g. Galván 2008, Stang and McRae 2009, Mumme 2014). Nevertheless, there are still few evidences supporting the use of chromatic signals in crepuscular and nocturnal birds, and they are focused on *Strigiformes* (e.g. Avilés and Parejo 2012, 2013).

1.2 The importance of a multi-scale approach for studying the evolutionary ecology of colour

Colours in birds are adaptations shaped by abiotic (i.e. light environment, climate, etc.) and biotic factors (i.e. predatory pressure, competitiveness between individuals, etc.) (Dalrymple et al. 2018)

and maintained through natural (Bortolotti 2006) and/or sexual selection (Dale et al. 2015) to fulfil different functions that can be summarized in three main categories: communication, camouflage and physical-physiological functions (Ortolani 1999).

Abiotic and biotic factors promoting colour variation are likely to vary spatially and temporally within and between populations of the same and different species (Cuthill et al. 2017). Therefore, in order to have a holistic knowledge of the evolution of colour diversity, it becomes crucial to adopt multi-scale approaches that allow studying the functional basis of colour variation at different spatial scales: from individuals within a population, to groups of species, or even to avian communities inhabiting contrasting environments.

This thesis aims to identify environmental factors promoting variation in plumage and iris colour in owls at different spatial scales (Fig.1). In a first stage, I will deal with interspecific variability in plumage colour at a world scale, that will allow respond the specific question of whether large-scale variation in plumage coloration, particularly melanin-based pigmentations, is associated with variation in environmental conditions over wide geographical scales. At a second level, I will investigate the most likely ecological factors leading to the evolution of plumage colour polymorphism and eye coloration in owls, with a particular emphasis on the role of heterogeneity in luminal environments. Finally, considering two owl species with different ecology, I aim to identify a potential role of iris coloration in communication.

By tackling colour variation on a variety of scales (see Fig. 1), I was able to integrate both field studies and modern comparative methods. Such an approach is the best way to assess if ecological factors promoting colour plumage variation on a worldwide scale may or not operate at a smaller niche scale or at population level. Furthermore, it makes possible to ascertain if different phenotypic traits are under similar selective pressures and likely maintained by the same adaptive advantage. Finally, phylogenetic analyses allow understanding the role of phylogenetic relatedness in the evolution of coloured trait variation.

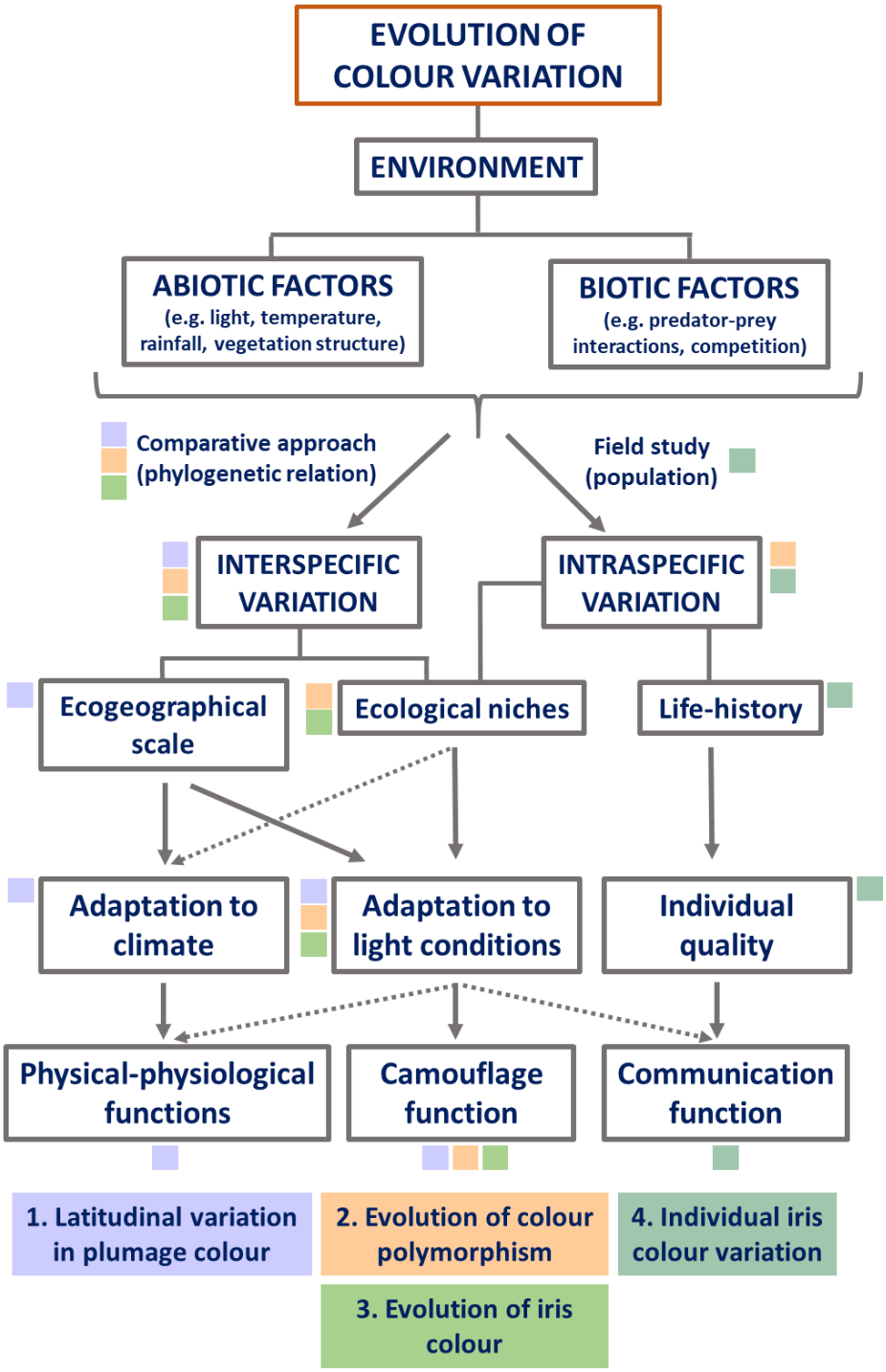

Figure 1. *Outline of thesis showing the multiple levels considered in the study of the evolutionary ecology of colour in owls and the objectives pursued at each stage. From global patterns of colour variation in an ecogeographical scale, considering climate and vegetation cover, descending to the study of evolutionary drivers of interspecific variation in different colour traits and to the study of the potential role of intraspecific colour variation in a communication context within a population. Dashed arrows indicate potential relationships that were discussed in the thesis but not supported by any analyses. The numbers in the colour boxes refer to the different chapters of the thesis.*

1.3 Owls as a study system for studying the evolutionary ecology of coloration

Owls constitute an ideal system to understand the evolutionary causes of phenotypic variation because the clade comprises a large number of species displaying a striking diversity in colour patterns and morphology associated to a complex ecology (König and Weick 2008).

Strigiformes is an order of mostly nocturnal birds of prey divided in two different families: *Tytonidae* (Barn and Bay owls) and *Strigidae* (the "true" owls). According to the comprehensive recent phylogeny by Jetz and co-workers (2012), the clade includes 206 species distributed in 26 different genera (see however (BirdLife International 2017 for a more recent split of subspecies)). Barn owls family, i.e. *Tytonidae*, includes 13 species separated in two sub-families, *Tytoninae* and *Phodilinae* with each one comprising the genus *Tyto* spp. and *Phodilus* spp., respectively. On the other hand, the *Strigidae* family represents the largest group, including the sub-families *Ninoxinae*, *Surniinae* and *Striginae* (Fig. 2). Owls are elusive birds of prey distributed worldwide, except in the Antarctica, occupying almost every type of terrestrial habitat (Mikkola 2014). Moreover, they differ greatly in their distributional ranges, inhabiting different ecological conditions, ranging from almost cosmopolitan species like the Barn Owl (*Tyto alba*) to species restricted to very small areas with narrow ecological niches like some *Ninox* species inhabiting remote Indian and Pacific islands (König and Weick 2008). Owls also present a huge range of body sizes, ranging from species the size of a sparrow, as in the genus *Glaucidium*, to species the size of a large eagle, as those in the genus *Bubo*. Therefore, owls occupy different trophic levels in food webs, from small predators, with a chiefly insectivorous diet as Scops owls (*Otus* spp.), to top predators as Eagle Owl (*Bubo bubo*) that can feed on fox-sized mammals. Owls are mostly sedentary, with few species undertaking true migration (e.g. Eurasian Scops-Owl *Otus scops*) or cyclic erratic movements following their main prey (e.g. Snowy Owl *Bubo scandiacus*) (König and Weick 2008). They are mostly territorial living solitary or in pairs that can display very aggressive intraspecific behaviours, including intra-guild predation (Lourenço et al. 2014). However, there are some species where individuals may roost together in small groups

during the winter (König and Weick 2008), or use conspecific and hetero-specific contact calls to assess predation risk before and during their reproduction (Parejo et al. 2012, Parejo and Aviles 2020). As in diurnal raptors, owls generally present inverse sexual dimorphism, with females being larger than males, and only a few species show sexual dichromatism (König and Weick 2008). Notably, although many owls are strictly nocturnal, some species extend their activities out of the night, and can be classified as mainly active at twilight or during the day (König and Weick 2008), providing a prime opportunity to assess the adaptive value of different colour patterns under a wide range of luminal conditions.

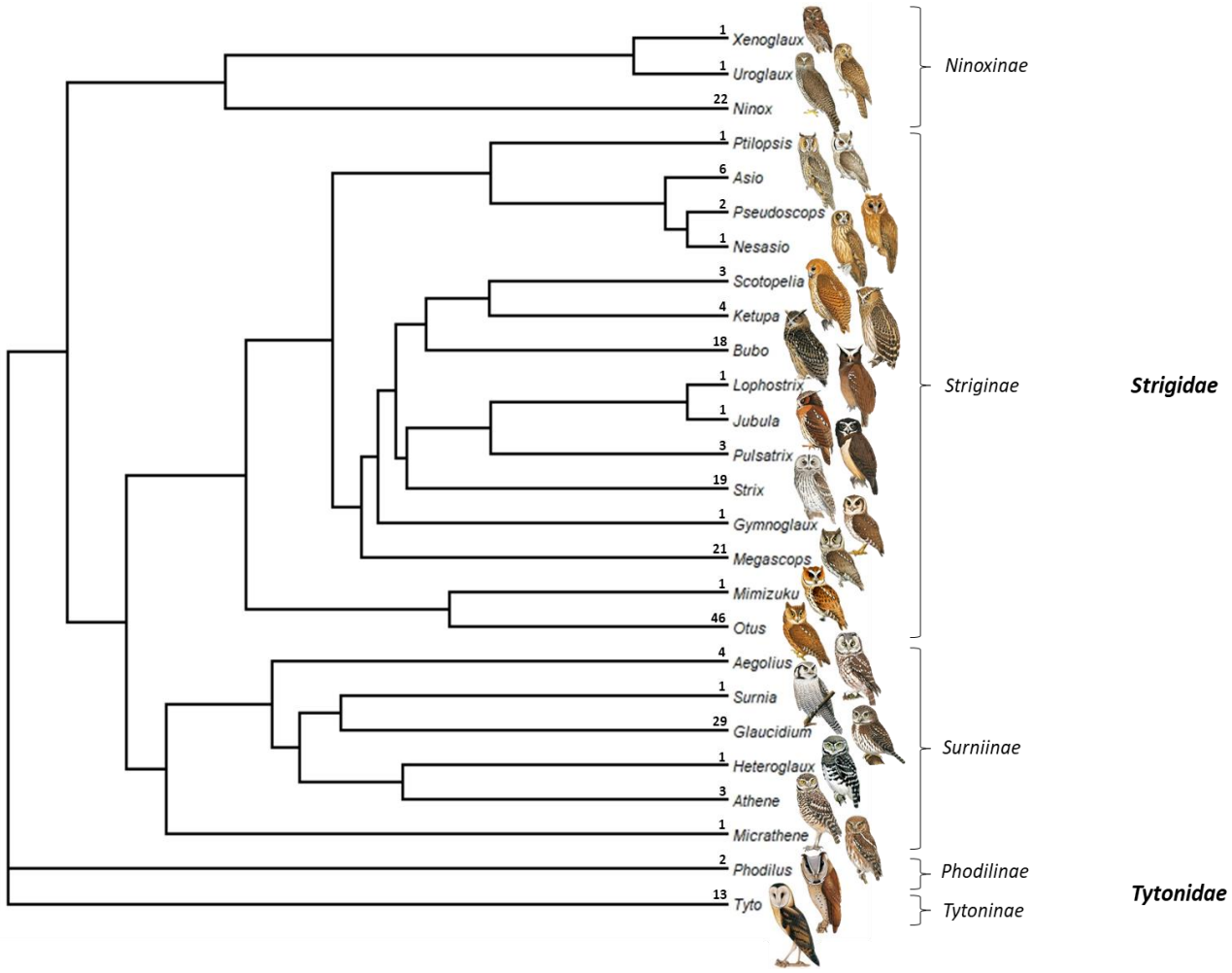

Figure 2. *Phylogenetic tree, randomly chosen from a sample of 1000 phylogenies among 10000 available, showing the relationship between the 26 genera included in the order Strigiformes (Jetz et al. 2012). Numbers at the end of the braches indicate the number of species belonging to each specific genus. The images of species in the tips of the phylogeny are not scaled by size of species as the aim was illustrating chromatic variability and were taken from the Handbook of the Birds of the World Alive (ed J. del Hoyo et al. 2017). Lynx Edicions, Barcelona. (Retrieved from www.hbw.com).*

Owl plumage show cryptic colour patterns mostly determined by melanins (e.g. Gasparini et al. 2009, Emaresi et al. 2011, Avilés et al. 2020). In contrast to other bird groups (e.g. passerines (Delhey et al. 2019)), the adaptive function of plumage colour in owls remains elusive. Melanins can play a role in strengthening feather structure (Bonser 1985) and improving resistance to abrasion and pathogen degradation (Kose and Møller 1999). In addition, because the palette of colours implied in camouflage is mostly dull and produced by melanin (Galván and Wakamatsu 2016), a concealment function might afford a plausible connection between colour and geographical clines in owls. So far, results are contradictory regarding large-scale associations between colour and environmental factors in owls. Melanic pigmentation was found to vary in an opposite manner to what predicted by Gloger's rule in barn owls (Roulin et al. 2009, Roulin and Randin 2015), whereas plumage redness increased with latitude as predicted by Gloger's rule in a comparative study based on 57 species (Roulin et al. 2011). However, in these studies the environmental factors promoting geographical clines in coloration were not identified, urging for a re-assessment of the role of climatic and environmental variables in promoting large-scale geographical colour patterns in the clade.

Species in which individuals within a population display multiple genetically-inherited colour variants, whose expression is independent of the environment and body condition, are considered to be polymorphic (White and Kemp 2016). Owls show one of the highest incidence of colour polymorphism among birds (Galeotti et al. 2003) and, non-surprisingly they have been the target of previous comparative work dealing with the evolution of such a prominent feature (Galeotti et al. 2003, Fowlie and Krüger 2003, Galeotti and Rubolini 2004). Colour polymorphism within a population can be discrete, implying extreme variation among the colour variants, or graded as in the Tawny Owl (Emaresi et al. 2013) and the Eurasian scops-owls (Parejo et al. 2018). However, mechanisms behind the evolution and maintenance of polymorphism in this group remain elusive as previous comparative work lead to contradictory results regarding the importance of the niche divergent hypothesis (Fowlie and Kruger 2003, Galeotti and Rubolini 2004). Indeed, these studies

were based on a limited number of species, and did not quantify and account for the tendency for related species to resemble each other (i.e. phylogenetic signal) (Revell et al. 2008) urging for a re-examination of the functional basis of colour polymorphism in owls in a comparative framework.

Beyond plumage, irises can present extreme bright colorations in owls, that, together with the frontal position of the eyes, make eyes and owls highly conspicuous (Fig. 3). Why some owl species display bright irises, where others do not, remains a mystery, and it might be related to camouflage in nocturnal light conditions because owls displaying dark eyes may disguise themselves while perching for hunting in the night, a strategy that might fool both predators and their prey. Although there are studies linking eye size and the activity rhythm (Lisney et al. 2012), also in other bird species (Thomas et al. 2002), the knowledge about the ecological factors driving the evolution of iris colour in an interspecific context remain largely overlooked (Negro et al. 2017). The remarkably conspicuousness of iris colour in the cryptic design of owls, may also suggest that it could play a signalling function in intraspecific framework. A number of studies in diurnal birds have suggested a possible signalling function or a role in mate recognition for eye coloration (e.g. Picozzi 1981, Davidson et al. 2014). This possibility might be plausible in owls as well (see Wails et al. 2018) since it has been observed that they can use coloration to obtain information in different contexts (Bortolotti et al. 2011). For example, in Eagle Owl (*Bubo bubo*), the white patch of the throat exhibited during territorial displays (Penteriani and Delgado 2009) is a likely signal of individual quality (Penteriani et al. 2006a, 2006b). In addition, white feathers around the owlets' mouth might play a role in parent-offspring communication (Penteriani et al. 2007). Moreover, other owl species could also rely on chromatic signals. In the Eurasian Scops-Owl (*Otus scops*) the cere of owlets shows a marked peak in the UV part of the spectrum, and parents biased in favour of lighter offspring which was simulated by reducing UV intensity (Parejo et al. 2010). Also in Little Owl (*Athene noctua*), studies on bill yellowness provide evidence for a role of chromatic signalling in sexual/social context (Avilés and Parejo 2012) and in parent-offspring communication (Avilés and Parejo 2013).

Figure 3. *Variation in plumage colour (a), degree of colour polymorphism (b) and iris colour (c) in Strigiformes. Species in panel (a) from left to right are Tyto alba, an almost ubiquitous species representing Tytonidae family, Strix nebulosa, Athene cunicularia, Ketupa zeylonensis and Strix (Ciccaba) nigrolineata. Examples of colour polymorphism in panel (b), from left to right, in Otus ireneae, Tyto alba, and Strix uralensis. Species illustrating iris colour variability in panel (c) are, from left to right, Ninox boobok, Asio flammeus, Ptilopsis granti and Strix aluco.*

OBJECTIVES

The main aim of this thesis is to understand the role of ecology in the evolution of coloration in the *Strigiformes* order, through a multi-scale analysis of the causes of variation of plumage and iris colour.

To achieve this goal, I will use comparative methods to study ecogeographical patterns of plumage colour at a world scale within the clade (**Chapter I**), as well as interspecific variation of plumage polymorphism (**Chapter II**) and iris coloration (**Chapter III**) in relation to different environmental variables and ecological traits of the species.

Aiming to deal with intra-specific variation in coloration, I also performed a field study and evaluate the potential role of iris colour as indicator of individual quality within a population in two social contexts (parent-offspring communication and between adults) in two owl species (Little Owl and Eurasian Scops-Owl) (**Chapter IV**).

GENERAL METHODS

Doctoral theses usually include a chapter about the general methods used. However, given that different analytical approaches have been used in each chapter, material and methods in this thesis are described in detail within each chapter. Therefore, in order to avoid any redundancy, they will not be reported in a separate section.

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SECTION 1:

GEOGRAPHIC PATTERNS OF

COLOUR VARIATION

Chapter I

Darker when warm and vegetated: ecogeographical rules in owl plumage coloration

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ABSTRACT

Ecogeographical rules associate animal colour patterns, particularly melanin-based pigmentations, with variation in environmental conditions over wide geographical scales. In particular, the Gloger's rule, coined for endothermic animals, suggests that the deposition of both eumelanin and pheomelanin would increase at high temperature, whereas an increase in environmental humidity would favor eumelanin deposition but would reduce pheomelanin. On the other hand, the Bogert's rule, predicts that darker colorations should be more frequent in animals inhabiting colder areas given their thermoregulation benefits. Here, we test these contrasting expectations on a world scale in owls, a group of nocturnal birds displaying huge variability in the degree of melanin-based plumage coloration and environmental specialization. We found that owls display darker phenotypes near the equator. In particular, owls inhabiting regions with high temperature and living in more densely vegetated areas were darker, which would exclude a key role of thermoregulation in promoting plumage variation in owls. Analyses on a pigment basis revealed that species inhabiting areas with a denser tree cover were more likely eumelanic, and that the extent of potential eumelanin colours increased at medium-high temperature but decreased at very high ones, which would agree with a potential role of eumelanin in background matching for camouflage. Finally, the proportion of pheomelanin colour was higher in species inhabiting warmer and wetter areas. Our results stress that several alternative selective forces may simultaneously be at work when studying ecogeographical patterns of colours where different melanin types are involved, and urge for experimental work to test the possible mechanisms behind the detected associations between owl colour and environmental variables.

INTRODUCTION

Understanding why phenotypic patterns differ across the environments has long puzzled evolutionary ecologists (Darwin 1859, Cott 1940), and remains a key challenge today, as it may help to understand adaptive responses to climate change (Radchuk et al 2019). In particular, latitudinal patterns of animal form aroused the greatest interest in the past, and have given birth to a series of pivotal assumptions termed as "ecogeographical rules". These rules are based on the observation that aspects of animal phenotype (e.g. size (Bergmann 1847) and shape (Allen 1877)) would be predictable across latitudinal clines due to the highly variable environments that animals face when spread across large spatial distances (Gaston et al. 2008). A paradigmatic example is the Gloger's rule (Gloger 1833), which postulates a relationship between broad-scale climatic gradients and pigmentation in endotherms, pointing out that, within populations of the same species, or, between different species, more heavily pigmented forms should be found in warm and humid regions while lighter forms should be more frequent in cold dry areas (see Delhey 2017). In contrast, the Bogert's rule (Bogert 1949), also termed "the thermal melanism hypothesis", chiefly postulated for ectotherms, states that animals inhabiting cold regions would benefit from having dark coloration as this would enhance their thermoregulation performance by absorbing more solar radiation (Clusella Trullas et al. 2007). Notably, while the Bogert's rule is based on the thermal properties of coloration establishing a causal link between temperature and coloration, the mechanisms underpinning the Gloger's rule could be diverse, but still to be defined (Delhey 2019).

Most of the light to dark colour variation in animals is due to melanins (McGraw 2006), which are the most common pigments coating animal integuments and feathers (McGraw et al. 2005), and that have primarily evolved as an adaptive mechanism of protection against UV radiation (Brenner and Hearing 2008). Melanins can be found in two main forms: eumelanin, mainly responsible for black and grey tones, and pheomelanin, which produce brown, fulvous and reddish colours. Melanins are particularly abundant in birds playing a key role in strengthening feather structure (e.g. Bonser

1995) and improving resistance to abrasion and pathogen degradation (e.g. Kose and Møller 1999). Melanins also enhance crypsis, because the colours produced by these pigments (i.e. different shades of black, grey and brown) are dull and and thus favor camouflage with surrounding environment (Galván and Wakamatsu 2016). Hence, a concealment function might afford a plausible connection between spatial colour variation and geographical clines since habitat structure is likely to vary across the globe in relation to climatic conditions (Delhey 2017). Denser canopies, which are usually found in warmer and wetter regions, are likely to provide darker backgrounds because of their lower irradiance levels (Endler 1993), which would promote the occurrence of better-concealed darker phenotypes. In the same vein, colder areas, commonly associated to open spaces, more subjected to strong light radiation, would favor lighter colour patterns.

So far, a large body of empirical work has found interspecific (e.g. Zink and Remsen 1986, Dalrymple et al. 2018, Delhey et al. 2019) and intraspecific colour patterns (e.g. Hogstad et al. 2009, Roulin and Randin 2015) fitting the Gloger´s rule in birds. Nevertheless, some studies have also yielded mixed support, revealing colour patterns fitting the Bogert's rule (Friedman and Remeš 2017, Delhey 2018), and, suggesting that the colour adaptation to environmental conditions may happen at more local geographical scales (Delhey 2018, Galván et al. 2018). Contradictory results might also be due to the need of recasting the Gloger's rule as originally proposed by Rensch (1929), separating temperature and humidity as well as the two types of melanin causing colour, since their deposition in feathers might be correlated with temperature and humidity in a different fashion (see Delhey 2017, 2019). Accordingly to this new view, eumelanin deposition would increase gradually with temperature whereas pheomelanin deposition would increase with temperature only after a certain treshold. More importantly, only eumelanin deposition would increase with humidity while pheomelanin deposition should increase in dryness (see Delhey 2019).

Owls constitute a world-wide-distributed clade of birds, providing an ideal study system to examine ecogeographical rules in a comparative framework. First, owls show huge variation in niche

specialization and distributional ranges with some species almost worldwide distributed, occupying diversified habitats as the Barn owl *Tyto alba*, while others, as several *Otus* species, inhabiting remote Indonesian islands and restricted to very specific environmental conditions (König and Weick 2008). Second, owl cryptic plumage colour is mostly composed by eumelanin and pheomelanin (Gasparini et al. 2009, Roulin et al. 2013), making this an ideal clade to examine the most complex version of the Gloger's rule recently proposed by Delhey (2019). Finally, owls provide a prime opportunity for testing the generality of ecogeographical rules in a mostly nocturnal clade of birds, in which ecological constraints acting on plumage expression might differ from those on diurnal species (e.g. Passarotto et al. 2018). Nothing is known on whether nocturnality influences the way that climatic conditions determine plumage colour adaptations over wide geographical scales.

Previous studies have analysed geographic variability of Barn Owl (*Tyto alba*) coloration, an almost ubiquitous taxon, and found individuals with darker pheomelanin-based coloration in colder regions (Roulin et al. 2009, Roulin and Randin 2015). However, barn owls represent a specific family that could have followed a different evolutionary pathway (del Hoyo et al. 1999). Also at an interspecific level, it was found that owl plumage redness was higher near the equator (Roulin et al. 2011). However, that study did not identify the climatic factors behind the latitudinal cline in colour, and was based only on four owl genera urging for a re-examination of Gloger's and Bogert's rules in *Strigiformes*. Here, we first analyse light-to-dark (i.e. lightness) plumage variation in relation to latitude while accounting for phylogenetic relationships among species. In a second step, we study lightness and the proportion of plumage colour potentially due to eu- and pheomelanin in relation to climatic variables (i.e. temperature and rainfall) and vegetation cover. According to Gloger's rule, we would expect that pigmentation become less intense in warm and humid areas. However, the complex version of the Gloger's rule would predict that owls inhabiting warmer and wetter areas would be more eumelanic. On the contrary, pheomelanin would be more frequent in owls living in dry environments, which would be predicted by a negative relationship between pheomelanin proportion

and rainfall. Finally, the Bogert's rule would predict that darker owl species would be found in colder regions.

MATERIAL AND METHODS

Plumage colour and melanization

We measured plumage colour variation on digitally scanned images from the plates in the Handbook of the Birds of the World (hereafter HBW) (del Hoyo et al. 1999) in 198 (96%) out of the 206 extant owl species considered in the most updated avian phylogeny currently available (Jetzt et al. 2012) (see Supplementary material). When different subspecies were represented on plates we systematically considered the nominal form, and for polymorphic species (N = 41 out of 198 sampled species) we averaged values of the morphs when they were drawn in plates. Sexual dichromatism in owls is uncommon and very rarely illustrated in the HBW (1% of sampled species).

Plates were scanned together with a colour reference card (X-Rite ColorChecker® Passaport) and standardized using the SpotEgg tool software (Gomez and Liñan-Cembrano 2016). For each species a stratified random sample of RGB (Red, Green, Blue) values was obtained by quantifying these into three subjectively selected polygons using the R package 'colorZapper' (Valcu and Dale 2014) within three characteristic body patches (i.e. head (including face and throat), front (including breast and belly) and back (including wings)). We quantified lightness as (R+G+B)/3, which varies from 0, indicating pure black, to 255, corresponding to pure white. The three lightness values obtained within each body patch were averaged to obtain a mean lightness per body patch, and a mean lightness for each species was subsequently calculated based on these (e.g. Delhey 2019).

In a second stage, aiming to disentangle the role of different melanin types in the detected ecogeographical patterns (see results), we quantified from the same scanned images the number of pixels potentially associated with eumelanin (i.e. black and grey) and pheomelanin (i.e. brown, rufous, chestnut and similar colours) using the plugin 'Threshold colour' in ImageJ program (https://imagej.nih.gov). Previous comparative studies have successfully estimated the presence and

relative importance of melanin based on colour plates (e.g. Bókony et al. 2003, Galván and Møller 2011, 2013). Specifically, we considered as potential eumelanin colours those whose RGB values simultaneously ranged from 0 to 59 for the R channel, from 0 to 106 for the G channel and from 0 to 104 for the B channel. Regarding potential pheomelanin colorations, we consider these to simultaneously have values ranging from 60 to 255 for the R channel, from 26 to 224 for the G channel, and from 5 to 110 for the B channel. Prior to this, we had removed from images all elements added by the artists that could potentially generate glare, as well as non-plumage traits (i.e. eyes and bill). Then, we calculated the proportion of pixels potentially associated with eumelanin and pheomelanin relative to the total amount of pixels in each body part and averaged these three values to obtain single values per species.

Plumage colour estimates in this study were derived from HBW plates rather than from objective spectrophotometry. Previous studies have shown that coloration estimated on plates strongly correlated with colour measures obtained from museum specimens through spectrophotometry (e.g. Badyaev and Hill 2000, Dale et al. 2015, Delhey et al. 2019). Moreover, a previous comparative survey reported significant positive across-species correlations between eu- and pheomelanin scores estimated on plates and the concentration of these two pigments in feathers (Galván et al. 2012), suggesting that our estimations of proportion of eu- and pheomelanin-based colorations would provide a reliable index of plumage melanization for comparative studies.

Climatic variables, vegetation cover and latitude

Based on distributional maps for each species provided by BirdLife International (2018), we obtained average species-specific information on the mean annual temperature (bio1, °C) and mean annual precipitations (bio12, mm/year) from CHELSA, a high-resolution climate data set (Karger et al. 2017). Percent of tree cover within each pixel was obtained from DeFries et al. (2000) and resampled to the spatial resolution of CHELSA data (i.e. 30 arc sec) using Google Earth Engine. All estimations were done in QGIS 3.0.

Previous tests of the Gloger`s rule have frequently relied on latitude as a proxy of temperature to assess ecogeographical patterns of colour, a premise worth testing in our data set. Hence, as in Passarotto et al. (2018), we estimated latitude (in degrees) as the average between the most northern and southern latitude of the distribution map of each species.

Statistical analyses

All variables were centered and scaled to improve the interpretability of regression coefficients (Schielzeth 2010). In a first step, we estimated the phylogenetic signal of plumage lightness and potential eu- and pheomelanin coloration by computing Pagel's λ (lambda) statistics (Pagel 1999) using the function 'phylosig' in the R package 'phytools' (Revell 2012). Pagel's λ values close to 0 are indicative that variation in a trait is random regarding the phylogeny. Conversely, values close to 1 are indicative of clumped trait variation across the phylogeny, and, hence that the trait evolved under a Brownian motion model. We calculated the phylogenetic signal for all 1000 phylogenies and then verified the distribution of values to determine the departure from both 0 and 1 (see Fig. 1S in Supplementary material).

Afterwards, we performed phylogenetic generalized least squares regressions (PGLS hereafter) using the R packages 'nlme' (Pinheiro et al. 2011) and 'GEIGER' (Harmon et al. 2007) to control for the possible effects of common ancestry on the relationship between plumage colour as dependent variables (i.e. lightness and proportion of eu- and pheomelanin coloration) and the environmental predictors (i.e. latitude in a first stage and then temperature, rainfall and tree cover). Since relationships might be not linear, we also included the quadratic effects for each variable (Delhey et al. 2019). All the analyses were based on a sample of 1000 phylogenetic trees obtained from birdtree.org (Jetz et al. 2012). We built a set of candidate models involving all possible combinations of predictors, and selected the best-fit models using Akaike's information criterion using the R package 'MuMIn' (Barton 2017) (Table 1S-3S in Supplementary material). All models were first run on a majority-rule consensus tree calculated on the 1000 random phylogenies using the function

'consensus.edge' in the R package 'phytools' (Revell 2012). After having selected the best-fit models, we reassessed all the relationships using the whole set of 1000 phylogenetic trees applying model averaging procedure (Symonds and Mousalli 2011) through the R package 'AICcmodavg' (Mazerolle 2011), which allowed calculating average effects and 95% confidence limits from the variance of model parameters across models while accounting for uncertainty due to phylogeny (Garamszegi and Mundry 2014).

RESULTS

Relationship between plumage colour variables

The extent of potential eumelanin-based colour was inversely related to the extent of pheomelanin-based colour across extant owls (coefficient (SE) = - 0.50 (0.05), t = - 10.51, $P < 0.0001$). Plumage lightness was negatively correlated with the extent of potential eumelanin-based colour (coefficient (SE) = - 0.75 (0.08), t = - 9.61, $P < 0.0001$) but not with the extent of potential pheomelanin-based colour (coefficient (SE) = - 0.02 (0.08), t = - 0.33, $P = 0.73$), meaning that darker owls would have a significant larger extent of potential eumelanin plumage colour.

Phylogenetic signal

Close relative owls are on average as similar as distant relatives ones are regarding plumage lightness (lambda = 0.31, $P = 0.08$) (Fig.1Sa in Supplementary material). However, both proportion of potential eu- and pheomelanin plumage colour have a significant phylogenetic signal (lambda = 0.69, $P < 0.001$ and lambda = 0.64, $P < 0.001$ respectively), meaning clumped trait variation across the phylogeny for these two traits (Fig.1Sb and 1Sc in Supplementary material).

Correlates of plumage lightness

Owls living near the equator are darker (quadratic latitude effect, coefficient (SE) = 0.26 (0.08), t = 3.34, $P < 0.0001$; Fig. 1a). The model selection procedure identified 2 best-fit models for plumage lightness (Table 1S in Supplementary material). Model averaging revealed that the strongest

environmental predictors of lightness were temperature and tree cover (Table 1). Owls inhabiting regions with high temperature are darker (quadratic temperature effect Table 1; Fig. 1d). Irrespective of the effect of temperature, owls living in regions with denser tree cover also have darker plumage (Fig. 1g).

Table 1. *Phylogenetic generalized least-square models testing the effect of climatic variables and vegetation cover on plumage lightness in owls in top models selected by multimodel inference. To deal with phylogenetic uncertainty, model average values of estimates and 95% CL are based on PGLS models with 1000 different phylogenetic trees. Bold type is used to remark support for* $\beta \neq 0$.

Model 1: Plumage lightness ~ temperature + temperature^2 + tree cover				
	β	SE	Lower CL	Upper CL
Intercept	0.01	0.24	-0.45	0.47
temperature	**-1.04**	**0.21**	**-1.45**	**-0.62**
temperature^2	**0.95**	**0.21**	**0.52**	**1.37**
tree cover	**-0.33**	**0.07**	**-0.46**	**-0.21**
Model 2: Plumage lightness ~ temperature + temperature^2 + precipitation + tree cover				
	β	SE	Lower CL	Upper CL
Intercept	0.01	0.24	-0.45	0.48
temperature	**-1.02**	**0.21**	**-1.44**	**-0.60**
temperature^2	**0.97**	**0.22**	**0.54**	**1.39**
precipitation	-0.11	0.12	-0.34	0.12
tree cover	**-0.26**	**0.10**	**-0.46**	**-0.06**

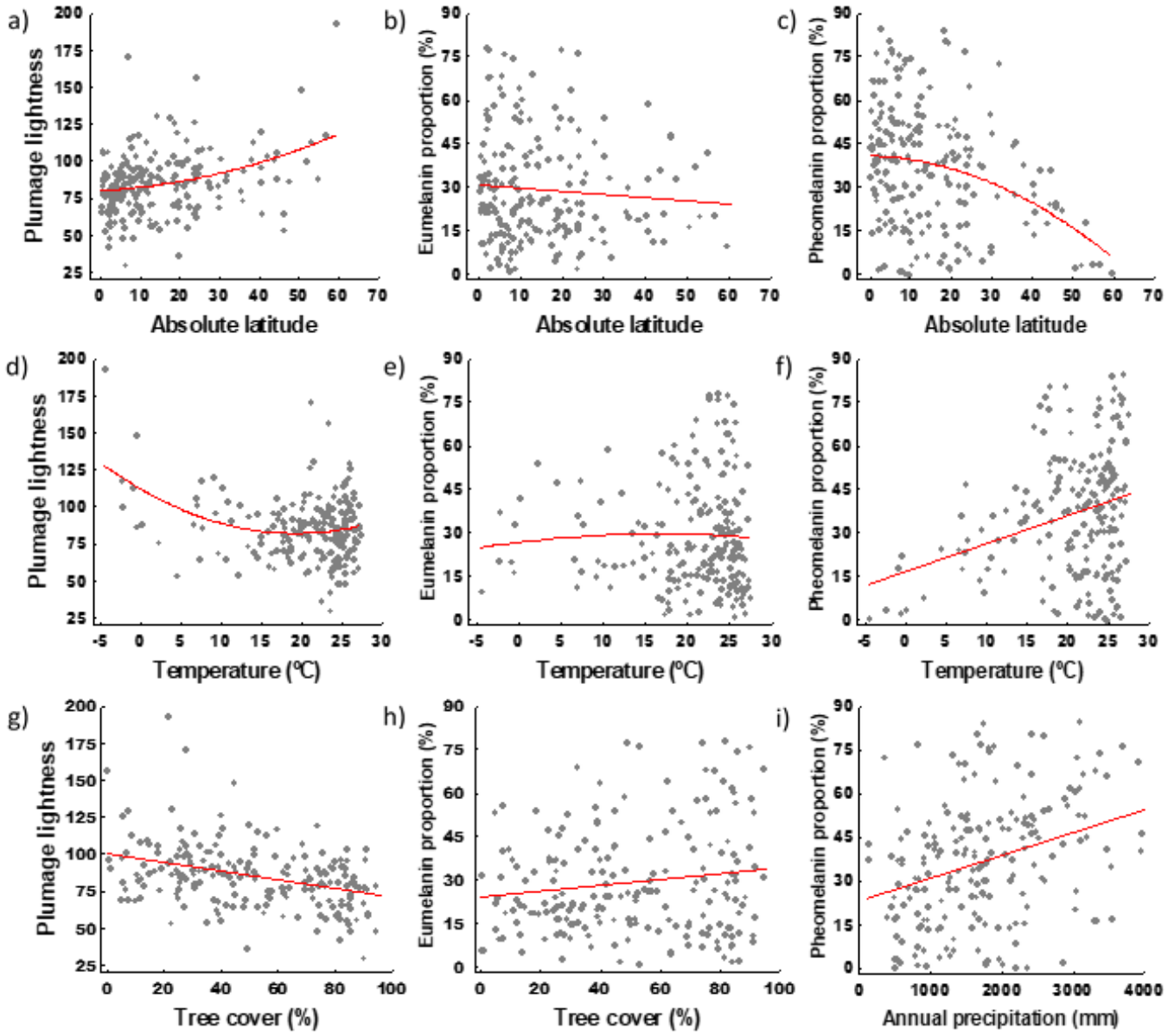

Figure 1. *Correlates of plumage lightness, and both potential eumelanin and pheomelanin plumage colour in owls (N = 198). On the left, scatterplots show plumage lightness in relation to quadratic effect of latitude (a) temperature (d), and tree cover (g). In the middle, scatterplots show correlates of eumelanin proportion with latitude (b), temperature (e) and tree cover (h). Finally, on the right, scatterplots show correlates of pheomelanin plumage with latitude (c), temperature (f) and precipitation (i).*

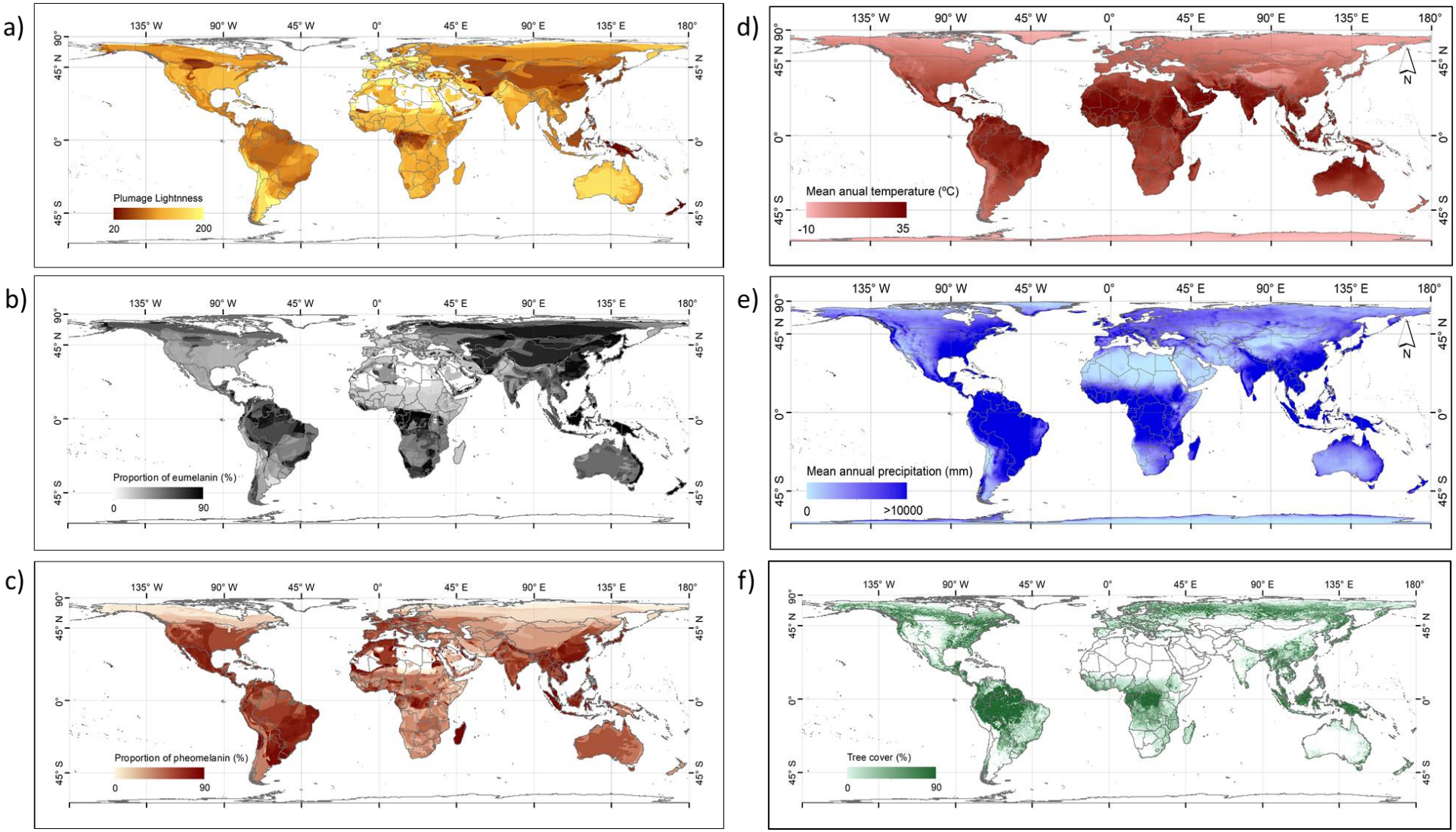

Figure 2. *Maps illustrating the world distribution of plumage lightness (a), potential eumelanin (b) and pheomelanin (c) owl coloration, mean annual temperature (d), annual precipitation (e) and percent tree cover (f). Final maps are calculated as the average value of all species within each of the 30 arc sec cells.*

Correlates of eumelanin-based colour proportion

The proportion of potential eumelanin-based colorations decreased from south to north (latitudinal effect: coefficient (SE) = - 0.14 (0.07), t = - 1.99, $P = 0.047$; Fig. 1b). Model averaging revealed that the proportion of potential eumelanin-based coloration was explained by tree cover and the quadratic effect of temperature (Table 2). Owls inhabiting areas with denser tree cover present more proportion of potential eumelanic coloration (Fig. 1e), whereas the extent of eumelanin coloration increases at medium-high temperature but slightly decreases at very high temperature (Fig. 1h).

Table 2. *Phylogenetic generalized least-square models testing the effect of climatic variables and vegetation cover on the proportion of potential eumelanin plumage in top models selected by multimodel inference. To deal with phylogenetic uncertainty, model average values of estimates and 95% CL are based on PGLS models with 1000 different phylogenetic trees. Bold type is used to remark support for* $\beta \neq 0$.

Model 1: Eumelanin proportion ~ tree cover				
	β	SE	Lower CL	Upper CL
Intercept	0.09	0.46	-0.81	0.99
tree cover	**0.16**	**0.06**	**0.04**	**0.28**
Model 2: Eumelanin proportion ~ temperature + temperature^2 + tree cover				
	β	SE	Lower CL	Upper CL
Intercept	0.07	0.47	-0.85	1.00
temperature	0.38	0.20	-0.02	0.78
temperature^2	**-0.42**	**0.21**	**-0.83**	**-0.02**
tree cover	**0.19**	**0.06**	**0.06**	**0.31**
Model 3: Eumelanin proportion ~ precipitation + tree cover				
	β	SE	Lower CL	Upper CL
Intercept	0.09	0.47	-0.83	1.01
precipitation	-0.03	0.10	-0.24	0.17
tree cover	0.19	0.10	-0.01	0.38
Model 4: Eumelanin proportion ~ temperature + tree cover				
	β	SE	Lower CL	Upper CL
Intercept	0.09	0.47	-0.82	1.00
temperature	-0.02	0.06	-0.14	0.10
tree cover	**0.17**	**0.06**	**0.04**	**0.29**

Correlates of pheomelanin-based colour proportion

We found a negative quadratic relationship between the extent of potential pheomelanin colour and latitude (coefficient (SE) = - 0.19 (0.06), t = - 2.95, $P < 0.001$; Fig. 1c), indicating that pheomelanin

was higher around the equator and decrease moving towards poles (Fig. 2c). The model averaging approach revealed that proportion of pheomelanin-based coloration was higher in species inhabiting warmer and wetter areas (Table3; Fig. 1f and 1i).

Table 3. *Phylogenetic generalized least-square models testing the effect of climatic variables and vegetation cover on the proportion of potential pheomelanin plumage in top models selected by multimodel inference. To deal with phylogenetic uncertainty, model average values of estimates and 95% CL are based on PGLS models with 1000 different phylogenetic trees. Bold type is used to remark support for* $\beta \neq 0$.

Model 1: Pheomelanin proportion ~ temperature + precipitation				
	β	SE	Lower CL	Upper CL
Intercept	0.09	0.48	-0.85	1.02
temperature	0.12	0.06	-0.01	0.24
precipitation	**0.16**	**0.06**	**0.04**	**0.29**
Model 2: Pheomelanin proportion ~ temperature + temperature^2 + precipitation				
	β	SE	Lower CL	Upper CL
Intercept	0.08	0.47	-0.84	1.00
temperature	0.35	0.19	-0.02	0.72
temperature^2	-0.25	0.19	-0.63	0.13
precipitation	**0.18**	**0.07**	**0.05**	**0.31**
Model 3: Pheomelanin proportion ~ temperature + precipitation + tree cover				
	β	SE	Lower CL	Upper CL
Intercept	0.09	0.48	-0.85	1.03
temperature	**0.13**	**0.06**	**0.00**	**0.25**
precipitation	0.10	0.10	-0.10	0.30
tree cover	0.09	0.09	-0.11	0.26

DISCUSSION

Our study provides support for the classic version of the Gloger's rule as we found darker (i.e. low lightness) owl phenotypes near the equator. Similar findings have been recently reported for diurnal passerines (Delhey et al. 2019). Moreover, a previous comparative study based on 4 owl genera found that dark reddish colorations were more frequent near the equator (Roulin et al. 2011). Analyses based on the environmental predictors *per se* showed that behind the latitudinal cline, there would be a concomitant effect of temperature and tree cover on owl plumage lightness: species living in regions with higher temperature and more vegetated (i.e. higher relative tree cover) have darker plumages.

The owl colour pattern here observed, show to follow the Rensch's prediction of lighter "polar coloration" at cold temperatures, with the emblematic case of Snowy Owl (*Bubo scandiacus*) (Fig. 1a). In addition, our results discard a possible thermoregulation role of owl plumage colour, such as proposed by the Bogert's rule, and rather suggest that some selective advantage might promote different fitness performances in lighter and darker plumage in relation to temperature, but not precipitation.

Contrary to expectation from the Gloger's rule, and to previous findings in diurnal passerines (Delhey et al. 2019), we failed to report an effect of rainfall on owl lightness. Galván et al. (2018) also found that, in Spanish birds, dark pigmentation was unrelated with rainfall but that it negatively related to temperature. Our results would suggest that plumage lightness in owls is not directly influenced by humidity. Here we have used rainfall as humidity proxy, but there could be a variety of other predictors as the presence of extensive bodies of water or evapotranspiration to name a few, which can act singularly or in a combination. Moreover, it must be stressed that, although our comparative analysis simultaneously assessed the relative effect of distinct environmental predictors on colour, climate variables can potentially constrain each other spatially. For instance, abundant rainfall are always associated to warm areas (e.g. tropics), whereas very cold regions are characterized by very low precipitation levels (e.g. tundra). Moreover, vegetation is shaped by temperature as well as precipitation and denser forests are thus commonly found in warm and wet areas. Hence, departures from Gloger's rule expectation might derive from an antagonistic or reinforcing effect between environmental variables (Delhey et al. 2019). Following this reasoning, owl plumage colour would suit prediction of Gloger's rule and be darker in warm and wet environments, since darker plumages are found in warm and densely wooded areas, which are likely to be highly moist as well.

Regarding the evolutionary processes behind the found colour patterns, multiple non-mutually exclusive selective pressures can explain melanin-based pigmentation. For instance, warm and humid environments are known to host a thriving bacterial community (Shawkey and Hill 2004), and,

therefore, species living in these regions might be under greater pressure from feather-degrading bacteria. In this scenario, owls leaving in warm and wet regions might achieve a selective advantage by displaying darker melanin-based colorations, as melanin increases the resistance of feathers to bacterial degradation (Burtt and Ichida 2004). Previous studies have argued that plumage coloration might be locally selected to enhance mechanical resistance in abrasive environments (Burtt and Gatz 1982). Indeed, experiments have shown that melanic feathers are significantly more abrasion resistant than feathers without melanin (Bonser 1995, Kose and Moller 1999). Following this argumentation, dense vegetation might represent a source of friction, which may have favoured the evolution of more abrasion-resistant dark owl phenotypes. Further experimental work is clearly needed to pinpoint the exact mechanisms behind the colour patterns here observed.

The found association between pigmentation and tree cover suggest that a concealment function might be a potential mechanism explaining ecogeographical colour patterns in owls. Camouflage is a visual disguise allowing animals to increase their survival by reducing detection from possible predators and prey (Ortolani 1999). It is plausible that in owls, which occupy different trophic levels, a good background matching might be involved in improving foraging efficiency but also in protection from intra-guild predation (Passarotto et al. 2018). Improved hunting success through lowering prey detection could be a valid explanation for our finding if a darker coloration proves to enhance camouflage in darker habitats (i.e. high percentage of tree cover), and vice versa for lighter colorations (Koskenpato et al. 2020). Indeed, in a recent study dealing with the relationship between environmental light and plumage coloration, Tate et al. (2016) found that darker morphs of Black Sparrowhawk (*Accipiter melanoleucus*) have higher foraging success in darker habitats, as they are better concealed. Since darker habitats are more likely to be more humid, this finding can relate to Gloger's rule. Indeed, Black Sparrowhawk dark morphs are more frequent (but not the exclusive morph) in wetter areas (Amar et al. 2014).

We found that pheomelanin showed a quadratic relationship with latitude, deposition concentrating in equatorial areas, and decreasing both north and south. Regarding eumelanin, we found a linear relationship showing that eumelanin deposition increased at lower latitude. Interestingly, eu- and pheomelanin seem to be differently influenced by environmental factors. Pheomelanin-based coloration increased with temperature but, contrarily to the complex version of Gloger's rule, also increased with rainfall. This result would be in line with previous comparative analyses showing that plumage redness (i.e. a potential proxy for pheomelanin) in 4 owl genera was more frequent near the equator (Roulin et al. 2011), and also fitted long-term intra-specific association between plumage redness and increased temperature and rainfall in the Eurasian Scops-Owl (*Otus scops*) (Galeotti et al. 2009). Similarly, in Eastern Screech-Owl (*Megascops asio*), redness was observed to be more frequent in climate presenting higher rainfall and humidity (Gehlbach 1994). A body of empirical work has shown that pheomelanic red morphs in Tawny Owl (*Strix aluco*) would be negatively selected in cold-dry years (Galeotti and Cesaris 1996, Karell et al. 2011), suggesting that pheomelanin may provide physiological benefits in terms of survival to warm and moist conditions. Covariation might stem indirectly from the relation of colour with other physiological traits, which may be differentially selected in the presence of wetter and warmer environments, like immune defense (Gasparini et al. 2009). This may have indirectly driven the evolution of higher proportion of pheomelanic coloration in wetter and warmer regions, e.g. around the equator, where immunity is likely to be strongly selected (Guernier et al. 2004). Another mechanism possibly involved might be diet. Food availability is likely to vary according to habitat quality, which in turn depends on climatic factors, thus diet may represent a selective pressure on pheomelanin-based coloration in warmer and wetter environments. Supporting this idea, Dalrymple et al. (2018), found more colourful birds in regions with greater net productivity production (NPP). Irrespective of the mechanism behind, which clearly deserve experimental work to be elucidated, global warming may

represent a challenge for pheomelanic species, since the increase of both temperature and rainfall may lead to a decrement in survival rate.

On the other hand, potential eumelanin colours were mostly related with tree cover tracing the opposite pattern of lightness. Eumelanic plumage was positively associated to higher tree cover in owls. Previous comparative work has stressed the importance of variation in luminal conditions due to vegetation cover (i.e. closed *vs* open habitats) in driving colour plumage polymorphism in owls (Passarotto et al. 2018). In agreement with those findings, our result may suggest a stronger selection on eumelanin to achieve background matching, although cryptic patterns probably involve a simultaneous presence of pheomelanin and eumelanin that cannot be elucidated here.

To summarise, our study provides further insight into the adaptive function of melanin-based plumage colour in owls and expand our knowledge on how variation in animal colour relates to broad-scale environmental variation. We found that owls follow Gloger's rule when considering plumage lightness, but that eumelanin and pheomelanin seem to be under different selective pressures, urging for further efforts to study the relative role of different pigments when studying eco-geographical colour patterns. Finally, our results attempt to further identify the adaptive value of melanin-based plumage in owls to better understand possible impact of human-induced changes, but the mechanism involved remains to be experimentally investigated.

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Supplementary material

Includes 3 tables and 1 figure

Table S1. *Results of AIC procedure concerning all the 25 models for plumage lightness. In bold are reported the best models (see the main text).*

Model	(Int)	temperature	temperature^2	precipitation	precipitation^2	tree cover	tree cover^2	df	logLik	AICc	ΔAIC	weight
Plumage lightness~temperature+temperature^2+tree cover	**0.062**	**-1.041**	**0.947**			**-0.335**		**6**	**-251.95**	**516.3**	**0**	**0.434**
Plumage lightness~temperature+temperature^2+precipitation+tree cover	**0.059**	**-1.028**	**0.969**	**-0.113**		**-0.257**		**7**	**-251.45**	**517.5**	**1.16**	**0.243**
Plumage lightness~temperature+temperature^2+tree cover+tree cover^2	0.063	-1.036	0.947			-0.248	-0.091	7	-251.89	518.4	2.04	0.156
Plumage lightness~temperature+temperature^2+precipitation+precipitation^2+tree cover	0.518	-1.013	0.961	-0.185	0.063	-0.009		8	-251.42	519.6	3.27	0.084
Plumage lightness~temperature+temperature^2+precipitation+precipitation^2+tree cover+tree cover^2	0.061	-0.983	0.947	-0.283	0.154	-0.080	-0.160	9	-251.31	521.6	5.25	0.031
Plumage lightness~temperature+temperature^2+precipitation	0.053	-0.991	0.975	-0.336				6	-254.69	521.8	5.48	0.028
Plumage lightness~temperature+temperature^2+precipitation+precipitation^2	0.053	-0.922	0.933	-0.648	0.313			7	-253.84	522.3	5.94	0.022
Plumage lightness~temperature+tree cover	0.020	-0.144				-0.307		5	-261.32	533	16.63	0
Plumage lightness~temperature+precipitation+tree cover	0.020	-0.126		-0.060		-0.265		6	-261.20	534.8	18.50	0
Plumage lightness~temperature+tree cover+tree cover^2	0.021	-0.139				-0.209	-0.102	6	-261.26	535	18.63	0
Plumage lightness~precipitation+precipitation^2+tree cover	0.027			-0.553	0.375	-0.170		6	-261.46	535.4	19.02	0
Plumage lightness~tree cover	0.035					-0.339		4	-263.60	535.4	19.08	0
Plumage lightness~precipitation+tree cover	0.028			-0.151		-0.224		5	-262.58	535.5	19.15	0
Plumage lightness~precipitation+precipitation^2+tree cover+tree cover^2	0.028			-0.721	0.561	0.276	-0.458	7	-260.48	535.5	19.21	0
Plumage lightness~precipitation+precipitation^2	0.022			-0.809	0.502			5	-262.68	535.7	19.33	0
Plumage lightness~temperature+precipitation+precipitation^2+tree cover	0.432	-0.095		-0.343	0.243	-0.008		7	-260.80	536.2	19.86	0
Plumage lightness~temperature+precipitation+tree cover+tree cover^2	0.021	-0.123		-0.056		-0.183	-0.088	7	-261.15	536.9	20.56	0
Plumage lightness~tree cover+tree cover^2	0.035					-0.125	-0.221	5	-263.31	536.9	20.60	0
Plumage lightness~temperature+precipitation+precipitation^2+tree cover+tree cover^2	0.024	-0.057		-0.562	0.445	0.156	-0.365	8	-260.29	537.3	21	0
Plumage lightness~precipitation+tree cover+tree cover^2	0.029			-0.141		-0.084	-0.152	6	-262.45	537.3	21	0
Plumage lightness~temperature+precipitation+precipitation^2	0.020	-0.037		-0.756	0.465			6	-262.56	537.6	21.23	0
Plumage lightness~precipitation	0.024			-0.328				4	-264.89	538	21.65	0
Plumage lightness~temperature+precipitation	0.018	-0.082		-0.290				5	-264.28	538.9	22.55	0
Plumage lightness~temperature+temperature^2	0.070	-1.004	0.831					5	-264.86	540	23.70	0
Plumage lightness~temperature	0.024	-0.213						4	-271.39	551	34.65	0

Table S2. *Results of AIC procedure concerning all the 25 models for plumage eumelanin proportion. In bold are reported the best models (see the main text).*

Model	(Int)	temperature	temperature^2	precipitation	precipitation^2	tree cover	tree cover^2	df	logLik	AICc	ΔAIC	weight
Eumelanin proportion~tree cover	**0.105**					**0.167**		**4**	**-250.32**	**508.8**	**0**	**0.174**
Eumelanin proportion~temperature+temperature^2+tree cover	**0.097**	**0.371**	**-0.414**			**0.187**		**6**	**-248.27**	**509**	**0.12**	**0.164**
Eumelanin proportion~precipitation+tree cover	**0.087**			**-0.053**		**0.207**		**5**	**-250.17**	**510.7**	**1.81**	**0.070**
Eumelanin proportion~temperature+tree cover	**0.095**	**-0.024**				**0.172**		**5**	**-250.25**	**510.8**	**1.96**	**0.065**
Eumelanin proportion~tree cover+tree cover^2	0.105					0.217	-0.052	5	-250.30	510.9	2.07	0.062
Eumelanin proportion~temperature+temperature^2+precipitation+tree cover	0.090	0.375	-0.409	-0.028		0.207		7	-248.23	511	2.20	0.058
Eumelanin proportion~temperature+temperature^2+tree cover+tree cover^2	0.097	0.373	-0.414			0.222	-0.037	7	-248.26	511.1	2.25	0.056
Eumelanin proportion~precipitation+precipitation^2+tree cover	0.100			0.209	-0.245	0.173		6	-249.60	511.6	2.79	0.043
Eumelanin proportion~temperature+temperature^2+precipitation+precipitation^2+tree cover	-0.207	0.314	-0.377	0.249	-0.242	0.006		8	-247.76	512.3	3.44	0.031
Eumelanin proportion~precipitation+precipitation^2	0.142			0.464	-0.369			5	-251.04	512.4	3.54	0.030
Eumelanin proportion~temperature+temperature2+precipitation+precipitation^2	0.126	0.247	-0.351	0.549	-0.403			7	-248.90	512.4	3.54	0.030
Eumelanin proportion~temperature+precipitation+tree cover	0.085	-0.012		-0.045		0.204		6	-250.16	512.8	3.91	0.025
Eumelanin proportion~precipitation+tree cover+tree cover^2	0.088			-0.051		0.233	-0.028	6	-250.17	512.8	3.93	0.024
Eumelanin proportion~temperature+tree cover+tree cover^2	0.095	-0.023				0.204	-0.034	6	-250.24	512.9	4.07	0.023
Eumelanin proportion~temperature+precipitation+precipitation^2	0.122	-0.088		0.585	-0.456			6	-250.28	513	4.16	0.022
Eumelanin proportion~precipitation	0.136			0.110				4	-252.46	513.1	4.28	0.020
Eumelanin proportion~temperature+temperature^2+precipitation	0.129	0.334	-0.402	0.148				6	-250.44	513.3	4.47	0.019
Eumelanin proportion~temperature+precipitation+precipitation^2+tree cover	-0.180	-0.051		0.318	-0.314	0.005		7	-249.37	513.3	4.49	0.018
Eumelanin proportion~precipitation+precipitation^2+tree cover+tree cover^2	0.101			0.261	-0.303	0.035	0.143	7	-249.49	513.6	4.72	0.016
Eumelanin proportion~temperature+temperature^2+precipitation+precipitation^2+tree cover+tree cover^2	0.097	0.279	-0.361	0.363	-0.348	-0.033	0.192	9	-247.60	514.2	5.30	0.012
Eumelanin proportion~temperature+precipitation	0.124	-0.044		0.129				5	-252.26	514.8	5.98	0.009
Eumelanin proportion~temperature+precipitation+precipitation^2+tree cover+tree cover^2	0.092	-0.078		0.472	-0.457	-0.124	0.267	8	-249.05	514.9	6.01	0.009
Eumelanin proportion~temperature+precipitation+tree cover+tree cover^2	0.120	0.357	-0.394		0.102			6	-251.53	515.5	6.66	0.008
Eumelanin proportion~temperature+temperature^2	0.099	0.335	-0.337					5	-252.67	515.7	6.81	0.006
Eumelanin proportion~temperature	0.102	0.011						4	-253.95	516.1	7.26	0.005

Table S3. *Results of AIC procedure concerning all the 25 models for plumage pheomelanin proportion. In bold are reported the best models (see the main text).*

Model	(Int)	temperature	temperature2	precipitation	precipitation2	tree cover	tree cover2	df	logLik	AICc	ΔAIC	weight
Pheomelanin proportion~temperature+precipitation	**-0.091**	**0.132**		**0.145**				**5**	**-239.72**	**489.7**	**0**	**0.209**
Pheomelanin proportion~temperature+temperature^2+precipitation	**-0.073**	**0.329**	**-0.205**	**0.155**				**6**	**-239.17**	**490.8**	**1.04**	**0.125**
Pheomelanin proportion~temperature+precipitation+tree cover	**-0.083**	**0.126**		**0.176**		**-0.037**		**6**	**-239.64**	**491.7**	**1.98**	**0.078**
Pheomelanin proportion~temperature+precipitation+precipitation^2	-0.091	0.136		0.111	0.034			6	-239.70	491.8	2.10	0.073
Pheomelanin proportion~precipitation	-0.140			0.200				4	-241.90	492	2.27	0.067
Pheomelanin proportion~temperature+tree cover	-0.133	0.175				0.092		5	-241.12	492.5	2.80	0.051
Pheomelanin proportion~temperature+temperature^2+precipitation+tree cover	-0.066	0.321	-0.202	0.181		-0.031		7	-239.11	492.8	3.07	0.045
Pheomelanin proportion~temperature+temperature^2+precipitation+precipitation^2	-0.073	0.343	-0.213	0.091	0.065			7	-239.12	492.8	3.09	0.045
Pheomelanin proportion~temperature	-0.141	0.191						4	-242.35	492.9	3.16	0.043
Pheomelanin proportion~precipitation+tree cover	-0.122			0.260		-0.078		5	-241.55	493.4	3.67	0.033
Pheomelanin proportion~temperature+precipitation+tree cover+tree cover^2	-0.088	0.131		0.183		0.093	-0.144	7	-239.47	493.5	3.78	0.032
Pheomelanin proportion~temperature+temperature^2+tree cover	-0.120	0.353	-0.184			0.100		6	-240.68	493.8	4.06	0.027
Pheomelanin proportion~temperature+precipitation+precipitation^2+tree cover	-0.014	0.126		0.177	-0.001	-0.001		7	-239.64	493.9	4.13	0.027
Pheomelanin proportion~precipitation+precipitation^2	-0.136			0.289	-0.093			5	-241.80	493.9	4.17	0.026
Pheomelanin proportion~temperature+temperature^2	-0.131	0.327	-0.138					5	-242.10	494.5	4.77	0.019
Pheomelanin proportion~temperature+tree cover+tree cover^2	-0.137	0.179				0.177	-0.091	6	-241.05	494.5	4.80	0.019
Pheomelanin proportion~precipitation+precipitation^2+tree cover	-0.107			0.437	-0.165	-0.102		6	-241.25	494.9	5.20	0.016
Pheomelanin proportion~temperature+temperature^2+precipitation+precipitation^2+tree cover	-0.025	0.332	-0.208	0.133	0.042	-0.001		8	-239.10	495	5.22	0.015
Pheomelanin proportion~precipitation+tree cover+tree cover^2	-0.126			0.265		-0.010	-0.076	6	-241.50	495.4	5.70	0.012
Pheomelanin proportion~temperature+precipitation+precipitation^2+tree cover+tree cover^2	-0.097	0.147		0.057	0.112	0.176	-0.212	8	-239.39	495.5	5.79	0.012
Pheomelanin proportion~temperature+temperature^2+tree cover+tree cover^2	-0.124	0.357	-0.184			0.186	-0.092	7	-240.62	495.8	6.08	0.010
Pheomelanin proportion~temperature+temperature^2+precipitation+precipitation^2+tree cover+tree cover^2	-0.082	0.378	-0.229	-0.018	0.184	0.236	-0.257	9	-238.74	496.4	6.69	0.007
Pheomelanin proportion~precipitation+precipitation^2+tree cover+tree cover^2	-0.106			0.443	-0.172	-0.118	0.016	7	-241.25	497.1	7.35	0.005
Pheomelanin proportion~tree cover	-0.232					0.125		4	-245.42	499	9.30	0.002
Pheomelanin proportion~tree cover+tree cover^2	-0.228					0.079	0.048	5	-245.40	501.1	11.37	0.001

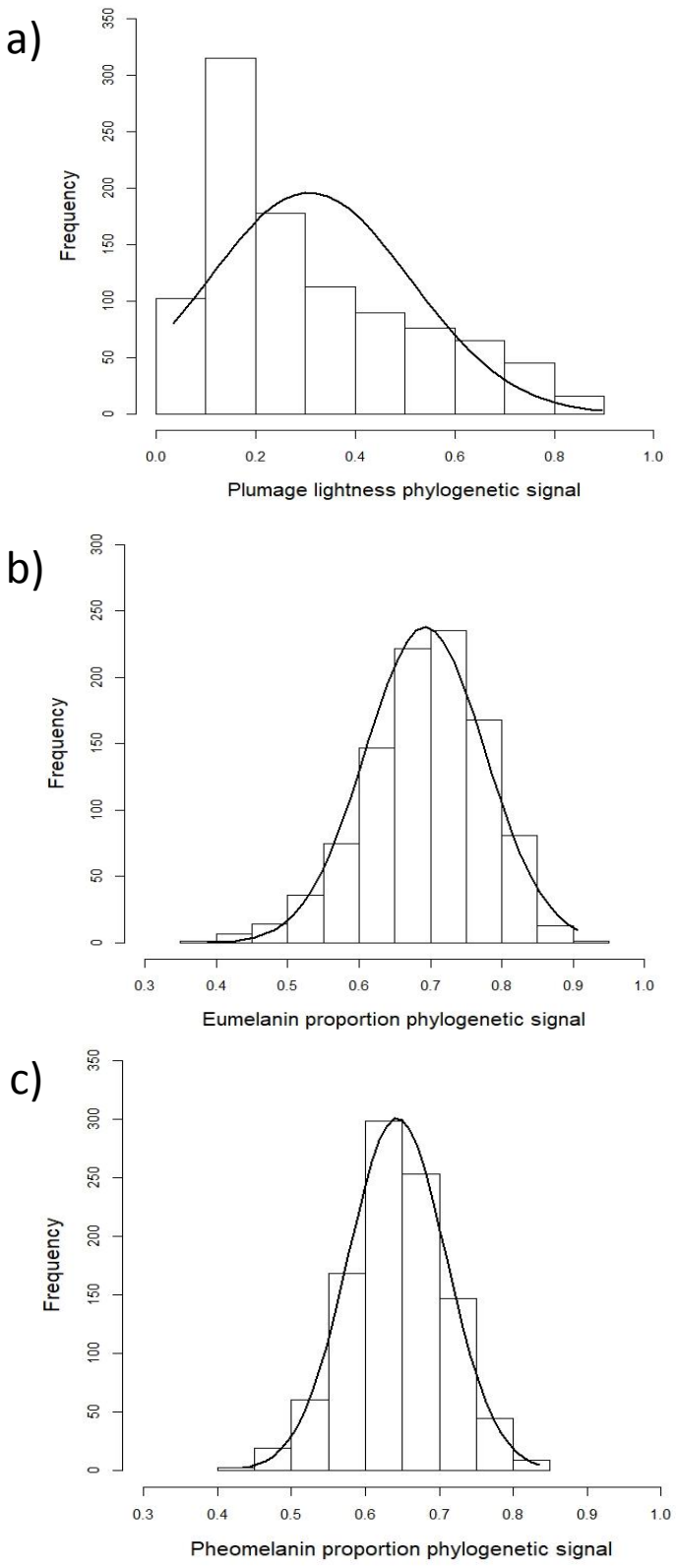

Figure 1S. *Probability distribution of λ phylogenetic signal based on 1000 randomly selected trees for plumage lightness (a), proportion of potential eumelanin-based colours (b) and proportion of potential pheomelanin-based tones (c). While plumage lightness present a great percentage of phylogenies reaching values close to 0, in both eu- and phemelanin proportion neither zero nor one were contained between the 99% Confidence Limits, thus providing support that* $\lambda \neq 0$ *and* $\lambda \neq 1$.

SECTION 2:

EVOLUTION OF INTER-SPECIFIC

COLOUR VARIATION

Chapter II

Colour polymorphism in owls is linked to light variability

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ABSTRACT

Owls show an astonishing variation in their degree of colour polymorphism, although the exact mechanisms driving such variation remain controversial. Here we address this fundamental question by considering information on all extant owls and recent advances in comparative methods in the frame of three mutually non-exclusive evolutionary scenarios. In addition, we study for the first time whether the evolution of influential ecological characters facilitated the evolution of colour polymorphism (or vice versa). In agreement with the niche divergence hypothesis, we found that species 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 colour polymorphism. Correlated evolution analyses revealed that a change in the luminal niche might be a fundamental requisite for the evolution of colour polymorphism. Moreover, polymorphism was more frequent among owl species occupying lower trophic levels, which could be explained by a particularly high selection for crypsis on small predator owls. Our results, thus, provide support for the idea that colour polymorphism in owls is an adaptive character likely maintained by the selective advantage of morphs under different environmental conditions via disruptive selection mechanisms.

INTRODUCTION

Colour polymorphism is widespread in several animal taxa including invertebrates and vertebrates (McLean and Stuart-Fox 2014), and long attracted evolutionary biologists interested in the understanding of evolution and maintenance of genetic and phenotypic diversity (Darwin 1859, Ford 1945, Huxley 1955, Milstead et al. 1974, Bond 2007, McKinnon and Pierotti 2010). Following the definition proposed by Roulin (2004), a species is polymorphic "when in a population individuals of the same age and the same sex display one of several coloration variants that are genetically inherited and for which the expression is sensitive neither to environment nor to body condition". Hence, polymorphism occurs when morphs occupy the same area at the same time, excluding geographical races and seasonal forms (White and Kemp 2016).

Colour polymorphism reaches high incidences in birds, being reported in 61% of avian orders (Roulin 2004). After Huxley (1955) acknowledged the relevance of birds for the study of polymorphism, this group has been the target of large body of empirical and comparative tests about the adaptive significance of colour polymorphism and the mechanisms facilitating its maintenance (e.g. Roulin 2004, Roulin et al. 2008, Roulin and Wink 2004, Galeotti et al. 2003, Galeotti and Rubolini 2004, Fowlie and Krüger 2003). However, the exact mechanisms promoting inter-specific variation in avian colour polymorphism are still under discussion.

So far, two main adaptive hypotheses for the evolution of bird colour polymorphism have been proposed (reviewed in Galeotti et al. 2003, Roulin 2004). The apostatic selection hypothesis has been invoked to explain colour polymorphism in avian predators (Ford 1945, Paulson 1973, Arnason 1978, Caldwell 1986) and brood parasites (Payne 1967), and it is based in negative frequency-dependent selection arising when rarity confers a selective advantage (see White and Kemp 2016). The hypothesis states that it would be advantageous for a predator to evolve a different, less frequent colour phenotype to result less recognizable to its prey and hence acquiring hunting benefits. Under this scenario, prey is the selective agent and predators would be more likely to evolve colour

polymorphism (Rohwer and Paulson 1987). Accordingly, this hypothesis would predict that top predators show higher occurrence of polymorphism than species occupying lower levels in the food web (i.e. small predators). In the same vein, the apostatic selection hypothesis would predict that species feeding more frequently on prey species able to form an avoidance image (i.e. birds and mammals, which have good memory and learning skills (e.g. Fowlie and Krüger 2003, Galeotti and Rubolini 2004)) are more likely polymorphic. Finally, Fowlie and Krüger (2003) also suggested that species displaying less frequent colour variants may attain hunting benefits during migration because their prey would not be able to form rapidly an avoidance image, hence predicting higher polymorphism in migratory than sedentary owls.

On the other hand, the niche divergence hypothesis is thought to lead to stable colour polymorphism through a mechanism of disruptive selection. It considers habitat heterogeneity experienced by individuals as a key prerequisite for the evolution of colour polymorphism, and states that species occupying heterogeneous environments will benefit by exhibiting different morphs because in that way individuals can better locally adapt to different ecological niches (Dreiss et al. 2012). Heterogeneity may involve various aspects of environmental complexity as climate and/or light conditions (Tate et al. 2016), both in space and time (Galeotti et al. 2003). Enhanced crypsis in different habitats within a population (i.e., resemblance with the habitat background) achieved through polymorphism may provide protection against predators by reducing prey detectability (Baker and Parker 1979) and, at the same time, by avoiding detection by prey (Götmark 1987) or competitors (Spear and Ainley 1993). In a comparative framework, this hypothesis would predict that species with broader ecological niches are more polymorphic than species occupying narrow niches (Van Valen 1965). Consequently, with regard to the light niche, the niche divergence hypothesis would predict that species living under more heterogeneous luminal conditions are more likely to evolve colour polymorphism than those inhabiting in more homogenous luminal conditions.

Finally, it has also been suggested that colour polymorphism may be simply a neutral trait, without an adaptive value (Fowlie and Krüger 2003). In this sense, colour polymorphism may represent a transient occurrence of certain alleles that are more likely expressed in large populations because they have a more diversified and wider gene pool promoting the occurrence of variations (Ford 1953, Galeotti et al. 2003, Roulin and Wink 2004). This hypothesis would predict that species with larger population sizes are more likely polymorphic than those with smaller population sizes.

Owls are a particularly suitable group of birds to test predictions about the apostatic selection hypothesis, given huge inter-specific variation in the degree of colour polymorphism (Mikkola 2014) and in their predator role, ranging from large top predators feeding on mammals and birds (i.e., prey that are assumed to have good learning capabilities and thus able to form avoidance-images) to small insectivorous species. Moreover, owls also show extraordinary variation in their degree of niche specialization with some species being restricted to very specific habitats and/or environmental conditions, as *Megascops* species in center and south of America, while others, as the Barn owl *Tyto alba*, being world-wide distributed (König and Weick 2008, Mikkola 2014). This also makes owls a suitable group to test how niche diversification may have driven the evolution of polymorphisms (niche divergence hypothesis). Non-surprisingly, owls have been the target of several comparative studies aiming to test the functional basis of colour polymorphism (Fowlie and Krüger 2003, Galeotti and Rubolini 2004), although such researches considered small subsamples of species, which could have determined that the conclusions are still controversial. Indeed, in a first comparative study based on 57 owl species, Fowlie and Krüger (2003) did not find support for the apostatic selection hypothesis, and interpreted a phylogenetically corrected correlation between the number of plumage morphs (including intra- and inter-population morphs) and the range size of species as evidence that polymorphism simply arises more likely in larger populations. In a second study, Galeotti and Rubolini (2004) compared ecological and behavioural traits of 31 closely related pairs of monomorphic and polymorphic species of owls. They found that number of used habitats was larger,

and the distributional range wider in polymorphic than in monomorphic species, which would agree with expectations from the niche divergence hypothesis. These results, however may be flawed as they vanished when correction for multiple testing was applied (Galeotti and Rubolini 2004), and given that pair species comparisons are not an appropriate approach to deal with phylogenetic relatedness (Harvey and Pagel 1991). Moreover, the results of these two studies were based on a limited number of species, and did not quantify and account for the tendency for related species to resemble each other (i.e., phylogenetic signal) (Freckleton et al. 2002, Revell et al. 2008), which impedes any strong inference about the evolution of polymorphism in owls.

Here we revisited the study of the functional basis of colour polymorphism in owls by retrieving data on polymorphism on all extant owls. We relied on a fully resolved avian phylogeny and benefited of recent advances in comparative methods to evaluate the association between colour polymorphism and predictors of the three above described mutually non-exclusive evolutionary scenarios. This approach is useful in identifying key correlations, but it does not inform about the evolutionary sequence of changes giving rise to colour polymorphism over time. Therefore, in a second set of analyses we reconstruct the ancestral state of colour polymorphism in owls, and used analyses of correlated evolution (Pagel 1994) to estimate transition rates from and to colour polymorphism under a range of different character states. All together, these analyses will allow identifying the main drivers of colour polymorphism in owls and whether the evolution of identified influential characters facilitates the evolution of colour polymorphism (or vice versa).

MATERIAL AND METHODS

Data collection

We collected data on colour polymorphism and behavioural and ecological predictors on 206 owl species by consulting the most complete literature currently available on this order (del Hoyo et al. 1999, König and Weick 2008, Mikkola 2014) (see Table S1). These 206 species are the entire owl number of species considered in the most updated birds' phylogeny of Jetz et al. (2012). We

considered as polymorphism only the occurrence of two or more coloration patterns in all individuals of both sexes and at any age within a given population (i.e. White and Kemp 2016), such as reported in König and Weick (2008). Therefore, our study deals with variation in sympatric colour polymorphism and excludes allopatric polymorphism that could be affected by different evolutionary processes (Bolton et al. 2015). We, however, considered *Tyto capensis* and *Tyto longimembris* as polymorphic rather than monomorphic as reported in König and Weick (2008) based on personal observation of A. Roulin. In a first step, we quantified the number of plumage morphs described for each species (see Table S1). However, the number of plumage morphs show very low variation in owls as most polymorphic species only display two (72.5 % of species), or three (26.1 % of species) morphs (see Table S1 and results). Furthermore, the number of morphs cannot be considered as a good proxy of colour polymorphism because in many species colour variation is in fact continuous rather than discrete. Therefore, polymorphism was classified here as a binary character with 1 indicating the presence of polymorphism and 0 its absence. The following 12 predictors were initially considered. As a proxy of biometrical measures, we gathered information on (1) body weight.

Because the apostatic selection hypothesis predicts that polymorphism would be more frequent among top predators, owls were classified regarding their (2) trophic level. We classified each species into one of three categories based on their diet and size relative to other species in the community. Accordingly, species included in the genera *Bubo*, *Scotopelia*, *Ketupa* and some species of genera *Strix* and *Ninox* were scored as top predators; medium-sized species were categorized as mesopredators as they can act as prey and predators in their communities; and small-sized species, which mainly prey on invertebrates and/or amphibians and reptiles, were considered as small predators (Table S1). In addition, we also classified each species regarding (3) the capability of its main prey to memorize and create an "avoidance image" as species feeding on invertebrates and amphibians and reptiles *versus* those feeding on other vertebrates (i.e. birds and mammals) (Table 1).

Niche breadth (4) was estimated as the number of different habitats used by a species (see Table 1 for a description of considered habitats);

Diet breadth (5) was estimated as the number of different prey a given species consumes (see Table 1 for a description of considered prey categories);

Migratory behaviour (6) species were classified as resident (i.e. species that live all the year in the same place; but also those showing nomadic or erratic short-distance non-reproductive movements in relation to prey availability), partially migratory (i.e. species with both migrant and resident population) and migratory (i.e. species that show regular migration). Moreover, following (del Hoyo et al. 1999, König and Weick 2008), we also classified owls regarding their (7) activity rhythm as strictly nocturnal species *versus* those that are also active during the day or at twilight.

We scored the extension of the distribution area of each species, i.e. their (8) distributional range, following Mikkola (2014)' maps, which were built as in Galeotti and Rubolini (2004). Species were classified as 1 = isolated; 2 = small and fragmented; 3 = small and continuous; 4 = wide and fragmented; and 5 = wide and continuous. A distributional area was considered "small" if it occupied less than 30% of the biogeographical region in which a given species inhabits, and "wide" if it occupied more than 30% of the biogeographical region. In addition, previous comparative work has shown that owl plumage coloration is related to latitude and hemisphere (Roulin et al. 2011). Therefore, based on Mikkola (2014)' maps, we calculated (9) latitude (in degrees) as the average between the most northern and southern latitude using Google Maps. Moreover, we divided species according to the (10) hemisphere in which they breed. We consider three categories: 0 = species breeding in the Northern hemisphere, 1 = species breeding in both hemispheres and 2 = species breeding only in the Southern hemisphere.

We also gathered information on (11) vegetation cover based on descriptions of the used habitats for each species. Briefly, vegetation cover was classed as "closed" when species only

inhabited habitats with dense canopy cover (including tropical, sub-tropical, cloud, rain, temperate and gallery forests and mangroves), or "open" when species only inhabited habitats without canopy cover or with a very scarce canopy (including wetlands, marshes, coastline, grasslands, prairies, meadows, rocky areas, deserts or semi-deserts). Vegetation cover was classed as "intermediate" for species either inhabiting habitats without a complete canopy cover (including savannah, woodlands, cultivated areas, bushy country with shuttered trees) and for species reported to use both "closed" and "open" habitats. Previous studies have relied on vegetation cover as an estimate of the luminal niche width potentially influencing the evolution of bird colour polymorphism (Galeotti et al. 2003). Open and closed habitats differ in the amount of light received because light is filtered by the canopy in closed habitats but not in open ones. Therefore, luminosity is lower in closed than in open habitats (Martin 2017). Also, it is known that in open habitats light is rich in almost all wavelengths because most light comes from the sun and open sky whereas in closed habitats light is rich in middle wavelengths (Endler 1993). Therefore, species living in "intermediate" conditions regarding vegetation cover are likely to experience a wider range of luminal conditions than those exclusively inhabiting "open" or "closed" habitats. Finally, since probability of reporting polymorphism is likely to differ among well-known and very rare species, we included study effort (12), calculated as the number of studies (log-transformed) for each species in Web of Science database in our comparative study (Table 1).

Table 1. *Definition of variables and details of scoring methods used to study colour polymorphism in owls.*

Variables	Description and categorization
Polymorphism	0 = monomorphic species
	1 = polymorphic species
Body weight	Average body weight of each species
Trophic level	1 = top-predators
	2 = mesopredators
	3 = small predators

Prey capability	0 = species mainly feeding on prey thought to be unable to form an avoidance image
	1 = species feeding on prey thought to be able to form an avoidance image
Niche breadth	Sum of the following categories:
	1 = tropical forest, cloud forest, rain forest
	2 = sub-tropical forest, temperate forest, forest
	3 = woodland, cultivated areas, gardens, open country with scattered trees, bushy country
	4 = freshwater habitats, wetlands, mangroves, coastlines, gallery forest
	5 = savannah
	6 = grassland, prairies, alpine meadows
	7 = rocky areas
	8 = semi-desert and desert habitats
Diet breadth	Sum of the different prey consumed by a species:
	1 = Arthropods (including crustaceans, spiders, scorpions and insects)
	2 = other invertebrates (snails, mollusks and worms)
	3 = Amphibians
	4 = Reptiles
	5 = Fish
	6 = Birds
	7 = Mammals
	8 = carrions
	9 = fruit
Migratory behaviour	0 = resident species
	1 = partially migratory species (including species presenting both resident and migratory populations)
	2 = migratory species (including species showing regular migratory movements)
Activity rhythm	0 = strictly nocturnal species
	1 = crepuscular species and species active also during the day
Distributional range	Extension of the distribution area:
	1 = isolated
	2 = small and fragmented
	3 = small and continuous
	4 = wide and fragmented
	5 = wide and continuous
Latitude	Average latitude between the most northern and southern latitude (Google Maps, degrees)
	Calculation based on distributional maps in Mikkola 2014
Hemisphere	0 = species breeding in the Northern hemisphere
	1 = species breeding in both hemispheres or cosmopolitan species
	2 = species breeding in the Southern hemisphere
Vegetation cover	0 = species using stable light environments (closed or open habitats)
	1 = species using unstable light environments (both closed and open habitats or intermediate habitats)
Study effort	Number of studies dealing with a specific species in Web of Science database

Phylogeny

The phylogenetic hypothesis was based on the most recent comprehensive time-calibrated set of complete phylogenies of extant bird species (Jetz et al. 2012). This phylogeny relies on the bird genome-based Hackett et al.´s (2008) phylogenies as a backbone for their phylogenetic reconstructions. We tested the sensitivity of our results to the phylogenetic hypothesis by using 1000 randomly extracted phylogenetic trees including all the considered species in our study from the site http://birdtree.org.

Ancestral character reconstruction

We reconstructed ancestral state of owl colour polymorphism on a majority-rule consensus tree, created on 1000 trees through the function consensus.edges in PHYTOOLS (Revell 2012) using the R function reroothingMethod in PHYTOOLS package. This approach allows estimating the marginal ancestral state for each internal node of the tree using likelihood and comparing the performance of various models of evolution. Specifically, we contrasted two different models: (1) the "Equal Rates" model (ER hereafter), which assumes colour polymorphism is lost or acquired at a similar rate over time; and (2) the "All Rates Different" model (ARD hereafter), which allows for differences in the rate of gain and loss of polymorphism. We used AIC (Akaike Information Criterion) to compare the models and select the best one, considering as support to our choice a difference of 4 or greater between models (Burnham and Anderson 2003).

Phylogenetic signal

We estimated the phylogenetic signal of colour polymorphism using the phylo.d function in CAPER package in R (Orme 2013), that specifically allows to compute it for binary variables. This approach is based on calculation of the statistic *D* (Fritz and Purvis 2010), whose value ranges continuously from 0 to 1. *D* values close to 1 are indicative that trait variation is random regarding the phylogeny. Instead, a *D* value next to 0 indicates the trait variation in the phylogeny is clumped and thus that the

character evolved under a Brownian model. We calculated the phylogenetic signal for all 1000 phylogenies and then we verified distribution of values to determine the departure from both 0 and 1.

Phylogenetic logistic regression

We ran a Phylogenetic logistic regression, using the function binaryPGLMM in APE package in R (Paradis et al. 2004) to control for the possible effects of common ancestry on the relationship between colour polymorphism and the above predictors. As our aim was assessing the relative importance of the three proposed evolutionary hypotheses in the evolution of polymorphism, this analysis was based on the subset of 196 species for which we gathered complete information (see Table S1). Before performing the logistic regression analyses, we determined the degree of multi-collinearity among predictors through estimation of VIF (Variance Inflation Factor) using the CAR package in R (Fox et al. 2010). Only body size and trophic level shared a large amount of variance (VIFs for log weight and trophic level 5.47 and 5.41, respectively). Therefore, we opted to retain trophic level instead of body mass as a predictor to avoid multi-collinearity, and, given that we have a clear-cut prediction regarding the role of trophic level on the evolution of colour polymorphism (see introduction).

Testing correlated evolution between characters

We used the program BayesTraits (Pagel and Meade 2013) to investigate whether colour polymorphism in owls correlated and coevolved with activity rhythm, trophic level and vegetation cover. As the method can only be applied to binary variables, we transform trophic level, initially categorized in three levels (see table S1), as upper tropic level including top- and mesopredator species *versus* lower trophic level including small predators. We used a Bayesian approach based on a Markov Chain Monte Carlo (MCMC) sampling algorithm and a Reversible-Jump (RJ) procedure which allow taking into account both uncertainty of the model estimates and phylogeny. This approach is based on the observation of transition rate of character' states under two contrasting

models. The dependent model assumes that the two traits co-evolved, and, thus the rate of change in one character is contingent on the state of the other character; conversely, under the independent model the transition from a state to another in one character is independent of the state of the other character. Each MCMC chain was run three times for all 1000 phylogenies for 1 010 000 iterations sampled every 1000 with the first 10000 excluded as burn-in period after visually confirming that convergence had been reached. We used a uniform prior for both the independent and the dependent model, which were compared by means of the Bayes Factor (BF) based on the harmonic means of the model likelihoods. By convention, a value >2 is taken as a positive evidence for hypothesized relationship and values >5 as a strong evidence (Pagel and Meade 2006).

After detecting coevolution between colour polymorphism and any of the predictors, we estimated the distribution of posterior probabilities of the values of the parameters, also referred to as *z*-scores, using the RJ MCMC procedure using an hyperprior (0, 100; 0, 100). The *z*-scores provide a means of analyzing the probability that the true value of the transition parameter between two character states is nonzero (i.e., the transition does not occur) based on the proportion of models visited by the Markov chain. The most likely evolutionary path from the hypothetic ancestral state to derived state of two discrete traits thus, can be inferred from the posterior probability distributions of the transition parameters in the model of evolution (Pagel and Meade 2006).

RESULTS

Species coverage

Colour polymorphism is found in 69 species belonging to 9 genera, whereas 137 species are classed as monomorphic (Table S1). Among the 69 polymorphic species, 50 showed 2 morphs (rufous and greyish), 18 showed 3 morphs (rufous, brown and greyish) and one species showed 5 morphs (Table S1 Supplementary material).

Ancestral character reconstruction and phylogenetic signal

The model “ARD” fitted the data considerably better than the model “ER” (ΔAIC = 28.90, see also Fig.1S). However, the potential of the ARD model to infer ancestral states is limited as it invariably assigned an equal likelihood for presence or absence of colour polymorphism for all internal nodes in the owl phylogeny (Fig. 1).

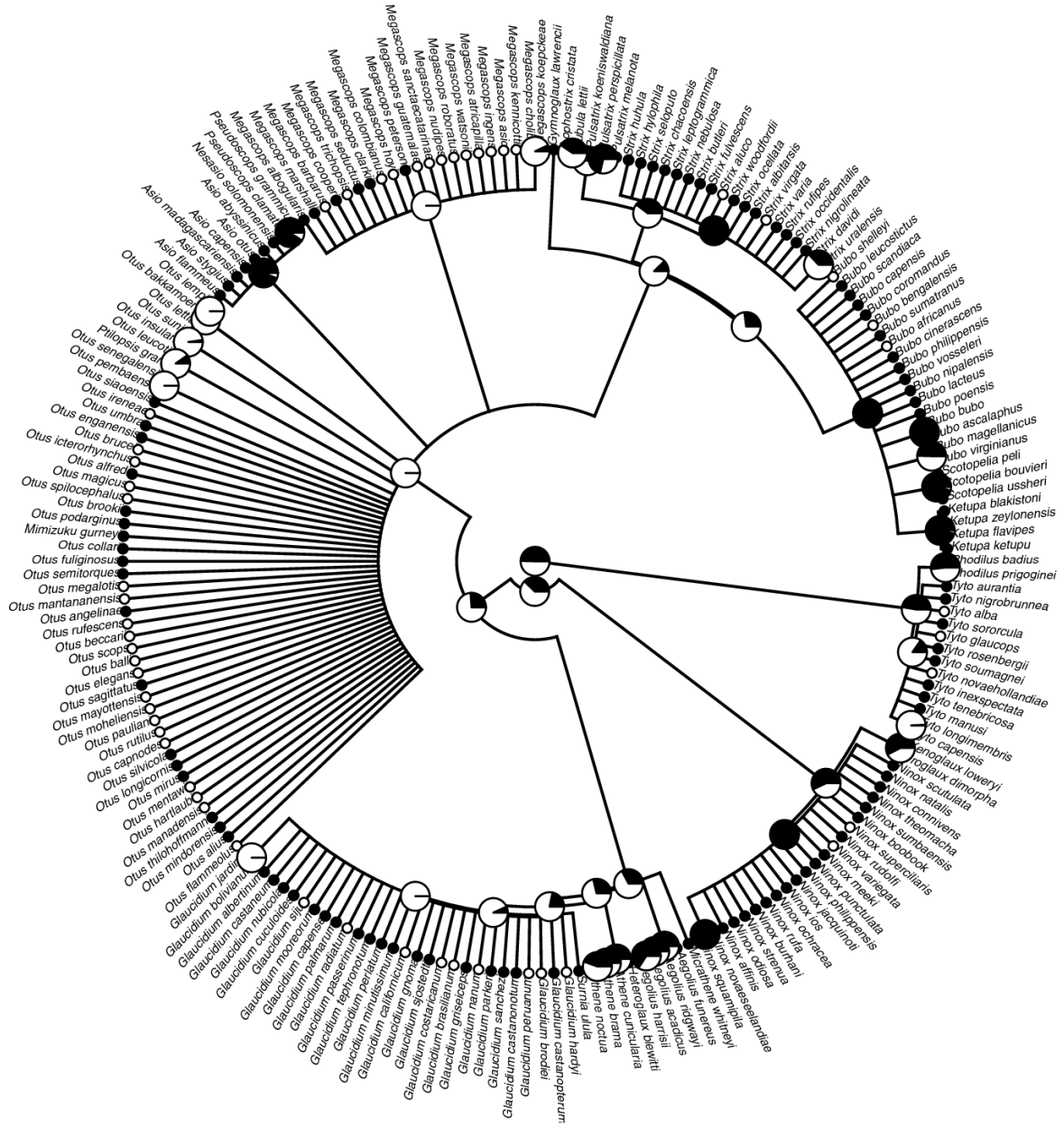

Figure 1*. Ancestral state reconstruction of the binary character colour polymorphism across a majority-rule consensus tree created on 1000 phylogenies using the ARD model. Pie charts at the nodes represent proportional maximum-likelihood support for the monomorphic (black) and polymorphic (white) character states from the ancestral state reconstruction.*

The average *D* phylogenetic signal calculated on the 1000 trees was 0.63, and differed significantly from 0 and 1 (Fig. S2)

Correlates of colour polymorphism

Phylogenetic logistic regression revealed that colour polymorphism was influenced by activity rhythm, trophic level and vegetation cover (Table 2). Specifically, species with a more diurnal activity rhythm were more likely to display colour polymorphism (Fig. 2a). In addition, colour polymorphism was relatively more frequent among small predators than among top- or mesopredators (Fig. 2b). Finally, species inhabiting in mixed habitats regarding vegetation cover were more likely polymorphic than those inhabiting exclusively open or closed habitats (Fig. 2c).

Table 2. *Results of the phylogenetic logistic regression to study colour polymorphism in owls. Significant results are reported in bold. N = 196 species with complete information for all predictors.*

	Coefficient	Standard Error	*Z*-score	*P*-value
(Intercept)	-7.64	1.86	-4.10	<0.001
Study effort	0.51	0.35	1.46	0.143
Trophic level	**1.89**	**0.48**	**3.96**	**<0.001**
Diet breadth	0.05	0.19	0.24	0.807
Niche breadth	-0.20	0.23	-0.84	0.401
Prey capability	0.19	0.82	0.23	0.816
Activity rhythm	**1.10**	**0.45**	**2.45**	**0.014**
Migratory behaviour	-0.50	0.56	-0.89	0.376
Distribution range	0.20	0.17	1.17	0.240
Latitude	0.00	0.02	0.13	0.893
Vegetation cover	**0.83**	**0.41**	**2.02**	**0.044**
Hemisphere	0.39	0.38	1.03	0.305

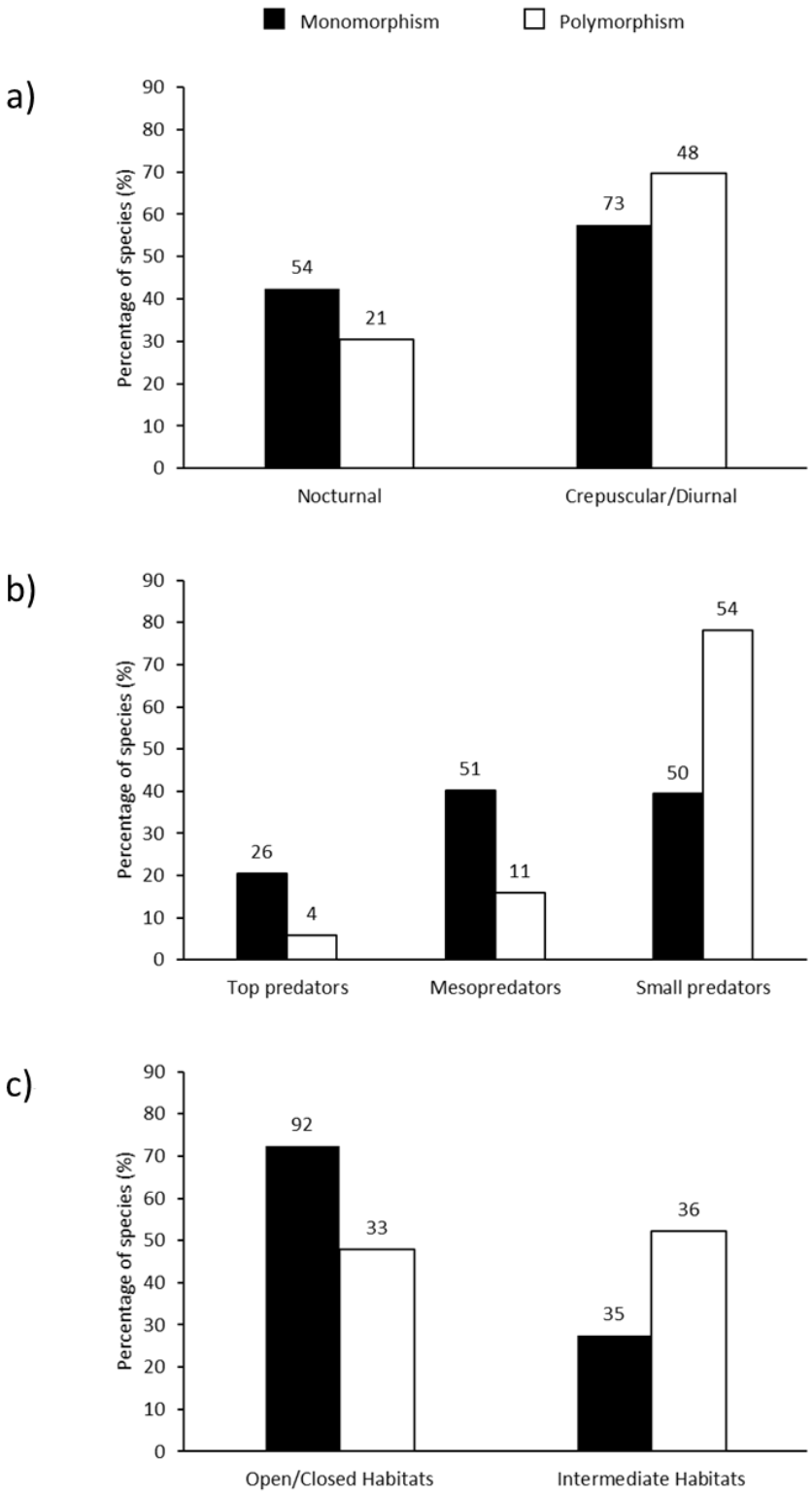

Figure 2. *Percentage of owl species exhibiting colour polymorphism and monomorphism in relation to activity rhythm (a), trophic level (b) and vegetation cover (c). Number of species is shown above bars.*

Coevolution between colour polymorphism and predictors

The evolutionary model in which colour polymorphism and activity rhythm evolved dependently was more likely than the model in which the two characters evolved independently (Bayes factor = 18.57). Comparison between *z*-scores at the transition *q*2,4 and at transition *q*1,3 revealed that there is a tendency of polymorphism to evolve more easily in species with both crepuscular and diurnal habits than in strictly nocturnal species (z-score value of *q*1,3= 0.000 *versus* z-score value of *q*2,4= 0.997, Fig 3). In addition, it is relevant to question which changed first: owl coloration followed by activity rhythm or vice versa. Comparison of these *z*-scores at the transition *q*1,2 with the alternative *q*1,3 suggests that both transitions are possible (Fig. 3a), and therefore that the first evolutionary step could not be unambiguously established. The analysis also shows that the transition probability from polymorphism to monomorphism was similar in species with crepuscular and diurnal habits (*z*-score at the transition *q*3,1 =0.000 *versus z*-score at the transition *q*4,2=0.000, Fig. 3a).

The evolutionary model in which colour polymorphism and trophic level evolved dependently did not significantly improve the fit of the model in which these two characters evolved independently (Bayes factor = -13.73) (Fig. 3b).

We found strong evidence of correlated evolution between colour polymorphism and vegetation cover (Bayes factor = 7.30, Fig. 3c). Comparison of the *z*-scores at the transition *q*2,4 with that of the alternative transition *q*1,3 suggests that polymorphism is more likely to evolve in intermediate than in open or closed habitats(*z*-score at *q*2,4 = 0.033 *versus z*-score at *q*1,3 = 0.842) (Fig. 3c). In addition, the analysis shows that the transition probability from polymorphism to monomorphism was similar in intermediate *versus* open and closed habitats (*z*-score at the transition *q*3,1 =0.093 *versus z*-score at the transition *q*4,2=0.000, Fig. 3c). Finally, comparison of *z*-scores at *q*1,2 with *q*1,3 provides strong evidence that changes in owl coloration only occurred after a change in habitat use (*z*-score at *q*1,2 = 0.000 *versus z*-score at *q*1,3 = 0.842) (Fig. 3c).

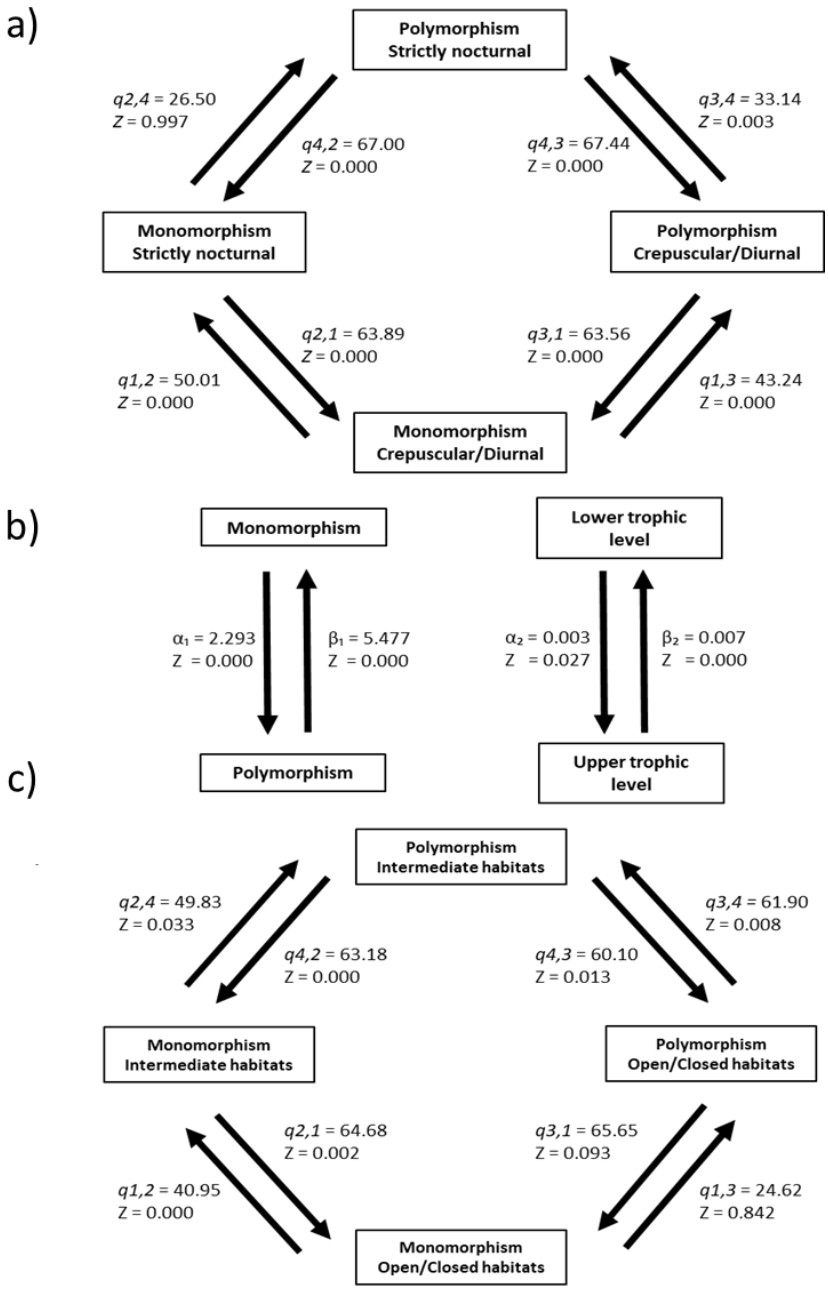

Figure 3. *Flow diagram showing the most probably evolutionary pathway for the evolution of owl polymorphism in relation to (a) activity rhythm, (b) trophic level and (c) vegetation cover. Changes between monomorphism and polymorphism occurred independently of changes in trophic level, but polymorphism evolved jointly with activity rhythm and vegetation cover (see results). Likelihood of all transitions based on the z-score (the higher the z-score, the more unlikely the transition; see Materials and Methods for details) are shown close to arrows.*

DISCUSSION

Our results show that colour polymorphism is an evolutionary labile trait in owls, with a trend to show similar values among related species, but that cannot be fully explained by the phylogenetic relatedness. Indeed, we were able to discriminate among the three evolutionary scenarios here considered to explain the appearance of colour polymorphism in owls, i.e. niche divergence hypothesis, apostatic selection hypothesis, and polymorphism by chance.

Scenario 1: the niche divergence hypothesis

Our results provide support for a role of ecology, namely activity rhythm and vegetation cover, in explaining colour polymorphism in owls. Specifically, species with diurnal and crepuscular habits and those inhabiting in a mixture of open and closed habitats are more likely polymorphic than strictly nocturnal species or those inhabiting only closed or open habitats. The niche divergence hypothesis would predict that species living under heterogeneous luminal conditions are more likely to evolve colour polymorphism than those inhabiting in homogenous luminal conditions. Compared to strictly nocturnal species, owls active at dawn and dusk experience highly variable luminal conditions (Martin 1990). In the same vein, species inhabiting in a mixture of open and closed habitats experience more variable luminal conditions due to the different structure of vegetation, daily variation in the incidence of sun light and weather conditions which affect propagation of coloured signals (Endler 1993). Therefore, our results suggest that the selection pressure due to detectability in different light conditions may be a key predictor of colour polymorphism, and thus that polymorphism in owls might be primarily driven by disruptive selection.

Our results contrast with previous comparative studies in owls that found only weak (Fowlie and Krüger 2003, Galeotti and Rubolini 2004) support for the disruptive selection hypothesis. There are several differences between our study and earlier ones that could explain different results. First, these earlier studies considered about ¼ of all extant owl species. It could thus be argued that

differences among studies might be due to non-intentional biases toward owls with non-representative ecological features in earlier studies. Second, and in contrast with our study, earlier analyses relied on a non-well resolved owl phylogeny. Finally, our study incorporates recent advances in comparative methods that allow for an appropriate treatment of binomial dependent variables as colour polymorphism in multiple-predictor models (Ives and Garland 2014). Our study, however, provides support for previous comparative studies with birds (Galeotti et al. 2003), and recent empirical evidence at the intra-specific level showing a role of light spectrum on promoting colour polymorphism (Tate et al. 2016). Individuals of the black morph in the Black sparrowhawk *Accipiter melanoleucus* provided more prey in lower light conditions whereas individuals of the light morph provided more prey in brighter conditions (Tate et al. 2016). Hence, the found relationships between polymorphism and activity rhythm and vegetation cover would suggest that morphs might function as an adaptation driven by light conditions to exploit varying niches in owls.

Alternatively, the found relationship between vegetation cover and polymorphism could be a side effect due to the fact that environmental conditions change across vegetation cover classes. A large body of empirical work has also shown that, in owls, different morphs may perform better under particular environmental conditions. For instance, in Eastern screech owl *Megascops asio*, redness was observed to be more frequent in climate presenting higher rainfall and humidity (Gehlbach 1994) and in closer habitats (Gehlbach and Gehlbach 2000). Similarly, the fluctuation of plumage redness in Eurasian Scops-Owl *Otus scops* seems to reflect the increasing of temperature and precipitations (Galeotti et al. 2009). In their study about polymorphism in Tawny owl *Strix aluco*, Galeotti and Cesaris (1996) found a significantly higher mortality rate of red morphs during cool-dry years, which could be due to the fact that different colour morphs have different ability to thermoregulate (Dreiss et al. 2016). Also, Roulin et al. (2011) in a comparative study observed that frequency of plumage redness in owls increased near the equator. Finally, a recent study highlighted that rufous tawny owls have lower survival in winters with deep snow compared to grey ones (Karell et al. 2011).

Analyses of correlated evolution strengthened the suggested key role of luminal conditions for the evolution of colour polymorphism in owls. Colour polymorphism and activity rhythm have evolved in concert in owls and the transition from monomorphism to polymorphism was more frequent in diurnal and crepuscular species than in nocturnal species. In addition, polymorphism was more likely to evolve in "intermediate" habitats regarding vegetation cover, where species are likely to experience more heterogeneous luminal conditions. It was evident that changes in owl coloration were triggered by a previous change in the luminal niche of species, supporting the hypothesis that a change in the luminal niche probably was an important requisite for the evolution of colour polymorphism. Nonetheless, the ancestral state of polymorphism cannot be unambiguously established in our study, and losses and gains occurred in the owl phylogeny (Fig. 1). Moreover, we found that species living under heterogeneous lighting conditions were more likely to show colour polymorphism, which could be explained by a loss of colour polymorphism in species living in homogenous light conditions. Hence, a plausible alternative interpretation is a neutral scenario for the appearance of colour polymorphism, which is negatively selected in some situations.

Scenario 2: the apostatic selection hypothesis

Our results do not support the idea that detection by prey played a key role in the evolution of colour polymorphism in owls through apostatic selection. Indeed, contrary to expectations from the apostatic selection hypothesis, occurrence of polymorphism was not explained by the relative importance of prey capable to form an avoidance image or by migratory behaviour. This hypothesis also predicts that higher occurrence of polymorphism may occur in larger predators because they prey more frequently on birds and mammals, which are said to have good memory and learning skills. Contrary to this expectation, we found that polymorphism was more frequent among owl species occupying lower tropic levels in their food webs. Fowlie and Krüger (2003) also found a negative relationship between number of morphs and body size. The most likely explanation for this result is that colour polymorphism in small owls was driven by intraguild predation risk, i.e. escape from visual predators

may favor the evolution of prey colour polymorphism (Bond and Kamil 2002, Bond and Kamil 2006). Intraguild predation is common in owls and: (a) larger owls often predates on smaller ones, the opposite being rare; and (b) small owls are frequently the target of diurnal birds of prey (Mikkola 1976, Lourenço et al. 2014). Therefore, selection for crypsis due to intraguild predation is expected to be larger in smaller owls. Moreover, examination of niche breadth in relation to trophic level shows that small owls occupy a larger number of habitats than larger ones (PGLS with niche breadth as dependent variable: trophic level: coefficient (SE): -0.39 (0.08), $t = -4.34$, $P < 0.0001$), which suggest that the prerequisite of high habitat diversity is fulfilled.

This hypothesis was primarily proposed to explain polymorphism in diurnal raptors and other birds active during daytime (Ford 1945, Paulson 1973, Arnason 1978, Caldwell 1986, Roulin and Wink 2004). However, many owl species are nocturnal, and, given the low performance of a vertebrate eye in detecting colour differences at night (Kelber et al. 2003), it is unlikely that owl prey may form an avoidance image under low light conditions.

Contrarily to diurnal raptors, in which polymorphism seems to be closely tied to migration (Roulin and Wink 2004), there is no relationship between colour polymorphism and migration in owls. Owls are an almost completely sedentary group of birds, with very few species undertaking true migration and others being characterized by a discontinuous nomadic behaviour, associated to fluctuation of their main preys' population (König and Weick 2008), which minimizes the chance for selection of polymorphism based on apostatic selection.

Scenario 3: polymorphism by chance

Our results revealed that colour polymorphism is not associated with the distributional range of species, which likely reflect the size of the populations of each species (see Fowlie and Krüger 2003), and thus the chance that polymorphism may have evolved randomly. This finding, together with the found associations between polymorphism and predictors of the amplitude of the luminal niche,

definitively undermines the possibility that colour polymorphism is a neutral trait without an adaptive value in owls (Fowlie and Krüger 2003).

Weakness of the used approach

This study, however, has several weaknesses worth mentioning here that may affect the strength of our conclusions. As above mentioned, polymorphism occurs when morphs occupy the same area at the same time, hence excluding geographical races and seasonal forms (White and Kemp 2016). Our measurement of polymorphism refers to variation within populations such as it would be required for testing the proposed evolutionary scenarios. However, predictor variables were collected from species descriptions and may include both inter- and intra-population variation that may potentially give rise to spurious relationships. Second, due to the absence of appropriate predictors measuring the intensity of sexual selection in owls, we have disregarded a potential role of sexual selection in driving colour polymorphism (Roulin and Bize 2007). However, most of owl species are long-lived species with a monogamous mating system (König and Weick 2008), which would suggest that the potential of sexual selection-based mechanisms to promote polymorphism might be particularly low in this group. Finally, the comparative method is in essence a correlative based approach in which inference about causality is established from statistical analyses in which multiple predictors are present (Bennett and Owens 2002).

CONCLUSIONS

This is the first comparative study simultaneously considering correlation and contingency analyses to test explicitly the three classic hypotheses on the evolution of colour polymorphism in owls. Our study provides evidence that variation in luminal conditions might be a key prerequisite promoting the evolution of colour polymorphism in this group of birds, in agreement with the niche divergence hypothesis. Our results, thus, provide support for the idea that colour polymorphism in owls is an adaptive character likely maintained by the selective advantage of camouflage under different light

regimes or in terms of physiological adaptation to environmental conditions via disruptive selection mechanisms.

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Author contribution statement. AP, DP, VP and JMA conceived the study design. AP collected data. AP and JMA performed the analyses. AP, DP, VP and JMA wrote the manuscript.

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Supplementary material

Includes 1 table, 2 figures and 1 file

Table S1. *Raw data used for comparative analyses on colour polymorphism in owls (see methods in the main text for explanation of categorization of each predictor). When information for a variable originated from sources different from del Hoyo et al. (1999), Konig and Weick (2008), and Mikkola (2014), it was denoted with a number above the data and the corresponding citation included in the list of references as a footnote. The asterisk refers to species for which body weight information was absent and thus estimated based on a regression between body mass and wing length (log body mass= -2.69 + 2.19 (log wing length)).*

Species	Colour Polymorphism	N. morphs	Body weight (Log10)	Trophic level	Prey capability	Niche breadth	Diet breadth	Migratory behaviour	Activity rhythm	Distributional range	Average latitude	Hemisphere	Vegetation cover	Study effort (Log10)
Tyto alba	polymorphic [1]	2	2.56	mesopredator	mammals/birds	5	5	partially migratory	crepuscular/diurnal	4	6.60	1	intermediate	3.62
Tyto glaucops	polymorphic	2	2.60	mesopredator	mammals/birds	1	5	resident	strictly nocturnal	1	18.85	0	intermediate	1.00
Tyto soumagnei	monomorphic	-	2.58	mesopredator	mammals/birds	3	5	resident	strictly nocturnal	3	-18.55	2	intermediate	1.23
*Tyto aurantia**	monomorphic	-	2.46	mesopredator	mammals/birds	2	1	resident	strictly nocturnal	1	-5.21	2	intermediate	0.30
*Tyto nigrobrunnea**	monomorphic	-	2.68	mesopredator	mammals/birds	1	5	resident	strictly nocturnal [2]	1	-1.83	2	open/close	0.70
Tyto longimembris	polymorphic	-	2.63	mesopredator	mammals/birds	2	5	resident	crepuscular/diurnal	4	-1.52	1	open/close	1.59
Tyto capensis	polymorphic	-	2.64	mesopredator	mammals/birds	2	3	resident	strictly nocturnal	4	-12.65	2	open/close	2.00
*Tyto sororcula**	monomorphic	-	2.52	mesopredator	mammals/birds	1	5	resident	strictly nocturnal	3	-5.42	2	open/close	0.30
Tyto novaehollandiae	polymorphic	2	2.96	mesopredator	mammals/birds	2	2	resident	strictly nocturnal	1	-25.42	2	intermediate	1.94
*Tyto manusi**	monomorphic	-	2.70	mesopredator	mammals/birds	1	5	resident	strictly nocturnal	1	-2.10	2	open/close	0.30
*Tyto rosenbergii**	monomorphic	-	2.87	mesopredator	mammals/birds	3	1	resident	crepuscular/diurnal	2	-1.38	2	intermediate	0.60
*Tyto inexspectata**	monomorphic	-	2.58	mesopredator	mammals/birds	2	5	resident	strictly nocturnal	3	0.43	1	open/close	0.70
Tyto tenebricosa	monomorphic	-	2.91	mesopredator	mammals/birds	2	2	resident	strictly nocturnal	4	-19.64	2	open/close	1.79
Phodilus prigoginei	monomorphic	-	2.29	mesopredator	-	1	-	resident	strictly nocturnal	1	-3.46	2	open/close	0.95
Phodilus badius	monomorphic	-	2.45	mesopredator	mammals/birds	3	5	resident	strictly nocturnal	4	10.02	1	intermediate	1.58
Otus sagittatus	monomorphic	-	2.09	small predator	other	2	1	resident	strictly nocturnal	3	19.15	0	open/close	0.30
Otus rufescens	polymorphic	2	1.88	small predator	other	2	1	resident	strictly nocturnal	1	0.83	1	open/close	0.90

*Otus thilohoffmanni**	monomorphic	-	1.97	small predator	other	1	1	resident	crepuscular/diurnal	1	6.50	0	open/close	0.60
Otus icterorhynchus	polymorphic	2	1.87	small predator	other	2	1	resident	crepuscular/diurnal	2	1.54	1	intermediate	0.60
Otus ireneae	polymorphic	3	1.70	small predator	other	1	1	resident	strictly nocturnal	2	-4.12	2	intermediate	1.23
*Otus balli**	polymorphic	2	2.00	small predator	other	2	1	resident	crepuscular/diurnal	2	12.46	0	intermediate	0.00
*Otus alfredi**	monomorphic	-	2.07	small predator	-	1	-	resident	crepuscular/diurnal	2	-8.51	2	open/close	0.70
Otus spilocephalus	polymorphic	2	1.90	small predator	other	2	1	resident	crepuscular/diurnal	4	14.77	1	open/close	1.00
Otus angelinae	monomorphic	-	1.92	small predator	other	1	2	resident	strictly nocturnal	2	-7.03	2	open/close	0.48
Otus mirus	monomorphic	-	1.81	small predator	other	1	1	resident	strictly nocturnal	1	7.89	0	open/close	0.48
*Otus longicornis**	monomorphic	-	2.04	small predator	other	1	1	resident	strictly nocturnal	1	16.54	0	open/close	0.60
*Otus mindorensis**	monomorphic	-	1.97	small predator	other	1	1	resident	strictly nocturnal	1	12.87	0	open/close	0.60
Otus hartlaubi	polymorphic	2	1.90	small predator	other	2	2	resident	crepuscular/diurnal	1	0.23	1	intermediate	0.70
Otus rutilus	polymorphic	3	2.00	small predator	mammals/birds	2	5	resident	crepuscular/diurnal	4	-18.93	2	intermediate	0.95
Otus mayottensis	polymorphic	2	2.08	small predator	mammals/birds	1	5	resident	strictly nocturnal	1	-12.83	2	open/close	0.60
Otus pauliani	polymorphic	2	1.85	small predator	other	1	1	resident	crepuscular/diurnal	1	-11.65	2	open/close	0.78
Otus capnodes	polymorphic	3	2.08	small predator	other	1	1	resident	crepuscular/diurnal	1	-12.23	2	open/close	0.90
Otus moheliensis	polymorphic	2	2.02	small predator	other	1	1	resident	strictly nocturnal	1	-12.32	2	open/close	0.70
*Otus pembaensis**	polymorphic	2	2.07	small predator	other	1	1	resident	crepuscular/diurnal	1	-5.17	2	intermediate	0.70
Otus scops	polymorphic	2	1.99	small predator	mammals/birds	3	5	totally migratory	strictly nocturnal	5	29.50	0	intermediate	2.61
Otus brucei	polymorphic	2	2.02	small predator	mammals/birds	4	4	partially migratory	crepuscular/diurnal	4	60.12	0	intermediate	1.41
Otus senegalensis	polymorphic	2	1.86	small predator	mammals/birds	4	5	resident	strictly nocturnal	5	-9.74	1	intermediate	1.46
Otus sunia	polymorphic	3	1.93	small predator	mammals/birds	3	5	partially migratory	crepuscular/diurnal	5	24.24	0	intermediate	1.49
Otus elegans	polymorphic	2	2.01	small predator	mammals/birds	1	5	partially migratory	strictly nocturnal	1	23.67	0	open/close	1.68
Otus magicus	polymorphic	5	2.14	small predator	mammals/birds	3	5	resident	crepuscular/diurnal	2	-3.23	1	intermediate	0.78
Otus beccarii	polymorphic	2	2.10	small predator	mammals/birds	2	5	resident	strictly nocturnal	1	-0.90	2	intermediate	0.30

Otus manadensis	polymorphic	3	1.94	small predator	mammals/birds	2	5	resident	crepuscular/diurnal	3	-2.37	1	intermediate	0.60
*Otus siaoensis**	monomorphic	-	1.90	small predator	mammals/birds	1	5	resident	crepuscular/diurnal	1	2.72	0	open/close	0.30
Otus collari	monomorphic	-	1.88	small predator	other	2	1	resident	strictly nocturnal	1	3.55	0	intermediate	0.60
Otus mantananensis	polymorphic	2	2.03	small predator	mammals/birds	2	5	resident	strictly nocturnal	2	8.16	0	intermediate	0.60
Otus insularis	monomorphic	-	2.16	small predator	other	2	3	resident	strictly nocturnal	1	-4.68	2	intermediate	1.23
*Otus alius**	monomorphic	-	2.16	small predator	other	1	2	resident	strictly nocturnal	1	7.00	0	intermediate	0.48
Otus umbra	monomorphic	-	1.98	small predator	other	2	1	resident	strictly nocturnal	1	2.63	0	intermediate	0.00
*Otus enganensis**	monomorphic	-	2.15	small predator	other	2	1	resident	strictly nocturnal	1	-5.40	2	open/close	0.00
*Otus mentawi**	polymorphic	2	2.15	small predator	other	2	1	resident	strictly nocturnal	1	-2.13	2	intermediate	0.30
Otus brookii	monomorphic	-	2.13	small predator	other	1	2	resident	-	2	0.35	1	open/close	0.30
Otus lempiji	polymorphic	3	2.06	small predator	mammals/birds	2	2	resident	strictly nocturnal [3]	5	0.42	1	intermediate	1.20
Otus lettia	polymorphic	2	2.14	small predator	mammals/birds	2	4	resident	strictly nocturnal	5	23.49	0	intermediate	0.95
Otus bakkamoena	polymorphic	2	2.14	small predator	mammals/birds	3	4	resident	strictly nocturnal	5	20.50	0	intermediate	1.85
Otus semitorques	monomorphic	-	2.11	small predator	mammals/birds	2	4	partially migratory	strictly nocturnal	3	40.25	0	intermediate	0.85
Otus megalotis	polymorphic	2	2.39	small predator	other	2	1	resident	strictly nocturnal	2	12.10	0	intermediate	1.26
*Otus fuliginosus**	monomorphic	-	2.03	small predator	other	2	1	resident	-	1	9.88	0	intermediate	0.30
Otus silvícola	monomorphic	-	2.33	small predator	other	3	1	resident	strictly nocturnal	2	-8.59	2	intermediate	0.00
Otus flammeolus	polymorphic	2	1.73	small predator	mammals/birds	1	2	partially migratory	crepuscular/diurnal	4	35.16	0	open/close	2.09
Otus leucotis	polymorphic	2	2.31	small predator	mammals/birds	3	4	resident	crepuscular/diurnal	5	10.00	0	intermediate	1.49
*Otus podarginus**	monomorphic	-	2.13	small predator	other	3	2	resident	crepuscular/diurnal	1	7.54	0	open/close	0.30
Megascops kennicottii	polymorphic	2	2.23	small predator	mammals/birds	3	5	resident	crepuscular/diurnal	5	37.68	0	intermediate	1.41
Megascops asio	polymorphic	3	2.27	small predator	mammals/birds	2	5	resident	crepuscular/diurnal	5	35.83	0	intermediate	1.99
Megascops cooperi	monomorphic	-	2.15	small predator	mammals/birds	3	5	resident	crepuscular/diurnal	3	13.53	0	open/close	0.00
Megascops trichopsis	polymorphic	2	1.98	small predator	mammals/birds	1	5	resident	crepuscular/diurnal	4	20.26	0	open/close	0.78

Megascops barbarus	polymorphic	2	1.84	small predator	other	2	1	resident	strictly nocturnal	3	16.64	0	open/close	0.48
Megascops seductus	monomorphic	-	2.21	small predator	mammals/birds	2	5	resident	strictly nocturnal	3	18.75	0	open/close	0.48
Megascops clarkii	monomorphic	-	2.20	small predator	mammals/birds	2	5	resident	crepuscular/diurnal	3	8.67	0	open/close	0.30
Megascops choliba	polymorphic	3	2.11	small predator	mammals/birds	2	5	resident	crepuscular/diurnal	5	-11.24	1	open/close	1.51
Megascops koepckeae	monomorphic	-	2.11	small predator	other	1	1	resident	strictly nocturnal	3	-10.26	2	intermediate	0.30
Megascops roboratus	polymorphic	2	2.08	small predator	other	1	1	resident	crepuscular/diurnal	3	-4.60	2	intermediate	0.00
Megascops hoyi	polymorphic	3	2.11	small predator	mammals/birds	1	2	resident	crepuscular/diurnal	3	-23.32	2	open/close	0.00
Megascops ingens	polymorphic	2	2.25	small predator	mammals/birds	1	5	resident	crepuscular/diurnal	3	-3.93	1	open/close	0.30
Megascops colombianus	polymorphic	2	2.26	small predator	mammals/birds	1	5	resident	strictly nocturnal	3	2.76	1	open/close	0.00
Megascops petersoni	monomorphic	-	1.98	small predator	mammals/birds	1	5	resident	strictly nocturnal	2	-4.81	2	open/close	0.60
Megascops marshalli	monomorphic	-	2.05	small predator	other	1	1	resident	strictly nocturnal	2	-14.61	2	open/close	0.30
Megascops watsonii	polymorphic	3	2.13	small predator	mammals/birds	1	5	resident	crepuscular/diurnal	5	-3.98	2	open/close	0.70
Megascops atricapilla	polymorphic	3	2.14	small predator	mammals/birds	1	5	resident	crepuscular/diurnal	3	-20.97	2	open/close	0.78
Megascops sanctaecatarinae	polymorphic	3	2.26	small predator	mammals/birds	1	5	resident	crepuscular/diurnal	3	-29.94	2	intermediate	0.78
Megascops guatemalae	polymorphic	2	2.03	small predator	mammals/birds	4	5	resident	strictly nocturnal	4	5.14	1	intermediate	0.85
Megascops nudipes	polymorphic	2	2.11	small predator	mammals/birds	1	5	resident	crepuscular/diurnal	1	18.22	0	intermediate	0.30
Megascops albogularis	monomorphic	-	2.20	small predator	mammals/birds	2	5	resident	crepuscular/diurnal	3	-3.70	1	open/close	0.48
Gymnoglaux lawrencii	monomorphic	-	1.90	small predator	mammals/birds	3	3	resident	crepuscular/diurnal	1	21.51	0	intermediate	0.48
Ptilopsis granti	monomorphic	-	2.36	small predator	mammals/birds	3	4	resident	strictly nocturnal	5	-13.97	2	intermediate	0.48
*Mimizuku gurneyi**	monomorphic	-	2.54	mesopredator	mammals/birds	2	3	resident	strictly nocturnal	3	8.02	0	open/close	1.08
Bubo scandiaca	monomorphic	-	3.26	top predator	mammals/birds	1	6	resident	strictly nocturnal	5	59.32	0	open/close	2.70
Bubo virginianus	monomorphic	-	3.24	top predator	mammals/birds	3	6	totally migratory	crepuscular/diurnal	5	14.47	1	intermediate	3.04
Bubo magellanicus	polymorphic	2	2.92	top predator	mammals/birds	4	3	resident	crepuscular/diurnal	5	-29.74	2	open/close	1.53
Bubo bubo	monomorphic	-	3.46	top predator	mammals/birds	2	6	partially migratory	crepuscular/diurnal	5	45.82	0	open/close	3.19
Bubo ascalaphus	monomorphic	-	3.32	top predator	mammals/birds	4	4	partially migratory	crepuscular/diurnal	4	24.85	0	open/close	1.67

Bubo bengalensis	polymorphic	2	3.04	top predator	mammals/birds	3	6	resident	crepuscular/diurnal	5	21.67	0	open/close	1.61
Bubo capensis	monomorphic	-	3.13	top predator	mammals/birds	3	6	resident	crepuscular/diurnal	2	-9.99	1	open/close	1.85
Bubo africanus	polymorphic	2	2.85	top predator	mammals/birds	4	5	resident	crepuscular/diurnal	4	-8.11	1	open/close	2.07
Bubo cinerascens	monomorphic	-	2.70	top predator	mammals/birds	3	5	resident	crepuscular/diurnal	5	7.39	1	open/close	0.60
Bubo poensis	monomorphic	-	2.84	top predator	mammals/birds	2	6	resident	crepuscular/diurnal	2	-1.67	1	open/close	1.00
Bubo vosseleri	monomorphic	-	2.95	top predator	mammals/birds	2	5	resident	crepuscular/diurnal	1	-5.64	2	open/close	1.00
Bubo lacteus	monomorphic	-	3.37	top predator	mammals/birds	3	7	resident	crepuscular/diurnal	4	-8.07	1	open/close	1.87
Bubo shelleyi	polymorphic	2	3.10	top predator	mammals/birds	1	2	resident	crepuscular/diurnal	1	1.78	1	open/close	0.70
Bubo sumatranus	monomorphic	-	2.79	top predator	mammals/birds	2	4	resident	strictly nocturnal	5	2.67	1	open/close	1.00
Bubo nipalensis	monomorphic	-	3.15	top predator	mammals/birds	3	4	resident	strictly nocturnal	4	19.35	0	open/close	1.26
*Bubo coromandus**	monomorphic	-	3.03	top predator	mammals/birds	2	6	resident	crepuscular/diurnal	4	17.34	0	open/close	0.95
Bubo leucostictus	monomorphic	-	2.74	top predator	other	3	1	resident	crepuscular/diurnal	3	3.34	1	open/close	0.48
*Bubo philippensis**	monomorphic	-	2.88	top predator	mammals/birds	3	2	resident	crepuscular/diurnal	2	12.10	0	open/close	0.85
Ketupa blakistoni	monomorphic	-	3.59	top predator	mammals/birds	2	3	resident	crepuscular/diurnal	3	47.28	0	open/close	1.72
Ketupa zeylonensis	monomorphic	-	3.08	top predator	mammals/birds	4	7	resident	crepuscular/diurnal	4	21.67	0	intermediate	1.54
Ketupa ketupu	monomorphic	-	3.19	top predator	mammals/birds	2	7	resident	crepuscular/diurnal	5	8.40	1	open/close	1.00
*Ketupa flavipes**	monomorphic	-	3.11	top predator	mammals/birds	2	6	resident	crepuscular/diurnal	5	23.77	0	open/close	1.20
Scotopelia peli	monomorphic	-	3.34	top predator	other	2	4	resident	crepuscular/diurnal	4	-6.30	1	open/close	1.65
Scotopelia ussheri	monomorphic	-	2.90	top predator	other	3	1	resident	crepuscular/diurnal	3	7.12	0	open/close	1.11
Scotopelia bouvieri	monomorphic	-	2.80	top predator	mammals/birds	2	5	resident	crepuscular/diurnal	2	0.30	1	open/close	0.90
Strix seloputo	monomorphic	-	3.00	mesopredator	mammals/birds	3	3	resident	strictly nocturnal	4	5.79	1	intermediate	0.95
*Strix ocellata**	monomorphic	-	2.87	mesopredator	mammals/birds	1	4	resident	crepuscular/diurnal	5	18.47	0	intermediate	0.48
Strix leptogrammica	monomorphic	-	2.97	mesopredator	mammals/birds	2	4	resident	strictly nocturnal	4	9.41	1	open/close	1.51
Strix aluco	polymorphic	3	2.72	mesopredator	mammals/birds	4	7	resident	crepuscular/diurnal	4	41.78	0	intermediate	3.22
Strix butleri	monomorphic	-	2.31	mesopredator	mammals/birds	3	4	resident	crepuscular/diurnal	2	23.76	0	open/close	1.57
Strix woodfordii	monomorphic	-	2.47	mesopredator	mammals/birds	3	5	resident	strictly nocturnal	4	-11.33	1	open/close	1.23
Strix virgata	polymorphic	2	2.42	mesopredator	mammals/birds	2	5	resident	crepuscular/diurnal	4	0.23	1	open/close	1.18
Strix rufipes	monomorphic	-	2.54	mesopredator	mammals/birds	2	5	resident	crepuscular/diurnal	3	-43.59	2	intermediate	1.76
Strix chacoensis	monomorphic	-	2.63	mesopredator	mammals/birds	1	5	resident	crepuscular/diurnal	3	-27.59	2	intermediate	0.90
Strix hylophila	monomorphic	-	2.53	mesopredator	mammals/birds	2	4	resident	crepuscular/diurnal	3	-23.36	2	open/close	1.00

Strix albitarsis	monomorphic	-	2.58	mesopredator	mammals/birds	2	2	resident	strictly nocturnal	3	-3.90	1	open/close	0.30
Strix nigrolineata	monomorphic	-	2.67	mesopredator	mammals/birds	3	4	resident	strictly nocturnal	5	8.49	1	open/close	0.70
Strix huhula	monomorphic	-	2.60	mesopredator	mammals/birds	3	5	resident	strictly nocturnal	5	-9.41	1	open/close	0.90
Strix occidentalis	monomorphic	-	2.81	mesopredator	mammals/birds	2	5	resident	crepuscular/diurnal	2	35.53	0	open/close	2.92
Strix fulvescens	monomorphic	-	2.78	mesopredator	mammals/birds	1	5	resident	strictly nocturnal	3	16.51	0	open/close	0.78
Strix varia	monomorphic	-	2.88	mesopredator	mammals/birds	2	6	partially migratory	strictly nocturnal	4	40.25	0	intermediate	2.66
*Strix davidi**	monomorphic	-	2.94	top predator	mammals/birds	2	1	resident	crepuscular/diurnal	3	29.74	0	intermediate	1.04
Strix uralensis	polymorphic	2	2.95	mesopredator	mammals/birds	2	4	resident	crepuscular/diurnal	4	50.29	0	open/close	2.65
Strix nebulosa	monomorphic	-	3.09	top predator	mammals/birds	3	4	partially migratory	crepuscular/diurnal	4	51.89	0	intermediate	2.56
Jubula lettii	monomorphic	-	2.26	mesopredator	mammals/birds	1	5	resident	crepuscular/diurnal	2	0.94	1	open/close	0.78
Lophostrix cristata	polymorphic	3	2.72	mesopredator	mammals/birds	1	5	resident	crepuscular/diurnal	4	2.56	1	open/close	1.26
Pulsatrix perspicillata	monomorphic	-	2.96	top predator	mammals/birds	3	5	resident	strictly nocturnal	5	-4.93	1	open/close	1.65
Pulsatrix koeniswaldiana	monomorphic	-	2.68	mesopredator	mammals/birds	2	5	resident	strictly nocturnal	3	-21.81	2	open/close	1.23
Pulsatrix melanota	monomorphic	-	2.66	mesopredator	mammals/birds	2	5	resident	strictly nocturnal	3	-8.74	1	open/close	0.48
Surnia ulula	monomorphic	-	2.48	mesopredator	mammals/birds	2	4	partially migratory	crepuscular/diurnal	4	56.51	0	intermediate	2.41
Glaucidium passerinum	monomorphic	-	1.81	small predator	mammals/birds	2	4	resident	crepuscular/diurnal	4	54.61	0	intermediate	2.69
Glaucidium perlatum	monomorphic	-	2.02	small predator	mammals/birds	2	5	resident	crepuscular/diurnal	5	-3.91	1	open/close	1.66
Glaucidium tephronotum	monomorphic	-	1.96	small predator	mammals/birds	2	3	resident	crepuscular/diurnal	2	2.14	1	open/close	1.18
Glaucidium brodiei	polymorphic	2	1.75	small predator	mammals/birds	2	4	resident	crepuscular/diurnal	4	15.26	1	open/close	1.04
Glaucidium californicum	polymorphic	3	1.85	small predator	mammals/birds	1	5	partially migratory	crepuscular/diurnal	4	44.21	0	open/close	0.85
Glaucidium gnoma	monomorphic	-	1.78	small predator	mammals/birds	1	4	resident	crepuscular/diurnal	3	26.88	0	open/close	1.85
Glaucidium nubicola	monomorphic	-	1.89	small predator	mammals/birds	1	4	resident	crepuscular/diurnal	3	1.87	1	open/close	0.60
Glaucidium costaricanum	polymorphic	2	1.88	small predator	mammals/birds	2	4	resident	crepuscular/diurnal	3	9.27	0	open/close	0.60
Glaucidium siju	polymorphic	2	1.87	small predator	mammals/birds	2	4	resident	crepuscular/diurnal	1	21.51	0	intermediate	1.00
Glaucidium sanchezi	monomorphic	-	1.72	small predator	mammals/birds	2	4	resident	crepuscular/diurnal	3	24.28	0	open/close	0.70
Glaucidium palmarum	monomorphic	-	1.66	small predator	mammals/birds	2	4	resident	crepuscular/diurnal	3	23.42	0	intermediate	0.60
Glaucidium griseiceps	monomorphic	-	1.73	small predator	mammals/birds	2	5	resident	crepuscular/diurnal	5	11.69	0	intermediate	0.85

Glaucidium minutissimum	monomorphic	-	1.70	small predator	mammals/birds	2	5	resident	crepuscular/diurnal	3	-20.77	2	open/close	1.36
Glaucidium hardyi	polymorphic	2	1.78	small predator	mammals/birds	1	4	resident	crepuscular/diurnal	5	-2.59	1	open/close	1.08
Glaucidium parkeri	monomorphic	-	1.79	small predator	other	2	1	resident	crepuscular/diurnal	3	-10.12	2	open/close	0.78
Glaucidium jardinii	polymorphic	2	1.82	small predator	mammals/birds	2	5	resident	crepuscular/diurnal	3	0.09	1	intermediate	1.08
Glaucidium bolivianum	polymorphic	3	1.79	small predator	mammals/birds	1	5	resident	crepuscular/diurnal	3	-18.15	2	open/close	0.30
Glaucidium peruanum	polymorphic	3	1.79	small predator	mammals/birds	3	5	resident	crepuscular/diurnal	3	-8.79	2	open/close	0.70
Glaucidium nanum	polymorphic	2	1.89	small predator	mammals/birds	3	4	partially migratory	crepuscular/diurnal	3	-45.82	2	intermediate	1.41
Glaucidium brasilianum	polymorphic	3	1.88	small predator	mammals/birds	4	4	resident	crepuscular/diurnal	5	0.10	1	intermediate	2.06
Glaucidium mooreorum	monomorphic	-	1.71	small predator	mammals/birds	1	5	resident	-	1	-35.58	2	open/close	0.70
Glaucidium sjostedti	monomorphic	-	2.15	small predator	mammals/birds	1	4	resident	crepuscular/diurnal	3	0.97	1	open/close	0.60
Glaucidium cuculoides	monomorphic	-	2.29	small predator	mammals/birds	3	4	resident	crepuscular/diurnal	5	23.77	0	intermediate	1.38
*Glaucidium castanopterum**	monomorphic	-	2.06	small predator	mammals/birds	4	4	resident	crepuscular/diurnal	2	-7.45	2	open/close	0.30
Glaucidium radiatum	polymorphic	2	2.00	small predator	mammals/birds	1	5	resident	crepuscular/diurnal	5	19.34	0	open/close	1.28
Glaucidium castanonotum	monomorphic	-	2.00	small predator	mammals/birds	1	4	resident	crepuscular/diurnal	1	6.83	0	open/close	0.48
Glaucidium capense	monomorphic	-	2.04	small predator	mammals/birds	2	5	resident	crepuscular/diurnal	4	-14.15	2	open/close	1.56
Glaucidium castaneum	monomorphic	-	1.99	small predator	mammals/birds	1	5	resident	crepuscular/diurnal	2	1.94	1	open/close	0.78
Glaucidium albertinum	monomorphic	-	1.86	small predator	mammals/birds	2	5	resident	crepuscular/diurnal	1	-0.88	2	open/close	0.48
Xenoglaux loweryi	monomorphic	-	1.68	small predator	other	1	1	resident	crepuscular/diurnal	1	-5.07	2	open/close	0.90
Micrathene whitneyi	monomorphic	-	1.62	small predator	mammals/birds	2	3	partially migratory	crepuscular/diurnal	4	27.63	0	open/close	1.65
Heteroglaux blewitti	monomorphic	-	2.38	small predator	mammals/birds	1	3	resident	crepuscular/diurnal	2	21.54	0	open/close	1.36
Athene noctua	polymorphic	2	2.26	small predator	mammals/birds	5	6	resident	crepuscular/diurnal	5	38.27	0	intermediate	3.11
Athene brama	monomorphic	-	2.05	small predator	mammals/birds	3	5	resident	crepuscular/diurnal	5	24.27	0	open/close	2.16
Athene cunicularia	monomorphic	-	2.29	small predator	mammals/birds	4	5	partially migratory	crepuscular/diurnal	4	-0.57	1	open/close	2.72
Aegolius funereus	monomorphic	-	2.18	small predator	mammals/birds	1	2	partially migratory	crepuscular/diurnal	4	52.88	0	open/close	3.04

Aegolius acadicus	monomorphic	-	1.94	small predator	mammals/birds	3	5	partially migratory	strictly nocturnal	4	40.07	0	open/close	2.49
Aegolius ridgwayi	monomorphic	-	1.90	small predator	mammals/birds	3	3	resident	strictly nocturnal	2	13.84	0	open/close	0.78
Aegolius harrisii	monomorphic	-	2.11	small predator	mammals/birds	3	5	resident	strictly nocturnal	2	-10.97	1	open/close	1.28
Ninox rufa	monomorphic	-	3.00	top predator	mammals/birds	3	3	resident	crepuscular/diurnal	2	-11.71	2	open/close	1.30
Ninox strenua	monomorphic	-	3.14	top predator	mammals/birds	4	2	resident	crepuscular/diurnal	3	-31.30	2	open/close	2.18
Ninox connivens	monomorphic	-	2.67	mesopredator	mammals/birds	4	3	resident	crepuscular/diurnal	4	-18.23	2	intermediate	1.76
Ninox rudolfi	monomorphic	-	2.35	mesopredator	other	3	1	resident	crepuscular/diurnal	1	-9.80	2	intermediate	0.70
Ninox boobook	monomorphic	-	2.40	mesopredator	mammals/birds	3	3	resident	crepuscular/diurnal	4	-22.10	2	intermediate	1.48
Ninox novaeseelandiae	monomorphic	-	2.26	mesopredator	mammals/birds	2	4	resident	crepuscular/diurnal	3	-40.41	2	intermediate	2.26
Ninox scutulata	monomorphic	-	2.27	mesopredator	mammals/birds	4	5	totally migratory	crepuscular/diurnal	5	23.42	1	open/close	1.92
Ninox affinis	monomorphic	-	2.12	mesopredator	other	1	1	resident	crepuscular/diurnal	1	12.04	0	open/close	0.85
Ninox superciliaris	polymorphic	2	2.37	mesopredator	mammals/birds	4	5	resident	crepuscular/diurnal	2	-19.63	2	intermediate	0.30
Ninox philippensis	monomorphic	-	2.05	mesopredator	mammals/birds	1	2	resident	strictly nocturnal	2	12.10	0	open/close	1.18
Ninox ochracea	monomorphic	-	2.20	mesopredator	other	2	1	resident	strictly nocturnal	3	-1.98	1	open/close	0.85
Ninox jacquinoti	monomorphic	-	2.24	mesopredator	mammals/birds	2	5	resident	strictly nocturnal	1	-7.92	2	open/close	0.60
Ninox theomacha	monomorphic	-	2.26	mesopredator	other	3	1	resident	strictly nocturnal	2	-6.00	2	open/close	0.70
Ninox punctulata	monomorphic	-	2.18	mesopredator	other	2	1	resident	strictly nocturnal	3	-1.98	1	intermediate	0.70
Ninox odiosa	monomorphic	-	2.32	mesopredator	mammals/birds	2	5	resident	strictly nocturnal	1	-5.21	2	intermediate	0.60
Ninox squamipila	monomorphic	-	2.24	mesopredator	other	3	1	resident	crepuscular/diurnal	1	-5.58	1	open/close	1.04
Ninox ios	monomorphic	-	1.89	mesopredator	-	1	-	-	strictly nocturnal	2	-0.07	1	open/close	0.95
Ninox burhani	monomorphic	-	2.00	mesopredator	-	3	-	resident	crepuscular/diurnal	1	-0.40	2	open/close	0.78
Ninox sumbaensis	monomorphic	-	1.95	mesopredator	-	1	-	resident	strictly nocturnal	1	-9.80	2	open/close	0.60
Ninox natalis	monomorphic	-	2.20	mesopredator	mammals/birds	2	3	resident	crepuscular/diurnal	1	-10.49	2	open/close	1.00
*Ninox meeki**	monomorphic	-	2.50	mesopredator	other	2	1	resident	-	1	-2.11	2	intermediate	0.30
*Ninox variegata**	polymorphic	2	2.39	mesopredator	other	1	1	resident	strictly nocturnal	1	-3.60	2	open/close	0.00
*Uroglaux dimorpha**	monomorphic	-	2.41	mesopredator	mammals/birds	3	3	resident	-	2	-5.52	2	open/close	0.70
Nesasio solomonensis	monomorphic	-	2.63	mesopredator	mammals/birds	1	2	resident	strictly nocturnal	1	-6.78	2	open/close	0.70
*Pseudoscops grammicus**	monomorphic	-	2.41	mesopredator	mammals/birds	1	5	resident	strictly nocturnal	1	18.19	0	intermediate	0.70

Pseudoscops clamator	monomorphic	-	2.65	mesopredator	mammals/birds	3	5	resident	crepuscular/diurnal	4	-5.98	1	intermediate	0.90
Asio stygius	monomorphic	-	2.80	mesopredator	mammals/birds	4	5	resident	strictly nocturnal	4	1.37	1	open/close	1.61
Asio otus	monomorphic	-	2.51	mesopredator	mammals/birds	3	5	partially migratory	crepuscular/diurnal	5	44.38	0	open/close	3.21
Asio abyssinicus	monomorphic	-	2.51	mesopredator	mammals/birds	4	5	resident	strictly nocturnal	2	5.20	1	open/close	0.60
Asio madagascariensis	monomorphic	-	2.89 [4]	mesopredator	mammals/birds	3	4	resident	strictly nocturnal	5	-18.77	2	open/close	1.00
Asio flammeus	monomorphic	-	2.53	mesopredator	mammals/birds	3	4	totally migratory	crepuscular/diurnal	5	9.38	1	open/close	2.97
Asio capensis	monomorphic	-	2.55	mesopredator	mammals/birds	4	5	resident	crepuscular/diurnal	4	2.55	1	open/close	1.79

1 = Roulin A (2004) Covariation between plumage colour polymorphism and diet in the Barn Owl *Tyto alba*. Ibis, 146:509–517.

2 = Davidson P, Stones T and Lucking R (1995) The conservation status of key bird species on Taliabu and the Sula Islands, Indonesia. Bird Conservation International, 5:1-20.

3 = Najmi-Hanis Z et al. (2016) Home range and activity patterns of Sunda scops owl in Peninsular Malaysia. Raffles Bullettin of Zoology 64:28–32.

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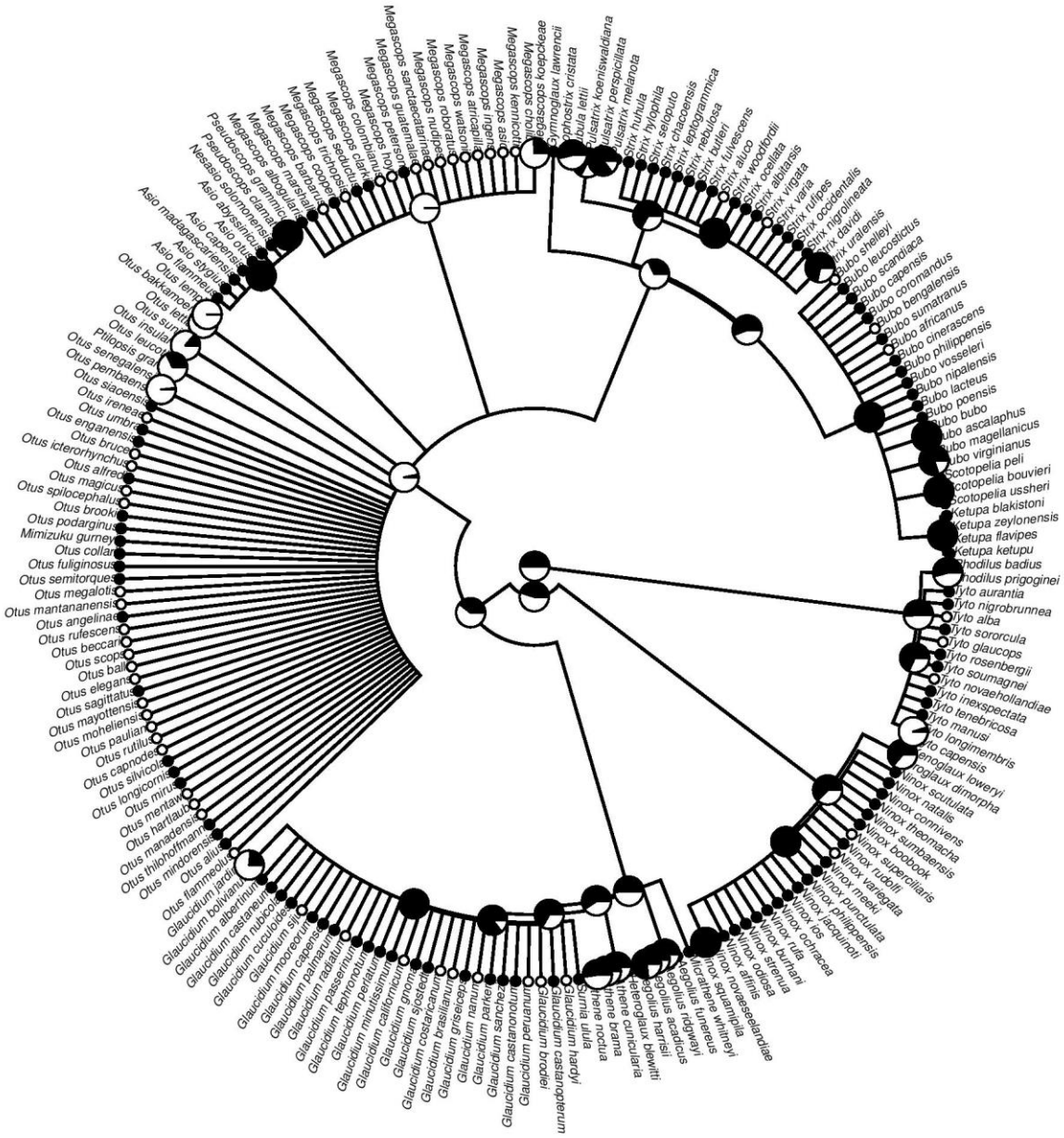

Figure 1S. *Ancestral state reconstruction of the binary character colour polymorphism across a majority-rule consensus tree created on 1000 phylogenies using the ER model (for comparison with ARD model in the main text). Pie charts at the nodes represent proportional maximum-likelihood support for the monomorphic (black) and polymorphic (white) character states from the ancestral state reconstruction.*

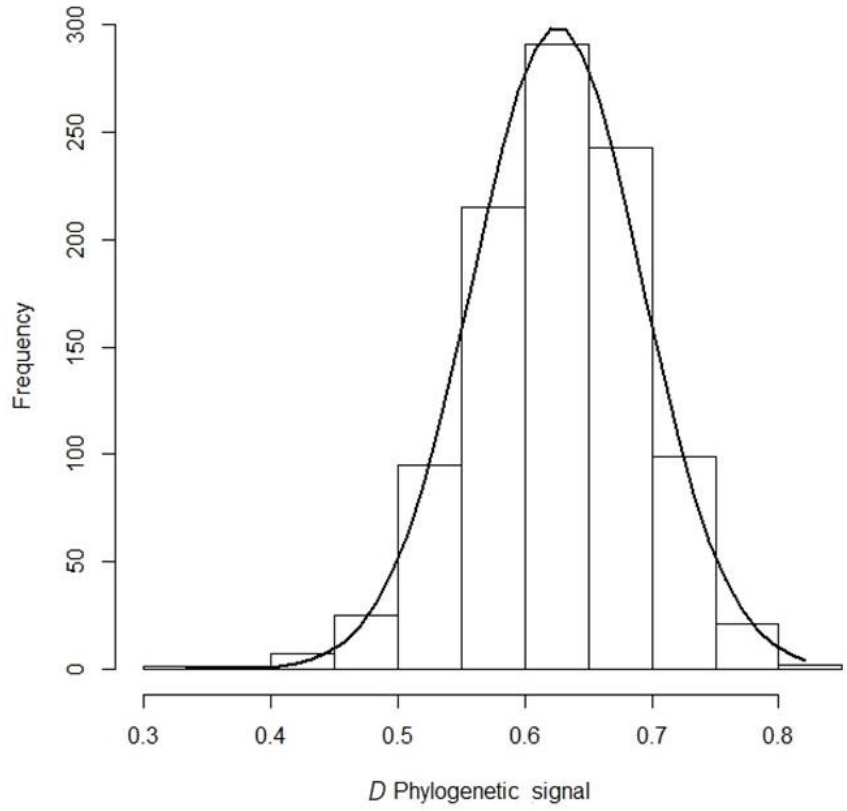

Figure 2S. *Probability distribution of D phylogenetic signal based on 1000 randomly selected trees. Neither zero nor one were contained between the 99% Confidence Limits, thus providing support that* $D \neq 0$ *and* $D \neq 1$.

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Chapter III

The evolution of iris colour in relation to nocturnality in owls

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ABSTRACT

Birds, due to their multiple colourful displays, constitute a classic paradigm for the study of colour evolution. Although avian eyes are remarkably coloured, the functional basis behind inter-specific variability in iris coloration remains poorly understood. Owls are an ideal system to shed light on the role of ecology in promoting iris colour evolution as they show inter-specific variation in iris colour and in niche specialization with some species being strictly nocturnal and others active during the day. Owls perching for hunting at night might be unnoticed by both predators and their prey if they had dark irises, which would predict that dark irises were more likely to evolve in strictly nocturnal species than in diurnal ones. Using phylogenetic comparative models, we tested the camouflage hypothesis for eye colour. The proportion of dark-eyed owl species is higher among strictly nocturnal owls than among diurnal ones. Ancestral state reconstruction revealed that the owl ancestor of the family *Strigidae* was more likely bright-irided whereas the ancestor of the family *Tytonidae* was more likely dark-irided. Our results show robust support for the coevolution of iris coloration and nocturnality in the owls, and suggest that shifting to a nocturnal niche would be a prerequisite leading to the evolution of dark eyes in owls. The specific evolutionary pathway by which iris coloration and activity rhythm coevolve, however, remains to be investigated further as we have found only partial support for the idea that dark irises in birds might be an adaptive feature evolved due to the selective advantage of concealment from undesired visual receptors.

INTRODUCTION

Understanding the functional basis of the formidable variation in animal colours remains a major challenge in evolutionary ecology (Cott 1940), and, birds, due to their multiple colourful displays, have constituted a classic paradigm for its study (Hill and McGraw 2006). Although a large body of empirical work has accumulated about the role of sexual and natural selection in promoting egg (e.g. Soler et al. 2005, Aviles et al. 2006), skin (Kilner 2006) and plumage colour variability (e.g. Hill and McGraw 2006, Dale et al. 2015), other conspicuous avian traits, such as the eyes, remain poorly studied.

Avian eyes are often conspicuously coloured and hence difficult to conceal to visually based receivers (Cott 1940). Studies at the intra-specific level have shown that iris coloration can change with age and sex and relates to individual quality in diurnal raptors (e.g. Picozzi 1981, Newton and Marquiss 1982, Bortolotti et al. 2003), penguins (Scholten 1999) and brood parasitic cuckoos (Yoo et al. 2017). Also, the greyish eye colour of Jackdaws (*Corvus monedula*) may serve as a warning signal to indicate that a nest is occupied and deter intrusions by conspecifics (Davidson et al. 2014), globally suggesting that iris coloration may play a role in social contexts (but see however Negro et al. 2017).

Iris coloration is highly variable across different avian species, although the functional basis behind inter-specific variation remains elusive. In an initial survey of Passerines' iris colour variability, Craig and Hulley (2004) found regional differences but not clear support for a role of ecology and sociability. More recently, Davidson et al. (2017) found that iris coloration in *Passeriformes* coevolved with cavity nesting habits and that cavity nesting species were more likely to have bright eyes than open-nesting species, which suggests that detection by predators in open nests may have driven iris colour evolution in this clade (Davidson et al. 2017). To our knowledge, the functional bases of inter-specific variation in eye coloration in clades others than *Passeriformes* remain unknown.

Owls display showy inter-specific variation in iris coloration and must resolve the visual challenge of finding food while remaining unnoticed by potential visually based receivers (i.e predators, prey and competitors) at night (König and Weick 2008). Comparative work has shown notable adaptations for avian eyes in terms of detecting prey and seeing in the dark. For instance eye morphology (size and shape) in birds is strongly related with nocturnality, with species adapted to scotopic environments exhibiting absolutely larger corneal diameters and axial lengths than do photopic adapted birds (Hall and Ross 2007, Lisney et al. 2012). More specifically owls present a binocular vision, large eyes and a rood-dominated retina that improve visual perception in darkness (Martin 1985). Iris is a fundamental part of the eye phenotype and although its coloration may play a key role in eye concealment (Bortolotti 2006), no study has tested for a possible relationship between iris colour and nocturnality yet.

Here we test the influence of nocturnality in driving the evolution of iris coloration in owls using phylogenetic comparative analyses. The camouflage hypothesis states that it would be advantageous for animals to evolve body colour traits allowing a *general colour resemblance* with the environmental background to be less recognizable to its prey, predators or competitors (Endler 1978). Accordingly, owls displaying dark eyes may disguise themselves while perching for hunting in the night, a strategy that might fool both predators and their prey. This hypothesis would specifically predict i) that iris coloration was evolutionarily correlated with activity rhythm in owls; ii) that dark irises were more likely to evolve in strictly nocturnal species than in diurnal ones; and, iii) that transition to nocturnality was a necessary prerequisite for the evolution of dark irises.

MATERIAL AND METHODS

We collated information on iris colour for all extant owls (206 species) included in a recent full updated avian phylogeny (Jetz et al. 2012). Iris colour was scored as a binary variable as dark (including black or dark brown eyes) *versus* bright (including yellow, orange, red and light brown eyes; see Davidson et al. (2017)) based on colour plates in del Hoyo et al. (1999). Moreover, species

were classified regarding activity rhythm as strictly nocturnal species *versus* species active also during the day or at dusk or dawn as in Passarotto et al. (2018). Activity rhythm information was available for 201 species only (see electronic supplementary material).

We reconstructed the ancestral state of iris colour on a maximum clade credibility tree, created using TREANNOTATOR in BEAST 1.8 with posterior probabilities set at 0.5 (Drummond et al. 2012). We considered 1000 trees sampled from a time-calibrated set of complete phylogenies (Jetz et al. 2012).This phylogeny relies on the bird genome-based Hackett et al.´s (2008) phylogenies as a backbone for their phylogenetic reconstructions. Ancestral character reconstruction was performed using the R function rerootingMethod in PHYTOOLS (Revell 2012): this approach allows estimating the marginal ancestral state for each internal node of the tree using likelihood and comparing the performance of two models of evolution: "Equal Rates" model (ER) that assumes the trait is lost or acquired at a similar rate over time and the "All Rates Different" model (ARD) that allows for differences in the rate of gain and loss of the trait. Then, we used AIC (Akaike Information Criterion) to select the best model, considering as support to our choice a difference of 4 or greater between models (Burnham and Anderson 2002). In order to improve the precision of ancestral state estimations, we included information for eye colour classified as in owls for three further avian orders as outgroups: *Cathartiformes* and *Accipitriformes* as the two closest sister clades of owls and *Falconiformes* as a more distant clade. Since some species of diurnal raptors display sexual dimorphism in eye colour, we decided to discard from analyses species where sexes show eye colour belonging to different categories (dark or bright), whereas coloration differences within the same category were included in analyses (see electronic supplementary material). Character-state transition rates under the selected model were calculated using stochastic character mapping with make.simmap function in the PHYTOOLS package in R (Revell 2012).

We used Pagel´s DISCRETE algorithm implemented in BayesTraits (Pagel and Meade 2013) to test whether iris colour and activity rhythm have evolved in concert. We used a maximum

likelihood approach based on the observation of transition rate of character' states under two contrasting models. The dependent model assumes that the two traits co-evolved, and, thus that the rate of change in one character is contingent on the state of the other character. Conversely, under the independent model, the transition from a state to another in one character is independent of the state of the other character (Pagel 1994). Each model was ran three times for all 1000 phylogenies and then averaged, and the choice of the best model was made using a likelihood ratio test.

In addition, once verified that the dependent model was the best one, we ran a set of target dependent models applying only the restrictions of direct relevance to our coevolutionary hypotheses (i.e. restricted model 1, bright to dark irises in diurnality = bright to dark irises in nocturnality (i.e. $q1,3 = q2,4$); restricted model 2, diurnality to nocturnality in presence of bright eyes=bright to dark irises in diurnality (i.e. $q1,2 = q1,3$); restricted model 3, diurnality to nocturnality in presence of bright eyes=, diurnality to nocturnality in presence of dark eyes (i.e. $q1,2 = q3,4$)). In an unrestricted dependent model there are eight parameters corresponding to all possible transitions, resulting from the states of two binary variables. They are indicated as $q_{i,j}$ where q characterizes the transition rate from one combination of eye colour and activity rhythm [*i*] to another eye coloration and activity rhythm combination [*j*]. Model restrictions are based on the reduction of parameters by constraining two transitions to assume the same value. Comparison between the unrestricted dependent model and the restricted ones using likelihood ratio tests would allow detecting trait evolution directionality, hence providing a test for the different evolutionary pathways (Pagel and Meade 2013). Specifically, comparison between the unrestricted dependent model and the restricted model 1 tests whether changes in iris colour is similar regardless of the environment it is in (in this case activity level); comparison between the unrestricted dependent model and the restricted model 2 allow testing which changed first: eye coloration followed by activity rhythm or vice versa; finally, comparison between the unrestricted dependent model and the restricted model 3 allow testing whether being dark-eyed is a pre-requisite for becoming nocturnal.

RESULTS

Dark eyes were found in 71 species belonging to 14 genera, whereas 135 species belonging to 20 genera were classed to have bright eyes (see Table S1 in electronic supplementary material). Dark irises were more frequent among strictly nocturnal owls (41 (58.57%) out of 70 nocturnal species) than among owls which are active during the day or in the last part of the day (37 (28.24%) out of 131 diurnal or crepuscular species) (Yates corrected χ^2=16.42, P=0.0001).

The model "ARD" does not improve the potential of the "ER" model to trace the evolution of iris coloration in owls (ΔAIC =1.52). Ancestral reconstruction revealed that the owl ancestor cannot be unambiguously established (proportional likelihood 0.58 for bright irides, 0.42 for dark irises) because the two owl families (i.e. *Tytonidae* and *Strigidae*) differ in the ancestral state of iris colour. Whereas the ancestor of *Strigidae* was most likely bright-eyed (proportional likelihood = 0.90 bright irises, 0.10 dark irises), the ancestor of *Tytonidae* was most likely dark-eyed (proportional likelihood = 0.72 dark irises, 0.28 bright irises) (Fig. 1). Stochastic character mapping revealed that the transition from a bright to a dark iris was more likely to occur than the opposite transition (total average changes between stages=59.06; 73.93 % of changes from bright to dark, 26.08% of changes from dark to bright).

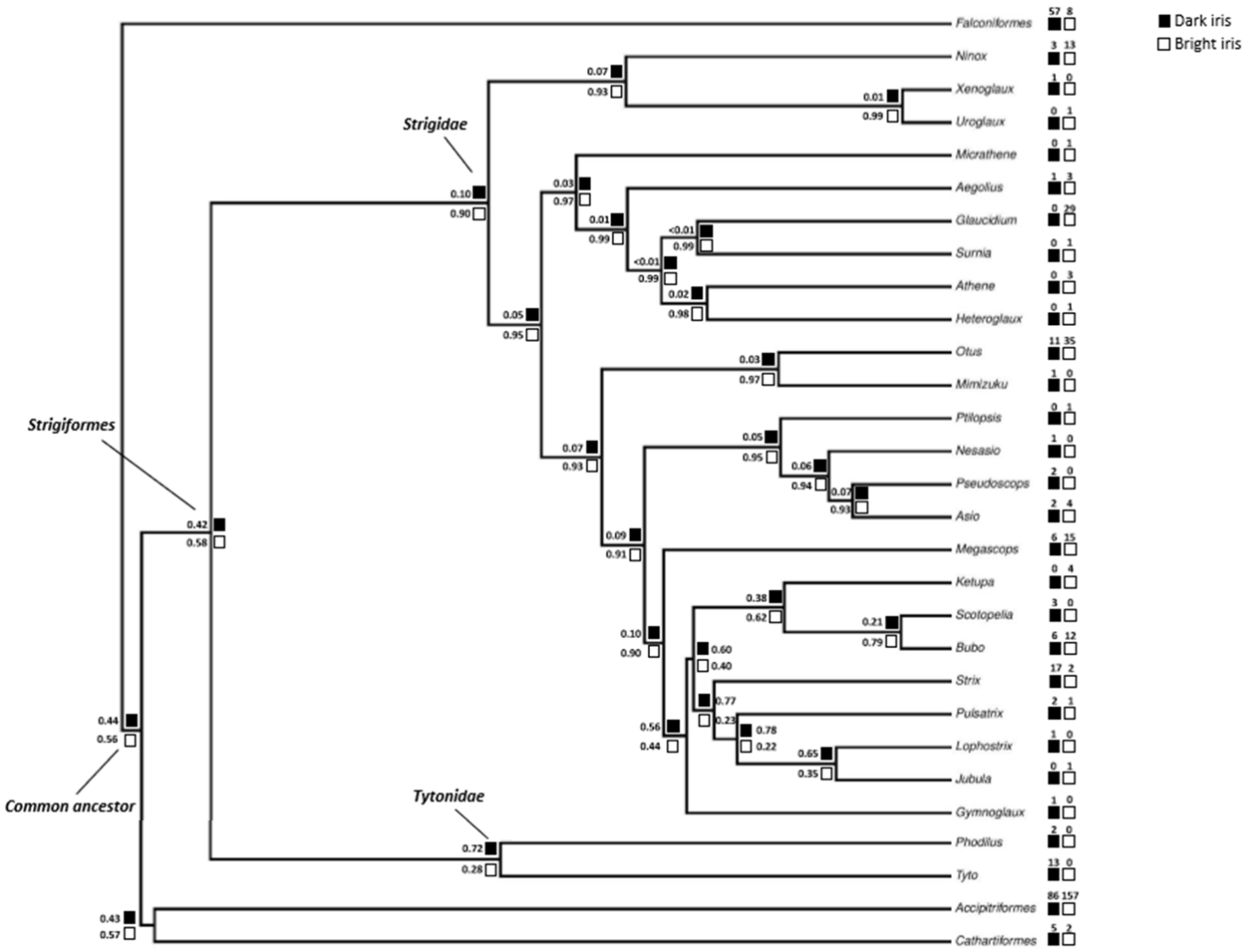

Figure 1. *Ancestral state reconstruction for iris colour in owls. Pie charts at the nodes represent proportional maximum-likelihood support respectively for the dark eyes (black) and bright eyes (white) character states from the ancestral state reconstruction. Number of species within each genus with dark and bright irises is shown on the tips.*

The analysis of correlated evolution revealed that iris colour and activity rhythm have more likely evolved in concert than independently in owls (log-likelihood for independent, -245.74 *versus* dependent model, -234.82; χ^2=21.9, d.f.=4, *P*=0.0006). Although transition rates from bright to dark irises are higher in owl species presenting strictly nocturnal habits than in diurnal species (*q*2,4 = 1.544 *vs q*1,3 = 0.194), this model of evolution is not significantly better than the restricted model 1, in which these transition rates are restrained to be equal across both activity rhythms (*q*1,3=*q*2,4, see methods; log-likelihood non-restricted model = -234.82, log-likelihood restricted model = -235.37;

χ^2=1.10, *P* = 0.29). Comparison between transition *q*1,2 and *q*1,3 suggests that the most likely evolutionary path for the evolution of iris colour and activity rhythm would be a change in the activity rhythm followed by a change in iris colour (*q*1,2 = 2.648 *vs* *q*1,3 = 0.194) (Fig. 2). The comparison between the unrestricted model and the restricted model 2 (*q*1,2=*q*1,3, see methods) was significantly different (log-likelihood non-restricted model = -234.82, log-likelihood restricted model = -237.83; χ^2=6.03, P = 0.014). Finally, comparison between transition q1,2 and q3,4 revealed that being dark-eyed was not a prerequisite for becoming nocturnal as there is no significant difference between the unrestricted dependent model and the restricted model 3, where *q*1,2 was constrained to be equal to *q*3,4 (log-likelihood non-restricted model = -234.82, log-likelihood restricted model = -236.13; χ^2= 2.61, p=0.11).

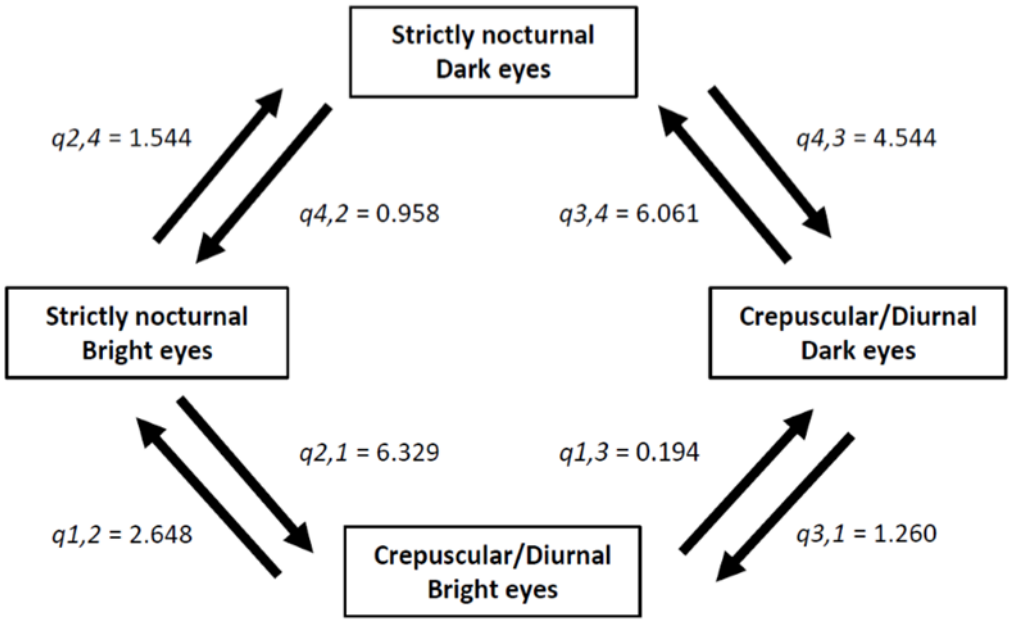

Figure 2. *Flow diagrams showing the most probably evolutionary pathway for the evolution of iris coloration in owls in relation to activity rhythm. Changes between bright and dark irises occurred in a correlated way with activity rhythm (see results) and were more likely in strictly nocturnal species than in species active during the day or at dusk or dawn. Likelihoods of all transitions are shown close to arrows.*

DISCUSSION

Our results provide strong support for the existence of an evolutionary correlation between iris coloration and activity rhythm in owls. Beyond that correlation, we did not find clear evidence that dark eyes are more likely to evolve in species presenting strictly nocturnal habits than in diurnal species. However, it was found that the most plausible evolutionary path leading to current high proportion of nocturnal owls with dark irises was a transition from diurnality to nocturnality followed by a change from light toward dark irises. Hence, these findings only partly agree with expectations from the camouflage hypothesis stating that owl species with nocturnal habits are under stronger selection to evolve dark eyes than those that are active at any time during the day.

A likely explanation for the found patterns would be that dark eyes might be better concealed at night and help to avoid prey or predator detection. Different experiments have shown that avian eyes constitute salient features attracting the attention of both predator and prey species (Scaife 1976a, Bones 1980, Curios 1975, Kerlinger and Lehrer 1982), and birds have evolved a wide array of plumage marking designs through or around the eye to hide this noticeable feature from undesirable receptors (Bortolotti 2006). In agreement, a recent comparative study has found that passerine species nesting in open nests, and, hence not having the concealment benefits of cavities, had more likely dark irises, which suggests a key role of predator detection on the evolution of iris coloration in passerines (Davidson et al. 2017). Nocturnal owls hunt at darkness and having dark inconspicuous irises may allow then to be unnoticed by their prey while approaching, which may provide them with foraging benefits. A similar mechanism was invoked to explain the high occurrence of protruding eyes among seabirds, which renders them inconspicuous to aquatic prey (Bortolotti 2006). Dark irises may also be beneficial while owls are resting during the day, in terms of reducing detection by potential predators or competitors. Indeed, since eye conspicuousness also depends on the visibility of the pupil (Scaife 1976a, Scaife 1976b, Bones 1980), dark eyes would be less detectable than bright ones since the pupil is not visible in the formers, avoiding the effect of a tracing gaze. However, the

camouflage hypothesis predicts that dark eyes are more likely to evolve in species presenting strictly nocturnal habits than in diurnal species, and we did not find support for that prediction. Moreover, there is no evidence that owls with brightly coloured irises do badly than dark-eyed species in terms of being detected in the dark by potential visually based receivers. It can be argued that iris colour would not be seen in total darkness anyway, as potential for detection of colour stimulus at night is very low in vertebrates (i.e. either prey, predator or competitor (Kelber et al. 2003). Also, most owl species have few predators and their typical prey (rodents and roosting birds) often do not perceive their approaching hunters until it is too late, which would suggest that selection toward dark iris in terms of detection would be weak at darkness.

An alternative possibility linking iris coloration and activity rhythm in owls is that iris coloration was linked to visual needs (Bortolotti 2006). Several source of evidence suggest that selection for visual perception in darkness may have promoted the evolution of changes in the owl visual apparatus (Martin 1985, Lisney et al. 2012), and in coloured features involved in intra-specific communication in owls, such as the beak or plumage patches (Parejo et al. 2010, Penteriani and Delgado 2017). Also, it has been suggested that light-coloured irises may allow more light to pass through than dark irises resulting in less sharp images in the retina, a possibility that would predict for a higher transition from light to dark iris in diurnal species (visual clarity hypothesis *sensu* (Savalli 1995)). However, this possibility seems unlikely as we found that rates away from bright irises were greater for nocturnal owls than diurnal owls. Alternatively, it could be argued that owls that have brightly coloured irises may benefit from better vision in twilight or in daytime hours whereas strictly nocturnal species may simply avoid investing in the production or acquisition of potentially costly pigments, and thus may have darker eyes for reasons other than camouflage. The colour of avian irises, however, is one of the most mechanistically complex aspects of avian phenotype (Prum 2006), and the relative importance and function of pigmentary and structural iris colour in visual perception at darkness remains to be determined.

The ancestral reconstruction revealed that the ancestor of the family *Strigidae* was more likely bright-eyed whereas the ancestor of *Tytonidae* was more likely dark-eyed. This finding may reflect the different evolutionary histories traced by the two main owl families. *Tytonidae* family might be under a stronger selective pressure for the occupancy of nocturnal niches and hence under selection of dark irises at an earlier stage of the evolutionary route than *Strigidae* family. Indeed, all extant *Tytoniade* species are nocturnal hunters and have dark irises (Fig. 1 and Table S1 in electronic supplementary material). Previous studies have suggested that diversity of *Tytonidae* in the Paleogene was notable, but that the group was partially superseded by *Strigidae* during the Neogene, which presented a greater diversification (del Hoyo et al. 1999). By contrast, extant *Strigidae* show greater diversity in eye coloration and activity rhythm and dark eyes seem to be a posterior acquisition in relation to the occupancy of dark luminal niches.

In conclusion, our results show robust support for the coevolution of iris coloration and nocturnality in the owls, and suggest that shifting to a nocturnal niche would be a prerequisite leading to the evolution of dark eyes in owls. The specific evolutionary pathway by which iris coloration and activity rhythm coevolve, however, remains to be investigated further as we have found only partial support for the idea that dark irises in birds might be an adaptive feature evolved due to the selective advantage of concealment from undesired visual receptors.

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Author contributions. AP, DP, and JMA conceived the study**.** AP and ACM collected data. AP and JMA performed the analyses. AP and JMA wrote the manuscript and all the authors contributed with comments that improved the final draft.

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Supplementary material

Includes 1 table

Table S1. *Raw data used for comparative analyses on eye colour. When information for a variable originated from sources different from del Hoyo et al. (1999), Konig and Weick (2008), and Mikkola (2014), it was denoted with a number above the data and the corresponding citation included in the list of references as a footnote.*

Species	Eye colour	Activity rhythm
Cathartes aura	dark	-
Cathartes burrovianus	bright	-
Cathartes melambrotus	dark	-
Coragyps atratus	dark	-
Sarcoramphus papa	bright	-
Gymnogyps californianus	dark	-
Pandion haliaetus	bright	-
Aviceda cuculoides	bright	-
Aviceda madagascariensis	bright	-
Aviceda jerdoni	bright	-
Aviceda subcristata	bright	-
Aviceda leuphotes	dark	-
Leptodon cayanensis	bright	-
Leptodon forbesi	bright	-
Chondrohierax uncinatus	bright	-
Chondrohierax wilsonii	bright	-
Henicopernis longicauda	bright	-
Henicopernis infuscatus	bright	-
Pernis apivorus	bright	-
Pernis celebensis	bright	-
Lophoictinia isura	bright	-
Hamirostra melanosternon	dark	-
Elanoides forficatus	dark	-
Macheiramphus alcinus	bright	-
Gampsonyx swainsonii	dark	-
Elanus caeruleus	bright	-
Elanus axillaris	bright	-
Elanus leucurus	bright	-
Elanus scriptus	bright	-
Chelictinia riocourii	bright	-
Rostrhamus sociabilis	bright	-
Helicolestes hamatus	bright	-
Harpagus bidentatus	bright	-
Harpagus diodon	bright	-
Ictinia mississippiensis	bright	-
Ictinia plumbea	bright	-
Milvus milvus	bright	-
Milvus migrans	dark	-
Haliastur sphenurus	dark	-
Haliastur indus	dark	-

Haliaeetus leucogaster	dark	-
Haliaeetus sanfordi	dark	-
Haliaeetus vocifer	dark	-
Haliaeetus vociferoides	dark	-
Haliaeetus leucoryphus	bright	-
Haliaeetus albicilla	bright	-
Haliaeetus leucocephalus	bright	-
Haliaeetus pelagicus	bright	-
Ichthyophaga humilis	bright	-
Ichthyophaga ichthyaetus	bright	-
Gypohierax angolensis	bright	-
Gypaetus barbatus	bright	-
Neophron percnopterus	dark	-
Necrosyrtes monachus	dark	-
Gyps africanus	dark	-
Gyps bengalensis	dark	-
Gyps indicus	dark	-
Gyps tenuirostris	dark	-
Gyps himalayensis	dark	-
Gyps fulvus	dark	-
Gyps coprotheres	bright	-
Aegypius monachus	dark	-
Torgos tracheliotos	dark	-
Trigonoceps occipitalis	dark	-
Circaetus gallicus	bright	-
Circaetus beaudouini	bright	-
Circaetus pectoralis	bright	-
Circaetus cinereus	bright	-
Circaetus fasciolatus	bright	-
Circaetus cinerascens	bright	-
Terathopius ecaudatus	dark	-
Spilornis cheela	bright	-
Spilornis klossi	bright	-
Spilornis kinabaluensis	bright	-
Spilornis rufipectus	bright	-
Spilornis holospilus	bright	-
Spilornis elgini	bright	-
Eutriorchis astur	bright	-
Circus ranivorus	bright	-
Circus spilonotus	bright	-
Circus approximans	bright	-
Circus maillardi	bright	-
Circus macrosceles	bright	-
Circus buffoni	bright	-
Circus assimilis	bright	-
Circus maurus	bright	-

Circus cyaneus	bright	-
Circus cinereus	bright	-
Circus macrourus	bright	-
Circus melanoleucos	bright	-
Circus pygargus	bright	-
Polyboroides typus	dark	-
Polyboroides radiatus	dark	-
Kaupifalco monogrammicus	dark	-
Melierax metabates	dark	-
Melierax poliopterus	dark	-
Melierax canorus	dark	-
Melierax gabar	dark	-
Accipiter poliogaster	bright	-
Accipiter trivirgatus	bright	-
Accipiter griseiceps	bright	-
Accipiter toussenelii	bright	-
Accipiter tachiro	bright	-
Accipiter castanilius	bright	-
Accipiter badius	bright	-
Accipiter butleri	bright	-
Accipiter brevipes	dark	-
Accipiter francesiae	bright	-
Accipiter trinotatus	dark	-
Accipiter novaehollandiae	dark	-
Accipiter fasciatus	bright	-
Accipiter melanochlamys	bright	-
Accipiter albogularis	bright	-
Accipiter haplochrous	dark	-
Accipiter rufitorques	bright	-
Accipiter henicogrammus	bright	-
Accipiter luteoschistaceus	bright	-
Accipiter imitator	bright	-
Accipiter poliocephalus	dark	-
Accipiter princeps	bright	-
Accipiter superciliosus	bright	-
Accipiter erythropus	bright	-
Accipiter collaris	bright	-
Accipiter minullus	bright	-
Accipiter gularis	bright	-
Accipiter virgatus	bright	-
Accipiter nanus	bright	-
Accipiter erythrauchen	bright	-
Accipiter cirrocephalus	bright	-
Accipiter brachyurus	bright	-
Accipiter rhodogaster	bright	-
Accipiter madagascariensis	bright	-

Accipiter ovampensis	bright	-
Accipiter nisus	bright	-
Accipiter rufiventris	bright	-
Accipiter striatus	bright	-
Accipiter chionogaster	bright	-
Accipiter ventralis	bright	-
Accipiter erythronemius	bright	-
Accipiter cooperii	bright	-
Accipiter gundlachi	bright	-
Accipiter bicolor	bright	-
Accipiter chilensis	bright	-
Accipiter henstii	bright	-
Accipiter gentilis	bright	-
Accipiter meyerianus	bright	-
Erythrotriorchis buergersi	bright	-
Erythrotriorchis radiatus	bright	-
Megatriorchis doriae	bright	-
Butastur rufipennis	bright	-
Butastur teesa	bright	-
Butastur liventer	bright	-
Butastur indicus	bright	-
Geranospiza caerulescens	bright	-
Leucopternis plumbeus	bright	-
Leucopternis schistaceus	bright	-
Leucopternis princeps	dark	-
Leucopternis melanops	bright	-
Leucopternis kuhli	dark	-
Leucopternis semiplumbeus	bright	-
Leucopternis albicollis	dark	-
Leucopternis occidentalis	dark	-
Leucopternis polionotus	dark	-
Buteogallus aequinoctialis	dark	-
Buteogallus anthracinus	dark	-
Buteogallus urubitinga	dark	-
Buteogallus meridionalis	bright	-
Buteogallus gundlachii	dark	-
Parabuteo unicinctus	dark	-
Busarellus nigricollis	dark	-
Geranoaetus melanoleucus	dark	-
Harpyhaliaetus solitarius	bright	-
Harpyhaliaetus coronatus	bright	-
Buteo magnirostris	bright	-
Buteo lineatus	dark	-
Buteo ridgwayi	bright	-
Buteo leucorrhous	bright	-
Buteo brachyurus	dark	-

Buteo albigula	dark	-
Buteo swainsoni	dark	-
Buteo albicaudatus	dark	-
Buteo galapagoensis	dark	-
Buteo polyosoma	dark	-
Buteo albonotatus	dark	-
Buteo solitarius	dark	-
Buteo jamaicensis	dark	-
Buteo ventralis	dark	-
Buteo buteo	dark	-
Buteo oreophilus	dark	-
Buteo brachypterus	bright	-
Buteo hemilasius	bright	-
Buteo regalis	bright	-
Buteo lagopus	dark	-
Buteo auguralis	dark	-
Buteo augur	dark	-
Buteo archeri	dark	-
Buteo rufofuscus	dark	-
Harpia harpyja	dark	-
Harpyopsis novaeguineae	dark	-
Pithecophaga jefferyi	bright	-
Ictinaetus malayensis	dark	-
Nisaetus floris	bright	-
Aquila pomarina	dark	-
Aquila hastata	dark	-
Aquila clanga	dark	-
Aquila rapax	bright	-
Aquila nipalensis	dark	-
Aquila adalberti	dark	-
Aquila heliaca	dark	-
Aquila wahlbergi	dark	-
Aquila gurneyi	bright	-
Aquila chrysaetos	dark	-
Aquila audax	dark	-
Aquila verreauxii	dark	-
Aquila fasciatus	bright	-
Hieraaetus spilogaster	bright	-
Hieraaetus pennatus	dark	-
Hieraaetus morphnoides	dark	-
Hieraaetus ayresii	bright	-
Hieraaetus weiskei	dark	-
Lophotriorchis kienerii	dark	-
Polemaetus bellicosus	bright	-
Spizaetus melanoleucus	bright	-
Lophaetus occipitalis	bright	-

Nisaetus cirrhatus	bright	-
Nisaetus nipalensis	bright	-
Nisaetus alboniger	bright	-
Nisaetus bartelsi	bright	-
Nisaetus lanceolatus	bright	-
Nisaetus philippensis	bright	-
Nisaetus nanus	bright	-
Spizaetus tyrannus	bright	-
Spizaetus ornatus	bright	-
Stephanoaetus coronatus	bright	-
Spizaetus isidori	bright	-
Sagittarius serpentarius	dark	-
Daptrius ater	dark	-
Ibycter americanus	dark	-
Phalcoboenus carunculatus	dark	-
Phalcoboenus megalopterus	dark	-
Phalcoboenus albogularis	dark	-
Phalcoboenus australis	dark	-
Caracara plancus	dark	-
Caracara cheriway	dark	-
Milvago chimachima	dark	-
Milvago chimango	dark	-
Herpetotheres cachinnans	dark	-
Micrastur ruficollis	bright	-
Micrastur plumbeus	bright	-
Micrastur gilvicollis	bright	-
Micrastur mintoni	bright	-
Micrastur mirandollei	bright	-
Micrastur semitorquatus	dark	-
Micrastur buckleyi	dark	-
Spiziapteryx circumcincta	bright	-
Polihierax semitorquatus	dark	-
Polihierax insignis	dark	-
Microhierax caerulescens	dark	-
Microhierax fringillarius	dark	-
Microhierax latifrons	dark	-
Microhierax erythrogenys	dark	-
Microhierax melanoleucos	dark	-
Falco naumanni	dark	-
Falco tinnunculus	dark	-
Falco newtoni	dark	-
Falco punctatus	dark	-
Falco araea	dark	-
Falco moluccensis	dark	-
Falco cenchroides	dark	-
Falco sparverius	dark	-

Falco rupicoloides	bright	-
Falco alopex	dark	-
Falco ardosiaceus	dark	-
Falco dickinsoni	dark	-
Falco zoniventris	bright	-
Falco chicquera	dark	-
Falco vespertinus	dark	-
Falco amurensis	dark	-
Falco eleonorae	dark	-
Falco concolor	dark	-
Falco femoralis	dark	-
Falco columbarius	dark	-
Falco rufigularis	dark	-
Falco deiroleucus	dark	-
Falco subbuteo	dark	-
Falco cuvierii	dark	-
Falco severus	dark	-
Falco longipennis	dark	-
Falco novaeseelandiae	dark	-
Falco berigora	dark	-
Falco hypoleucos	dark	-
Falco subniger	dark	-
Falco biarmicus	dark	-
Falco jugger	dark	-
Falco cherrug	dark	-
Falco rusticolus	dark	-
Falco mexicanus	dark	-
Falco peregrinus	dark	-
Falco pelegrinoides	dark	-
Falco fasciinucha	dark	-
Tyto alba	dark	diurnal
Tyto glaucops	dark	nocturnal
Tyto soumagnei	dark	nocturnal
Tyto aurantia	dark	nocturnal
Tyto nigrobrunnea	dark	nocturnal[1]
Tyto longimembris	dark	diurnal
Tyto capensis	dark	nocturnal
Tyto sororcula	dark	nocturnal
Tyto novaehollandiae	dark	nocturnal
Tyto manusi	dark	nocturnal
Tyto rosenbergii	dark	diurnal
Tyto inexspectata	dark	nocturnal
Tyto tenebricosa	dark	nocturnal
Phodilus prigoginei	dark	nocturnal
Phodilus badius	dark	nocturnal
Otus sagittatus	dark	nocturnal

Otus rufescens	dark	nocturnal
Otus thilohoffmanni	bright	diurnal
Otus icterorhynchus	bright	diurnal
Otus ireneae	bright	nocturnal
Otus balli	bright	diurnal
Otus alfredi	bright	diurnal
Otus spilocephalus	bright	diurnal
Otus angelinae	bright	nocturnal
Otus mirus	dark	nocturnal
Otus longicornis	bright	nocturnal
Otus mindorensis	bright	nocturnal
Otus hartlaubi	bright	diurnal
Otus rutilus	bright	diurnal
Otus mayottensis	bright	nocturnal
Otus pauliani	bright	diurnal
Otus capnodes	bright	diurnal
Otus moheliensis	bright	nocturnal
Otus pembaensis	bright	diurnal
Otus scops	bright	nocturnal
Otus brucei	bright	diurnal
Otus senegalensis	bright	nocturnal
Otus sunia	bright	diurnal
Otus elegans	bright	nocturnal
Otus magicus	bright	diurnal
Otus beccarii	bright	nocturnal
Otus manadensis	bright	diurnal
Otus siaoensis	bright	diurnal
Otus collari	bright	nocturnal
Otus mantananensis	bright	nocturnal
Otus insularis	bright	nocturnal
Otus alius	bright	nocturnal
Otus umbra	bright	nocturnal
Otus enganensis	bright	nocturnal
Otus mentawi	dark	nocturnal
Otus brookii	bright	-
Otus lempiji	dark	nocturnal[2]
Otus lettia	dark	nocturnal
Otus bakkamoena	dark	nocturnal
Otus semitorques	bright	nocturnal
Otus megalotis	dark	nocturnal
Otus fuliginosus	dark	-
Otus silvicola	bright	nocturnal
Otus flammeolus	dark	diurnal
Otus leucotis	bright	diurnal
Otus podarginus	dark	diurnal
Megascops kennicottii	bright	diurnal

Megascops asio	bright	diurnal
Megascops cooperi	bright	diurnal
Megascops trichopsis	bright	diurnal
Megascops barbarus	bright	nocturnal
Megascops seductus	dark	nocturnal
Megascops clarkii	bright	diurnal
Megascops choliba	bright	diurnal
Megascops koepckeae	bright	nocturnal
Megascops roboratus	bright	diurnal
Megascops hoyi	bright	diurnal
Megascops ingens	dark	diurnal
Megascops colombianus	dark	nocturnal
Megascops petersoni	dark	nocturnal
Megascops marshalli	dark	nocturnal
Megascops watsonii	bright	diurnal
Megascops atricapilla	dark	diurnal
Megascops sanctaecatarinae	bright	diurnal
Megascops guatemalae	bright	nocturnal
Megascops nudipes	bright	diurnal
Megascops albogularis	bright	diurnal
Gymnoglaux lawrencii	dark	diurnal
Ptilopsis granti	bright	nocturnal
Mimizuku gurneyi	dark	nocturnal
Bubo scandiaca	bright	nocturnal
Bubo virginianus	bright	diurnal
Bubo magellanicus	bright	diurnal
Bubo bubo	bright	diurnal
Bubo ascalaphus	bright	diurnal
Bubo bengalensis	bright	diurnal
Bubo capensis	bright	diurnal
Bubo africanus	bright	diurnal
Bubo cinerascens	dark	diurnal
Bubo poensis	dark	diurnal
Bubo vosseleri	bright	diurnal
Bubo lacteus	dark	diurnal
Bubo shelleyi	dark	diurnal
Bubo sumatranus	dark	nocturnal
Bubo nipalensis	dark	nocturnal
Bubo coromandus	bright	diurnal
Bubo leucostictus	bright	diurnal
Bubo philippensis	bright	diurnal
Ketupa blakistoni	bright	diurnal
Ketupa zeylonensis	bright	diurnal
Ketupa ketupu	bright	diurnal
Ketupa flavipes	bright	diurnal
Scotopelia peli	dark	diurnal

Scotopelia ussheri	dark	diurnal
Scotopelia bouvieri	dark	diurnal
Strix seloputo	dark	nocturnal
Strix ocellata	dark	diurnal
Strix leptogrammica	dark	nocturnal
Strix aluco	dark	diurnal
Strix butleri	bright	diurnal
Strix woodfordii	dark	nocturnal
Strix virgata	dark	diurnal
Strix rufipes	dark	diurnal
Strix chacoensis	dark	diurnal
Strix hylophila	dark	diurnal
Strix albitarsis	dark	nocturnal
Strix nigrolineata	dark	nocturnal
Strix huhula	dark	nocturnal
Strix occidentalis	dark	diurnal
Strix fulvescens	dark	nocturnal
Strix varia	dark	nocturnal
Strix davidi	dark	diurnal
Strix uralensis	dark	diurnal
Strix nebulosa	bright	diurnal
Jubula lettii	bright	diurnal
Lophostrix cristata	dark	diurnal
Pulsatrix perspicillata	bright	nocturnal
Pulsatrix koeniswaldiana	dark	nocturnal
Pulsatrix melanota	dark	nocturnal
Surnia ulula	bright	diurnal
Glaucidium passerinum	bright	diurnal
Glaucidium perlatum	bright	diurnal
Glaucidium tephronotum	bright	diurnal
Glaucidium brodiei	bright	diurnal
Glaucidium californicum	bright	diurnal
Glaucidium gnoma	bright	diurnal
Glaucidium nubicola	bright	diurnal
Glaucidium costaricanum	bright	diurnal
Glaucidium siju	bright	diurnal
Glaucidium sanchezi	bright	diurnal
Glaucidium palmarum	bright	diurnal
Glaucidium griseiceps	bright	diurnal
Glaucidium minutissimum	bright	diurnal
Glaucidium hardyi	bright	diurnal
Glaucidium parkeri	bright	diurnal
Glaucidium jardinii	bright	diurnal
Glaucidium bolivianum	bright	diurnal
Glaucidium peruanum	bright	diurnal
Glaucidium nanum	bright	diurnal

Glaucidium brasilianum	bright	diurnal
Glaucidium mooreorum	bright	-
Glaucidium sjostedti	bright	diurnal
Glaucidium cuculoides	bright	diurnal
Glaucidium castanopterum	bright	diurnal
Glaucidium radiatum	bright	diurnal
Glaucidium castanonotum	bright	diurnal
Glaucidium capense	bright	diurnal
Glaucidium castaneum	bright	diurnal
Glaucidium albertinum	bright	diurnal
Xenoglaux loweryi	bright	diurnal
Micrathene whitneyi	bright	diurnal
Heteroglaux blewitti	bright	diurnal
Athene noctua	bright	diurnal
Athene brama	bright	diurnal
Athene cunicularia	bright	diurnal
Aegolius funereus	bright	diurnal
Aegolius acadicus	bright	nocturnal
Aegolius ridgwayi	dark	nocturnal
Aegolius harrisii	bright	nocturnal
Ninox rufa	bright	diurnal
Ninox strenua	bright	diurnal
Ninox connivens	bright	diurnal
Ninox rudolfi	dark	diurnal
Ninox boobook	bright	diurnal
Ninox novaeseelandiae	bright	diurnal
Ninox scutulata	bright	diurnal
Ninox affinis	bright	diurnal
Ninox superciliaris	dark	diurnal
Ninox philippensis	bright	nocturnal
Ninox ochracea	bright	nocturnal
Ninox jacquinoti	bright	nocturnal
Ninox theomacha	bright	nocturnal
Ninox punctulata	dark	nocturnal
Ninox odiosa	bright	nocturnal
Ninox squamipila	bright	diurnal
Ninox ios	bright	nocturnal
Ninox burhani	bright	diurnal
Ninox sumbaensis	bright	nocturnal
Ninox natalis	bright	diurnal
Ninox meeki	bright	-
Ninox variegata	bright	nocturnal
Uroglaux dimorpha	bright	-
Nesasio solomonensis	bright	nocturnal
Pseudoscops grammicus	dark	nocturnal
Pseudoscops clamator	dark	diurnal

Asio stygius	bright	nocturnal
Asio otus	bright	diurnal
Asio abyssinicus	bright	nocturnal
Asio madagascariensis	dark	nocturnal
Asio flammeus	bright	diurnal
Asio capensis	dark	diurnal

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SECTION 3:

IRIS COLOUR AS AN INDICATOR

OF INDIVIDUAL QUALITY

Chapter IV

Iris yellowness relates to age and individual quality in two owl species

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ABSTRACT

Birds show huge variation in colour displays evolved for communication. However, among coloured phenotypic traits, eyes remain largely overlooked, with only a few studies suggesting a potential signaling function or a role in mate recognition and crypsis. Iris colour is a remarkably striking feature in the wholly cryptic pattern of many owls, and may potentially play a signaling function, a possibility so far neglected. Here, we studied variation and potential signaling of iris yellowness as an indicator of quality in parent-offspring communication and other social contexts in Little Owl *Athene noctua* and Eurasian Scops-Owl *Otus scops*. Yellowness did not differ between the sexes; however, adults of the two species had more intensely coloured yellow irises than owlets. Most of variation in iris yellowness of owlets occurred between rather than within nests and seemed to be linked to parental qualities in Little Owls, but was unrelated with condition among Eurasian Scops-Owl owlets. In adults, however, we found that iris yellowness of females was positively associated with nest success (an index of female fitness) in Little owls, but not in Eurasian Scops-Owls. This study suggests that iris colour variation is unlikely to play a role in a parent-offspring communication in these two owl species, but that iris yellowness in female Little Owls may potentially play a signaling role in social contexts, a possibility that needs to be studied in the future.

El color amarillo del iris se relaciona con la edad y la calidad de los individuos en dos especies de búhos

RESUMEN

Las aves muestran una gran variabilidad en los patrones de color implicados en la comunicación. Sin embargo, entre los rasgos coloreados, pocos estudios han investigado la función del color de los ojos, habiéndose sugerido un posible papel en la señalización, en el reconocimiento entre parejas o en el camuflaje. El color del iris es un rasgo muy llamativo en el diseño mayoritariamente críptico de muchas especies de búhos y, por tanto, podría jugar un papel en la comunicación basada en señales cromáticas, una posibilidad aún no suficientemente explorada en este grupo de aves. En este trabajo, estudiamos la variación en la coloración amarilla del iris en el mochuelo europeo *Athene noctua* y el autillo europeo *Otus scops* para comprobar si este rasgo podría ser un indicador la calidad de los individuos en un contexto de comunicación paterno-filial y/o en otro contexto social. El color del iris no difiere entre los dos sexos en las dos especies, pero los adultos exhiben coloraciones amarillas más intensas que los pollos. En el mochuelo, la variabilidad en el color del iris de los pollos se da más entre nidos que dentro de ellos, y el color amarillo del iris se relaciona con la calidad de los padres. En los pollos de autillo no encontramos ninguna relación. En los adultos, encontramos una relación positiva entre éxito del nido y el color del iris en las hembras de mochuelo, pero no en autillos. Este estudio sugiere que es improbable que la coloración amarilla del iris actúe en la comunicación entre padres e hijos en estas dos especies, no obstante, el color del iris podría desempeñar un papel en un contexto social en el mochuelo europeo que es necesario explorar más en el detalle en estudios futuros.

INTRODUCTION

Animals have extraordinary variability in chromatic patterns evolved in communicative contexts. For instance in birds, a massive body of empirical evidence has shown that individual birds can assess the quality of others by using information encoded in plumage and/or the colour bare patches (e.g. Hill and McGraw 2006, Kilner 2006, Velando et al. 2006, Avilés and Parejo 2013). Surprisingly, although avian eyes are often remarkably conspicuous, and hence potentially detectable by visually based receivers (Cott 1940), the possible role of iris colour in communication has been mostly neglected, probably due to a poor understanding of the mechanisms underlying iris colour variation (Prum 2006).

Iris coloration varies across avian species and may range from the dark colours or black found in many species to the vividly yellow, orange or red colored eyes of some passerines and owls (Davidson et al. 2017, Passarotto et al. 2018). Iris colour variation is also present among populations of the same species (del Hoyo et al. 2001), or between individuals within the same population (Snyder and Snyder 1974, Picozzi 1981, Scholten 1999). Indeed, in diurnal species, iris colour may differ between sexes (Snyder and Snyder 1974, Bortolotti et al. 2003), change with age (Snyder and Snyder 1974, Picozzi 1981, Sweijd and Craig 1991, Wilson and Hartley 2006), and/or correlate with breeding success (Newton and Marquiss 1982). In addition, the grayish eye colour of Eurasian Jackdaws (*Corvus monedula*) may funtion as a warning signal toward conspecifics, deterring intrusions (Davidson et al. 2014). All of these examples suggest that avian iris coloration, at least in diurnal birds, may potentially serve a communicative role (but see Negro et al. 2017).

Iris colour is a candidate trait for pigment-based signaling in birds. The brightness of avian irises is due to structural elements (i.e., stromal purine crystals in reflecting organelles or superficial blood vessels) as well as pigments (Ferris and Bagnara 1972, Oliphant 1987, Oliphant and Hudon 1993). Chromatographic analyses have revealed that pteridines (also called pterines) and purines are the most widely distributed pigments in the irises of 28 bird species with bright coloured eyes,

although carotenoids were also present in families such as *Strigidae, Ardeidae* or *Anatidae* (Oliphant 1987). High-performance liquid chromatography has shown that the absolute and relative amounts of light-absorbing compounds in the iris varies among species of blackbirds, and markedly within one species between sexes and age classes that vary in eye colour (Hudon and Muir 1996).

Among pigments coloring avian irises, carotenoids cannot be synthesized *de novo* and must be ingested in the diet; therefore, carotenoid colours may potentially provide information about an individual's capacity to acquire food rich in carotenoids and to assimilate and process those nutrients (Hill 1991). Carotenoids also have important antioxidant and inmunostimulant properties (Møller et al. 2000), which may also confer honesty to colour signals (von Schantz et al. 1999). Indeed, in humans, low plasma lutein and zeaxanthin concentrations or dietary intake are associated with low macular pigment density and increased risk of age-related macular degeneration (Semba and Dagnelie 2003). Eye colour in humans is also a cue for the perception of age, health and attractiveness (Russell et al. 2014). Pterines may play analogous physiological functions, as immune cells are known to stimulate the release of pteridines that act as oxidative stress reducers during inflammation (McGraw 2005). Hence pterines might potentially be involved in physiological trade-offs to solve the challenge of allocating resources to either immunity or to coloration (Grether et al. 2001, Weiss et al. 2011).

Here we aim to study for the first time whether yellow iris coloration may play a signaling function in two owl species with brightly pigmented irises, the Little Owl (*Athene noctua*) and the Eurasian Scops-Owl (*Otus scops*). In Northern Saw-whet Owls (*Aegolius acadicus*), eye colour and body condition, based on fat and keel scores, were strongly correlated, suggesting that iris colour might potentially provide information about individual quality (Wails et al. 2018). In mammals, eye colour provides detectable colour cues for health (e.g. Kaplan et al. 2004), a possibility so far neglected for birds. Little Owls and Eurasian Scops-Owls both potentially could use eye chromatic information revealing variation in quality, given that variation in coloration of the bill and cere might

play a role in mate choice for Little Owls (Avilés and Parejo 2012) and in parent-offspring communication for both Eurasian Scops and Little Owls (Parejo et al. 2010, Avilés and Parejo 2013).

Here we report a two-stage study. In the first stage, we tested for age and sex differences in iris colour of the two owl species. In the second stage, we tested whether yellow iris coloration of owlets may potentially function as a chromatic stimulus revealing aspects of offspring quality to parents. To achieve this latter goal, we assessed owlet iris colour variation between and within nests, and studied its covariation with owlet quality (as indexed by body size). The possibility that iris coloration serves a communication function predicts that (1) eye coloration will vary more among siblings than among owlets raised in different nests, and that (2) iris coloration will covary with quality of owlets within nests. Finally, aiming to test for a potential role of iris colour in social contexts, we analyzed whether iris colour of breeding individuals covaried with individual quality (as indexed by size, condition, and reproductive outcome) in adult birds.

MATERIAL AND METHODS

Study system

We collected data in the region surrounding Baza (37°18' N, 3°11' W), southeastern Spain, in April-July during two years (2017-2018) in the context of a long-term monitoring program to study the evolution and maintenance of plumage colour polymorphism of small cavity-nesting owls (Parejo et al. 2018). Vegetation of the study area is scattered holm oak (*Quercus ilex*) forest interspersed with cereal fields; most Little Owls and scops-owls breed in cork nest boxes installed on trees (see Parejo and Avilés 2011 and Rodríguez et al. 2011 for details).

In April each year, we visited nest boxes once per week until we noted the presence of an owl egg, and we recorded the approximate laying date. After that, we visited the nests every second day until clutch completion, when we trapped females and recorded both clutch size and egg size (i.e., length and width in mm). Based on egg width and length, we calculated egg volume using Hoyt's

equation (Hoyt 1979). We trapped males at nests of the two species after the young had hatched (Parejo et al. 2018), and monitored nests regularly to record hatch date, the number of hatchlings, and the number of fledglings. Based on this information, we calculated nest success (defined as the percentage of fledglings relative to number of hatched eggs) and breeding success (defined as the percentage of fledglings relative to number of laid eggs). Because laying date and hatching date were strongly correlated for Little Owls ($r_s = 0.95$, $n = 16$, $P < 0.0001$) and Eurasian Scops-Owls ($r_s = 0.92$, $n = 33$, $P < 0.0001$), we used only hatching date in our analyses because it was more precisely estimated than laying date.

Field data collection

We collected data on owlet characteristics when the oldest young in each nest were 20-21 days old. Upon capture, each Little Owl and Eurasian Scops-Owl adult and owlet was individually marked with a metal ring, weighed with a Pesola spring balance to the nearest 0.5 g, and measured with a ruler (wing) and a caliper (i.e., bill and tarsus) to the nearest 1 mm. For eye colour determination, we photographed the faces of all individuals with a flash (aperture: 4.5, shutter speed: 1/200, ISO: 800) 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. During photography, owls were gently placed in a harness inside a neutral-coloured box that ensured stable light conditions, with the head placed next to a colour chart (X-Rite ColorChecker® Passport, Grand Rapids, Michigan, United States).

For genetic sexing of owlets, we collected a drop of blood by brachial venipuncture. We sexed adults based on the presence/absence of a brood patch, which only develops in females (Parejo et al. 2018). Genetic sex determination of a sample of adults indicated that the assessment of sex by the presence/absence of a brood patch is 100% reliable in our population (J.M. Avilés unpubl. data).

Owlet sex determination

Owlets were genetically sexed using a polymerase chain reaction (PCR) amplification based on the technique used by Fridolfsson and Ellegren (1999). DNA was isolated from the red blood cells by processing samples in 100 µl of 50 mM NaOH for 20 min in a thermocycler. PCR amplification was performed in 20 µl volumes on an Eppendorf Mastercycler Nexus. Final concentrations were: 5 mM MgCl2, 0.2 mM dNTPs (each; Bioron GmbH), 1µM (each) 2550F/2718R primers (Fridolfsson and Ellegren 1999), 0.098 mg/ml BSA (Amersham Biosciences), 0.5 U Taq DNA polymerase (Bioron GmbH) and 1 µl raw extract. The thermal profile used was: 94ºC for 2 min, 55ºC for 30 sec, 72ºC for 1 min, followed by 36 cycles (92ºC for 30 sec, 52ºC for 30 sec, 72ºC for 45 sec), and a final 72ºC for 5 min step. PCR products were separated in 2% agarose gels run in standard TE buffer and visualized by SyBRSafe (Invitrogen) staining. Except for three Little Owl nestlings for which it was not possible to determine the sex, all the other owlets were successfully sexed.

Iris colour quantification

Photos of owl faces were standardized using the Adobe® Photoshop Lightroom 6 plugin and analyzed using the R package colorZapper (Valcu and Dale 2014). From each photograph, a stratified random sample of colour of the iris of each eye was obtained by dividing the iris in two bands around the transverse axis of the eye. RGB components (i.e., the intensity of red, green and blue primary colours) were measured three times in each of these two bands. Repeatability analyses revealed high consistency in RGB values obtained from the three measurements within each band (R–$F_{43,91}$ = 22.52, R= 0.88, P < 0.001; G–$F_{43,91}$ = 49.88, R= 0.94, P < 0.001; B–$F_{43,91}$ = 29.53, R= 0.90, P < 0.001), so we averaged values derived from the three measurements. Subsequently, we estimated repeatability within the eye on the averaged values for each band and found that it was very high for Little Owls (R–$F_{15,\ 48}$ = 32.99, R= 0.89, P < 0.001; G–$F_{15,\ 48}$ = 26.04, R= 0.86, P < 0.001; B–$F_{15,\ 48}$ = 48.33, R= 0.92, P < 0.001), but not for Eurasian Scops-Owls, (R–$F_{19,100}$ = 8.28, R= 0.55, P < 0.001; G–$F_{19,100}$ = 6.66, R= 0.49, P < 0.001; B–$F_{19,100}$ = 1.60, R= 0.09, P = 0.07). For this reason, we decided to consider

only the upper part of the iris in Eurasian Scops-Owls. Average values obtained for the left and right eye proved to be repeatable in both species (Little Owl: R–$F_{27,32}$ = 30.88, R= 0.93, P <0.001; G–$F_{27,32}$ = 13.35, R= 0.85, P < 0.001; B–$F_{27,32}$ = 19.80, R= 0.92, P <0.001; Eurasian Scops-Owl: R–$F_{29,32}$ = 8.61, R= 0.79, P < 0.001; G–$F_{29,32}$ = 7.57, R= 0.76, P <0.001; B–$F_{29,32}$ = 6.02, R= 0.71, P <0.001), indicating that the two eyes had the same coloration.

To quantify the intensity of yellow iris coloration, we calculated a chromatic index (hereafter yellowness) by dividing the value of the R component by the average value of the three RGB components (Villafuerte and Negro 1998). Higher values of yellowness are associated with more intense and saturated yellow tending to orange, whereas lower values would correspond with greenish or grayish yellow. Yellowness is commonly used to study carotenoid-based signals, often referred as to "redness". Though we are unsure about the pigments coloring the iris in Little and Eurasian Scops-Owls, yellowness can be successfully applied to other yellow carotenoid-like pigmentations as an index of bright colour intensity without speculating on the true nature of pigmentation (Andersson and Prager 2006).

Statistical analyses

Analyses were conducted using STATISTICA 7 (StatSoft Inc. 2005). In a first stage, we studied iris yellowness variation relative to age and sex in the two owl species. For that purpose, we performed two linear mixed models (hereafter LMMs; normal error distribution and identity function) in which yellowness was the dependent variable, and age and sex were considered as fixed factors while study year was a random factor. We could not include nest as a random factor in these two models because many nests (50 % of Scops-Owl nests and 38.5 % of Little Owl nests) had only a single sampled owlet (either because only one owlet survived long enough to photograph [age 20–21 d], or because the photos for some owlets lacked sufficient quality). However, we also ran two LMMs using only one randomly selected owlet per nest and found that the patterns detected were qualitatively identical

to those based on all owlets (see Tables S1), suggesting that pseudoreplication had a negligible effect on our results.

In a second stage, we investigated the potential signaling role of iris coloration in parent-offspring communication. We estimated the percentage of total variation in owlet yellowness within nests compared to variation between nests from ANOVA means of squares using a variance method (see Avilés and Parejo 2013). Afterwards, to ascertain the potential informative content of iris coloration within nests, we ran two LMMs (one for each species) with mass as the dependent variable and iris yellowness as predictor. Tarsus length was entered as a predictor to control for differences in owlet body size and nest ID was considered as a random factor to account for the non-independence of owlets from the same nest. In addition, to control for possible sexual differences in size, we entered sex and the interaction between sex and tarsus in the two models. Finally, to explore whether between-nest variation in iris yellowness of owlets reflected parental qualities, we ran Spearman's correlations between the average yellowness of each nest and hatching date, clutch size, egg volume, breeding success and nest success. We opted to use average yellowness in these analyses due to a low variation within nests in the number of sampled owlets, which precluded LMMs with the nest as a random factor.

To test the potential of iris colour in predicting body condition for adults, we performed a LMM in which body weight was the dependent variable and yellowness and tarsus length were entered as two covariates, while year was a random factor. Sex and the interaction between sex and tarsus length were also included to control for possible sexual size dimorphism. Finally, we studied examined female eye yellowness relative to reproductive variables (i.e. clutch size, egg volume, hatching date and nest success) by using Spearman´s rank correlations. We did not test this association for adult males due to small sample size (Little Owl: $n = 7$; Scops Owl: $n = 11$).

Among Little Owls, we found some relationships between owlet iris yellowness and reproductive parameters (as index to fitness; see Results) that might have arisen from different

feeding efforts and/or diet composition related to different breeding periods. To test this possibility, we used recordings of feeding behavior to determine diet and feeding time. When the first-hatched owlet of each nest reached 8 d old, we placed a small infrared camera in the upper part of the nest box, carefully hidden to avoid interfering with feeding activity. The device was set to automatically record for 90 min starting at 21:00 H. We marked females at the time of capture with a white stripe of correction fluid (which does not harm the animals; Expósito-Granados et al. 2016) on the head to facilitate determining the sex of the individual delivering prey in the recordings. Each nest was recorded only one night. We noted the type of prey, the total number of feedings per hour, and the contribution of each sex. Prey included earthworms, grasshoppers, crickets, moths, beetles, mice and small birds but centipedes (*Scolopendra* spp.) and spiders (*Lycosa* spp.) accounted for the most variation, so we assessed possible differences in diet related to breeding period by calculating the percentage of these two prey (of the total number of prey recorded for each nest). Through Spearman correlation analyses, we assessed the relationship between hatching date and the percentage of the most common prey (i.e., centipedes and spiders) as well as the number of feedings per hour and the number of prey brought by both the female and the male. As a proxy of parental effort, we also considered the time adults took to return to the nest after we installed the camera (i.e. latency). Finally, because identification of prey was not possible in several cases, we included in the analyses the percentage of successfully identified prey on the total number of feeds, to control for possible biases (Table S3).

RESULTS

We monitored 21 Little Owl nests (10 in 2017 and 11 in 2018) at which we captured 29 adults and sampled 39 owlets. On the other hand, we monitored 58 Scops Owls nests (32 in 2017 and 26 in 2018) in which we captured 79 adults and sampled 84 owlets. Because some adults (2 Little Owls and 12 scops-owls) nested in both years, we used only the first year in the analysis, to avoid pseudoreplication. In addition, some nests failed before we could assess their reproductive outcome

(Little Owl: 50.0% and 9.1% of nests in 2017 and 2018, respectively; Scops-Owl: 46.9% and 34.6% of nests in 2017 and 2018, respectively); most of these apparently failed due to predation. Finally, some photos lacked the needed quality to reliably estimate eye colour (13.2% and 16.0% of photographs of Little Owls and Eurasian Scops-Owls, respectively). Hence, for Little Owls we analyzed eye colour of 30 owlets (76.9% of sampled owlets) from 13 nests (61.9% of sampled nests; 7 fledglings in 3 nests in 2017 and 23 owlets in 10 nests in 2018) and 20 adults (69% of sampled adults; 7 in 2017 and 13 in 2018). Our sample size for scops-owls was 40 owlets (47.6% of sampled owlets; 8 owlets from 7 nests in 2017 and 32 owlets from 17 nests in 2018) and 27 adults (34.2% of sampled adults; 13 in 2017 and 14 in 2018).

Age and sex variation in iris coloration

There was extensive variation in iris yellowness in both owlets and adults of Little Owls (Fig. S2) and Eurasian Scops-Owls (Fig. S3) (Fig.1). In the two species, adults had more vividly yellow irises than owlets (Fig. 1, Table 1). However, yellowness did not differ between sexes in either of the species once the significant effect of year was taken into account (Table 1, Fig. S1).

Table 1. *Results of LMMs analyzing the effect of year, age and sex on iris yellowness in Little Owls and Eurasian Scops-Owls. Bold font indicates significant effects.*

SPECIES	DEPENDENT VARIABLE	PREDICTORS	EFFECT	COEFFICIENT	SE	df	F	*P*
Little Owl[a]	Iris yellowness	**Year**	**random**	**-0.35**	**0.07**	**1,42**	**26.77**	**< 0.0001**
	(*n* = 47)	**Age**	**fixed**	**0.99**	**0.10**	**1,42**	**176.14**	**< 0.0001**
		Sex	fixed	-0.02	0.09	1,42	2.14	0.151
		Sex*Age	fixed	-0.15	0.12	1,42	1.45	0.235
Eurasian Scops-Owl[b]	Iris yellowness	**Year**	**random**	**-0.28**	**0.10**	**1,62**	**8.80**	**0.004**
	(*n* = 67)	**Age**	**fixed**	**0.65**	**0.13**	**1,62**	**65.88**	**< 0.0001**
		Sex	fixed	-0.07	0.12	1,62	0.08	0.781
		Sex*Age	fixed	0.14	0.13	1,62	1.14	0.290

a. Adjusted $R^2 = 0.80$, $F_{4,42} = 47.39$, $P < 0.0001$

b. Adjusted $R^2 = 0.49$, $F_{4,62} = 16.95$, $P < 0.0001$

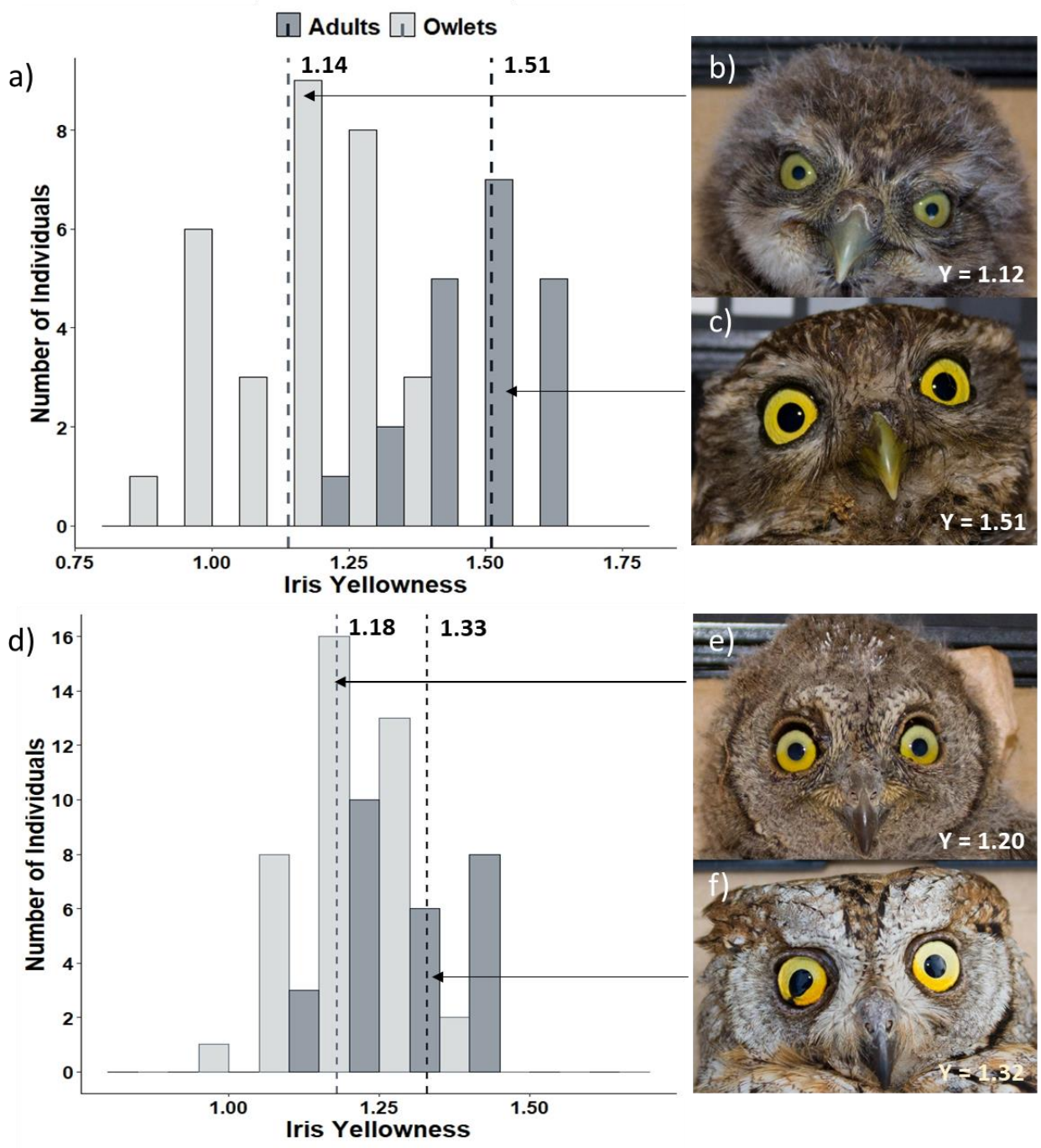

Figure 1. *Iris colour (i.e. yellowness) variation in relation to age of (a) Little Owl and (d) Eurasian Scops-Owl. The dashed lines indicate average yellowness for each age class (relative values are shown next to the lines). Photographs of owlets (b and e) and adults (c and f) of the two species show approximate average values of yellowness.*

Iris colour variation and quality in owlets

Owlet yellowness varied more between than within nests in the Little Owls ($F_{12,17} = 11.67$, $P < 0.0001$), and the total variation in yellowness within nests was 17.0%, indicating that owlets raised in the same nests tended to share similar values of yellowness. By contrast, in the Eurasian Scops-Owl, variation in iris yellowness was not larger between than within nests ($F_{23,16} = 1.34$, $R = 0.17$, $P = 0.27$).

Yellowness variation was not related to owlet body mass within nests either in Little Owls or in Scops-Owls (Table 2). Little Owl owlet yellowness was not significantly related to clutch size ($r_s = - 0.19$, $n = 13$, $P = 0.54$), egg volume ($r_s = 0.09$, $n = 13$, $P = 0.77$) or nest success ($r_s = - 0.41$, $n = 13$, $P = 0.16$). However, the average iris yellowness of Little Owl owlets was higher in later broods ($r_s = 0.60$, $n = 13$, $P = 0.032$) (Fig. 2a). Also we found a significant negative relationship between owlet yellowness and breeding success ($r_s = - 0.59$, $n = 13$, $P = 0.038$), indicating that owlets in nests with fewer fledglings had higher average yellowness (Fig. 2b). Finally we observed a near-significant inverse relationship between hatching date and breeding success ($r_s = - 0.49$, $n = 13$, $P = 0.088$), indicating that later nests may be associated with lower percentage of fledglings.

Based on videorecordings, we found that owlets of later nests received more food deliveries per hour than those in earlier nests ($r_s = 0.60$, $n = 13$, $P = 0.037$, Table S3). However, correlation analyses did not reveal significant seasonal differences in diet and parent contribution (Table S3). Nor did we find correlations among yellowness and fitness variables among Scops-Owl owlets (Table S2).

Table 2. *Results of LMMs analyzing the association between iris yellowness and body condition in Little Owl and Eurasian Scops-Owl owlets. Models include tarsus length as a covariate, sex as a fixed term and the interaction between sex and tarsus length to control for sexual differences in size, and nests as a random factor. Bold font indicates significant effects.*

SPECIES	DEPENDENT VARIABLE	PREDICTORS	EFFECT	COEFFICIENT	SE	df	F	*P*
Little Owl[a]	Body weight (*n* = 30 individuals, 13 nests)	Nest	random	-0.28	0.29	12,10	1.98	0.143
		Sex	fixed	2.16	2.81	1,10	0.59	0.459
		Iris yellowness	fixed	-0.73	0.41	1,10	3.17	0.106
		Tarsus length	**fixed**	**0.86**	**0.35**	**1,10**	**5.87**	**0.036**
		Sex*Tarsus length	fixed	-2.13	2.80	1,10	0.58	0.464
Eurasian Scops-Owl[b]	Body weight (*n* = 40 individuals, 24 nests)	Nest	random	-0.05	0.13	23,12	2.38	0.061
		Sex	fixed	3.39	2.56	1,12	1.76	0.210
		Iris yellowness	fixed	-0.07	0.13	1,12	0.29	0.601
		Tarsus length	**fixed**	**1.19**	**0.35**	**1,12**	**28.67**	**< 0.001**
		Sex*Tarsus length	fixed	-3.47	2.49	1,12	1.95	0.188

a. Adjusted $R^2 = 0.63$, $F_{16,10} = 3.74$, $P = 0.020$

b. Adjusted $R^2 = 0.80$, $F_{27,12} = 6.69$, $P < 0.001$

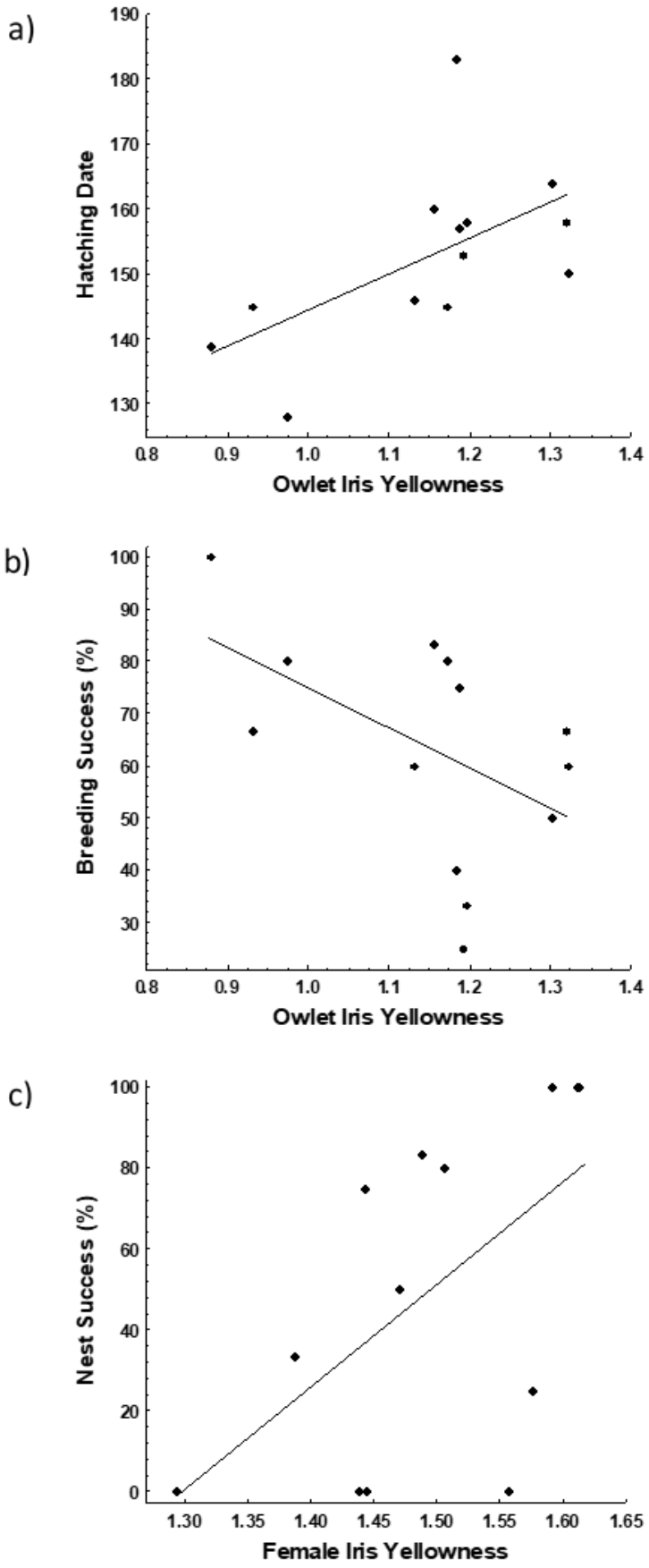

Figure 2. *Relationship between average iris yellowness of owlets and (a) hatching date (in days from 1 January) and (b) breeding success (i.e., percentage of fledglings relative to number of eggs laid) in Little Owls (n = 13 nests). Panel (c) shows the relationship between adult female iris yellowness and nest success (i.e., percentage of fledglings relative to number of hatched eggs) in Little Owls (n = 13).*

Iris colour variation and quality in adult females

Yellowness variation was not related to adult condition (i.e., body mass) either in Little Owls or in Eurasian Scops-Owls (Table 3). Although the iris yellowness of adult Little Owl females was positively associated with nest success (r_s = 0.66, n =13, P = 0.014; Fig. 2c), there was no significant relation with clutch size (r_s = 0.23, n = 13, P = 0.45), egg volume (r_s = 0.28, n = 12, P = 0.38) or hatching date (r_s = - 0.36, n = 11, P = 0.28). In Scops-Owl females, iris yellowness showed no correlation with any measure of reproductive rate (Table S2).

Table 3. *Results of LMMs analyzing the association between iris yellowness and body condition in Little Owl and Eurasian Scops-Owl adults. Models include tarsus length as a covariate, sex as a fixed term and the interaction between sex and tarsus length to control for sexual differences in size, and nest as a random factor.*

SPECIES	DEPENDENT VARIABLE	PREDICTORS	EFFECT	COEFFICIENT	SE	df	F	*P*
Little Owl[a]	Body weight (n = 20)	Year	random	-0.31	0.20	1,14	2.48	0.137
		Sex	fixed	3.72	5.82	1,14	0.41	0.533
		Iris yellowness	fixed	-0.11	0.23	1,14	0.23	0.641
		Tarsus length	fixed	0.22	0.32	1,14	0.26	0.620
		Sex*Tarsus length	fixed	-3.16	5.85	1,14	0.29	0.598
Eurasian Scops-Owl[b]	Body weight (n = 27)	Year	random	0.03	0.15	1,21	0.03	0.867
		Sex	fixed	1.07	3.57	1,21	0.09	0.767
		Iris yellowness	fixed	-0.02	0.16	1,21	0.02	0.890
		Tarsus length	fixed	1.17	0.20	1,21	0.63	0.437
		Sex*Tarsus length	fixed	-1.86	3.57	1,21	0.27	0.607

a. Adjusted R^2 = 0.38, $F_{5,14}$ = 3.36, P = 0.033

b. Adjusted R^2 = 0.64, $F_{5,21}$ =10.29, P < 0.0001

DISCUSSION

Iris colour and parent-offspring communication in owls

Our results provide weak support for a potential role of iris colour in parent-offspring communication in owls. In Little Owls, owlets raised in the same nest showed low variation in iris yellowness, and variation in owlet iris colour was not related to body condition in either Little or Scops-Owls.

Therefore, it seems unlikely that adult owls might use iris yellowness as an indicator of young's condition or rely on it to adjust their parental effort, as sometimes happens with other coloured traits of owlets such as bill and cere (Parejo et al. 2010, Avilés and Parejo 2013), or the white feathers around the mouth of the Eurasian Eagle-Owl (*Bubo bubo;* Penteriani et al. 2007). However, we cannot rule out the possibility that iris yellowness reflects aspects of owlet quality that were not considered in this study.

Interestingly, rather than indicating their own quality, owlet iris colour may be an indicator of parent quality in Little Owls, as we found owlets with more-yellow irises later in the breeding season. This apparent finding contradicts a large body of empirical evidence suggesting that individuals of better quality usually are those raised in early broods (Verboven and Verhulst 1996, Arnold 2006, Saino et al. 2012), which would predict high yellowness values in early Little Owl clutches. However, we also reported that nest-associated yellowness was inversely related to breeding success, and that breeding success tended to decrease as the season progressed, which in combination suggest that high values of iris yellowness in the late Little Owl nests might result from a higher brood reduction in those nests. Therefore, one possibility is that negative selection against owlets in the poorest condition (i.e. those with low yellowness) at the end of the breeding season resulted in an increase in average nest-associated yellowness. A mutually non-exclusive possibility is that the pattern resulted from differences in parental food allocation strategies in later clutches, as adults could distribute food among fewer owlets in these cases, which may promote an increase in yellowness due to a greater amount of food. Supporting this premise, we found that nests hatched later in the season had more frequent feedings. Owlet iris variation could possibly be related to diet, if parents of late nests "specialized" in providing types of prey richer in coloring pigments; thi was not supported in our study, however, as we found that diet did not change seasonally in our sample of nests. Finally, we cannot dismiss the possibility that differences in colour might arise because of different environmental conditions and/or exposure to contaminants inhibiting colour expression (e.g.

Bortolotti et al. 2003, García-Heras et al. 2017) experienced by the later nests compared to earlier clutches.

We found remarkable variation in iris yellowness between Scops Owl owlets raised in the same nest that was uninformative regarding owlet body condition. A previous study found that female scops-owls might be able to adjust the sex of their offspring at egg production (Blanco et al. 2001). Hence, it could be argued that in this species natural selection may favor prenatal control over subsequent parental feeding adjustments (based on iris coloration) that affect offspring survival and growth. Prenatal control, however, does not exclude parental feeding adjustments in this species; there is evidence that parents may preferentially feed lighter offspring with an experimentally UV reduced cere (Parejo et al. 2010). Another possibility is that the great difference in iris yellowness within nests was derived from a high extra-pair paternity rate in the study population, a possibility worth exploring in future studies on this species.

Iris colour in adults

Among adults, we did not find any direct relationship between iris colour and body condition; however, Little Owl females with more-yellow irises had higher nest success (i.e. fledglings per hatched eggs). Several potential mechanisms may explain this result. Firstly, covariation may be due to condition-dependence of expression of iris coloration in a signaling scenario. Two alternative interpretations are possible: a) female iris coloration is a true sexually selected trait used by females in territorial context and/or by males in mate choice to assess female quality (Amundsen 2000, Doutreland et al. 2008), or b) covariation is driven by males through the correlation between the expression of female and male coloured traits (the true subject of sexual selection; Lande 1980). Yellow irises may function as pigment-based signals of individual quality as their yellowness is partly due to the presence of pigments such as pteridines and purines, and/or carotenoids that may play key physiological functions (see Introduction). Oliphant (1987) analysed the iris stroma of Eastern Screech-Owls (*Megascops asio*), Northern Saw-whet Owls (*Aegolius acadicus*), Short-eared Owls

(*Asio flammeus*) and Great Horned Owls (*Bubo virginianus*) and found carotenoids, in addition to pteridines and purines, in all species except Great Horned Owls, whose iris pigment was principally xanthopterin (Oliphant 1981). Little and scops-owls are small predators frequently preying on different *Orthoptera* spp., which are known to accumulate plant carotenoids (Ogilvy et al. 2012). Moreover, a relationship of iris colour with body condition could also result from quality-related differences in the rearrangement of stromal purine crystals or superficial blood vessels (Oliphant and Hudon 1993). A second mechanism promoting covariation might be that the colour of the iris is a signal rather than a byproduct of other intrinsic measures of female quality (Ducrest et al. 2008). For instance, pleiotropic effects of the genes regulating the expression of pigments or the arrangement of crystals might potentially account for the covariance between iris coloration and other phenotypic traits in Little Owls. Finally, it may be that age-related variation of iris coloration and fitness measures promote covariation (Delhey and Kempenaers 2006). In the Hen Harrier (*Circus cyaneus*), older females with more-intense yellow irises have higher nest success and are preferred by males, suggesting that iris colour could serve as an index of experience (Picozzi 1981). Unfortunately, our low sample size precludes any sound analysis about age-related variation in adults.

Disentangling the exact mechanism promoting the link between yellow iris coloration and female fitness/reproductive success in Little Owls deserves further research effort. However, our findings confirm the existence of such a link, which could be the premise for using iris yellowness as an indicator of individual quality in mate choice and/or rival assessment in intra-sexual contests. The high territoriality and the monogamous mating system of Little Owl (Holt et al. 2019) may suggest a potential implication of iris colour in territory defense as a deterrent of conspecifics.

We have found that juveniles of both species have less-intense pigmentation in their irises than do adults. Although variation in iris colour with age is relatively widespread in birds (Snyder and Snyder 1974, Sweijd and Craig 1991, Wilson and Hartley 2006), mechanisms promoting age-induced change in iris pigmentation are still poorly understood (Prum 2006). Previous studies found that iris

color correlated with sex, age and maturity stage, potentially suggesting hormonal control of iris colour (Trauger 1974, Picozzi 1981, Newton and Marquiss 1982, Nelson 1983, Scholten 1999), a possibility worth exploring in future work.

Our study confirms that iris yellowness changes with ontogeny in Little and Eurasian Scops-Owls (Mikkola 1983). Furthermore, the possibility that owlet iris colour was involved in parent-offspring communication seemingly is low, both because of the low variability between siblings and because of the apparent lack of relationship with quality indicators in the two studied species. Among adults, although we did not find any direct relationships with body condition, our results suggested that iris colour might potentially function as a signal of quality in Little Owls. However, we note that our study was correlative and we recommend experimental studies to determine whether there is a causative link between iris colour and quality signaling in adult Little Owls.

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Supplementary material

Includes 3 tables and 3 figures

Table S1. *Results of LMMs analyzing the effect of year, age and sex on iris yellowness in Little Owls and Eurasian Scops-Owls when considering one randomly chosen owlet per nest (see Methods for further explanation). Significant effects are depicted in bold.*

Little Owl: Adjusted $R^2 = 0.79$, $F_{4,28} = 30.65$, $P < 0.0001$							
Dependent variable	Predictors	Effect	Coefficient	SE	df	F	p
Iris yellowness	**Year**	**random**	**-0.31**	**0.08**	**1,28**	**13.46**	**0.001**
(n = 33)	**Age**	**fixed**	**0.97**	**0.13**	**1,28**	**116.89**	**< 0.0001**
	Sex	fixed	-0.06	0.13	1,28	1.94	0.174
	Sex*Age	fixed	-0.13	0.17	1,28	0.56	0.460
Scops Owl: Adjusted $R^2 = 0.51$, $F_{4,46} = 14.17$, $P < 0.0001$							
Dependent variable	Predictors	Effect	Coefficient	SE	df	F	p
Iris yellowness	**Year**	**random**	**-0.33**	**0.11**	**1,46**	**9.89**	**0.003**
(n = 49)	**Age**	**fixed**	**0.62**	**0.15**	**1,46**	**52.08**	**< 0.0001**
	Sex	fixed	-0.09	0.15	1,46	0.01	0.355
	Sex*Age	fixed	0.18	0.17	1,46	1.15	0.753

Table S2. *Relationships between iris yellowness and fitness prospect in owlets (a) and adult females (b) Eurasian Scops-Owls. See main text for variable explanation. Analyses were based on average yellowness due to a low variation within nests in the number of sampled owlets, which impedes us running LMMs with the nest as a random factor.*

a)

Life-history traits	n	r_s	t	P
Clutch size	24	0.28	1.37	0.183
Egg volume	23	0.21	1.01	0.325
Hatching date	24	-0.14	-0.65	0.521
Nest success	24	0.08	0.36	0.723

b)

Life-history traits	n	r_s	t	P
Clutch size	16	-0.16	-0.60	0.556
Egg volume	16	0.27	1.06	0.305
Hatching date	16	0.35	1.42	0.177
Nest success	16	-0.12	-0.46	0.654

Table S3. *Correlation analyses aiming to explore relationship between hatching date and both food and parent feeding effort in Little Owl. Latency refers to the time that one of the two adults took to return to the nest after installing the video camera. Furthermore we controlled for the percentage of prey we were able to identify (i.e. percentage of identified prey). See main text for a detailed explanation of variables. Significant correlations are shown in bold. (n = 12 nests)*

Variables	*n*	r_s	*t*	*P*
Latency	12	-0.35	-1.16	0.272
Percentage of identified prey	12	0.25	0.80	0.443
Percentage of centipedes	12	-0.34	-1.14	0.281
Percentage of spiders	12	0.15	0.48	0.643
Feedings per hour	**12**	**0.60**	**2.40**	**0.037**
Female feedings per hour	11	0.15	0.46	0.658
Male feedings per hour	11	0.22	0.69	0.507

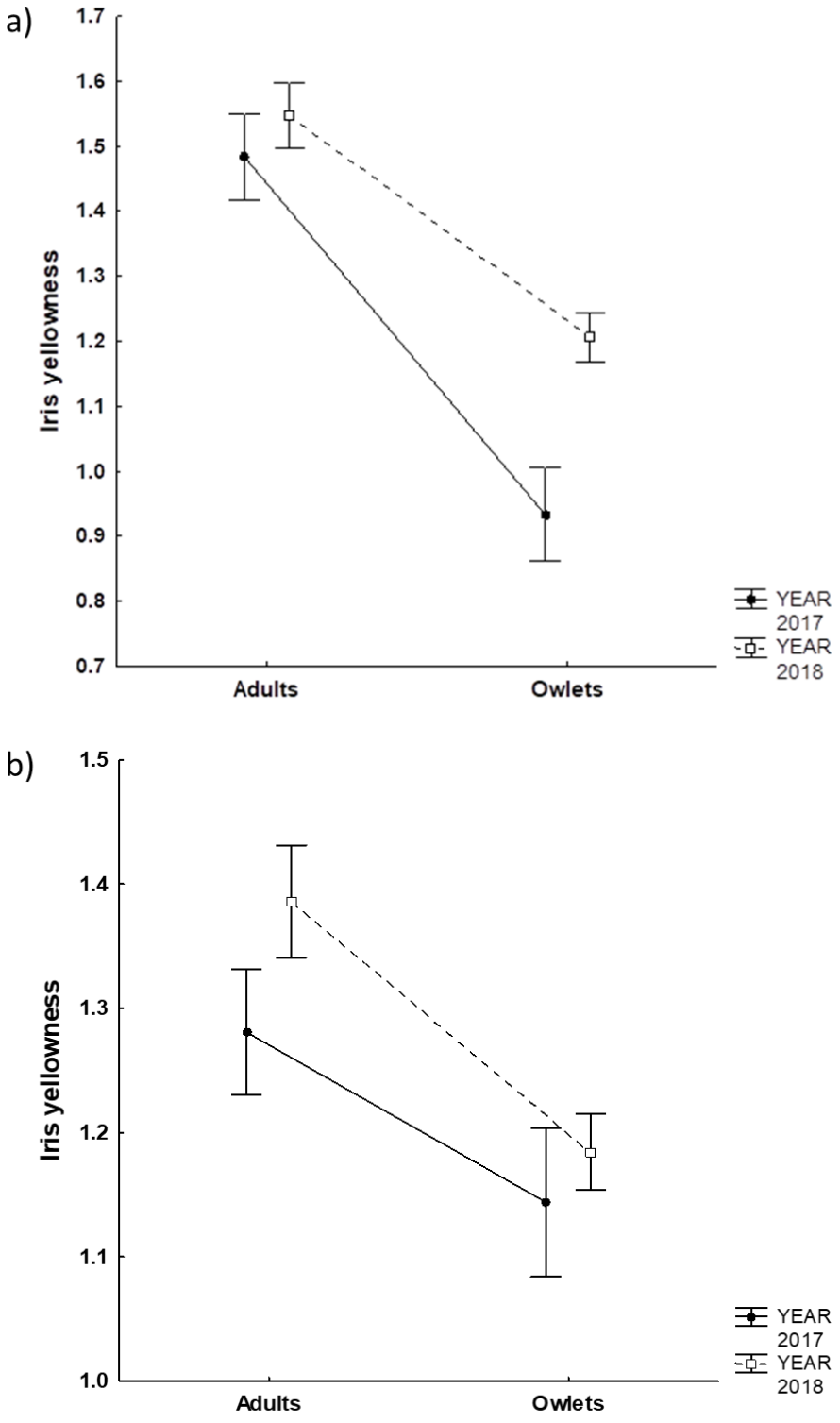

Figure S1. *Variation in iris yellowness in relation to years and age in Little Owl (a) and Eurasian Scops-Owl (b). Interactions are shown only for graphical purposes.*

Figure S2. *Pictures showing the range of iris yellowness in Little Owl in both owlets (a) and adults (b). From left to right representative individuals displaying the lowest, the middle and the highest values, respectively.*

Figure S3. *Pictures showing the range of iris yellowness in Eurasian Scops-Owl in both owlets (a) and adults (b). From left to right representative individuals displaying the lowest, the middle and the highest values, respectively.*

INTEGRATED DISCUSSION

Elucidating how colour variation has evolved in birds, in response to environmental variation, is a fundamental question in evolutionary ecology. This thesis provided novel findings about the role of ecology in promoting the evolution of different colour patterns in *Strigiformes*, shedding light on their adaptive function at both global (**chapter I**) and local scale (**chapter II** and **III)**. The used methods, as well as the insights gained through this research, are widely applicable to the study of colour diversity in other animal groups, both nocturnal and diurnal species, and could thus be used to contextualize the evolution of colour variation in other avian orders. Notably, these findings have contributed to identify potential selective forces acting on the evolution of interspecific iris colour variation (**chapter III**), and fill the gap on the knowledge on how iris colour may potentially function in communicative context in owls (**chapter IV**).

In order to understand how ecological factors determine the evolution of colour variation in different traits in owls, first it was necessary to assess whether variation in those traits depended on degree of species relatedness. To do that, the evolution of colour polymorphism (**chapter II**) and iris colour (**chapter III**) was traced along the owl phylogeny. This allowed to compute the phylogenetic signal for geographic plumage variability (**chapter I**), and to estimate the relative importance of phylogenetic relatedness in trait variation. Reconstructions of ancestral character in the phylogeny failed to find robust support for the most likely common ancestor in the two features, but provided new insights on character lability. Plumage polymorphism (**chapter II**) was identified as a labil trait that evolved repeatedly in distant taxa. In addition, plumage polymorphism had a clumped distribution along the owl phylogeny, concentrating in genera as *Otus*, *Megascops*, *Strix*, *Glaucidium*, and *Tyto*, which indicates it is a feature with strong phylogenetic signal. This finding might have important implications in our understanding of the role of colour polymorphism in speciation, as

theory suggests that it may promote and accelerate processes generating and maintaining speciation (Hugall and Stuart-Fox 2012).

Phylogenetically related owl species are more likely to display similar levels of both potential eu- and pheomelanin-based colours, whereas plumage lightness showed a weak phylogenetic signal (**chapter I**). On the other hand, the two owl families included in the order seemed to diverge in their ancestral iris colour phenotype (**chapter III**). While the ancestor of the family *Strigidae* was more likely bright-eyed, the ancestor of *Tytonidae* was more likely dark-eyed, possibly reflecting the different evolutionary histories undertaken by the two families. *Tytonidae* family might be under a stronger selective pressure for the selection of dark irises at an earlier stage of the evolutionary route compared to *Strigidae* family (del Hoyo et al. 1999). By contrast, *Strigidae* species showed greater variability in iris coloration and ecology than *Tytonidae* species. This might suggest that dark eyes in this family are a posterior acquisition.

In adittion, we successfully applied modern comparative techniques to study the influence of classic ecological drivers in promoting the evolution of colour polymorphism (**chapter II**) and iris colour (**chapter III**) (or vice versa). Coevolution analyses provided strong support for the existence of an evolutionary correlation between activity rhythm and both colour polymorphism and iris coloration in owls. While a change in the luminal niche likely preceded the evolution of colour polymorphism, it remains open the question of which was the exact evolutionary pathway for iris colour. More diurnal species showed a higher propensity to evolve polymorphic plumage and bright irises compared to strictly nocturnal species, among which monomorphism and dark eyes were more frequent. Hence, the exploitation of divergent luminal niches, triggered by different activity rhythms, might be a key evolutionary driver of different colour patterns in more than a trait in owls.

Phylogenetic relatedness, however, did not fully explain colour variation in owls. In **chapter I**, it was found that owl plumage patterns varied according to a latitudinal cline, so that species

inhabiting equatorial areas display more frequently darker phenotypes. This pattern is consistent with the simple version of Gloger's rule predicting heavier pigmentation in warm and humid regions (Delhey 2019). The pattern seem to result from a simultaneous effect of temperature and tree cover since species occupying warmer and more densely vegetated areas, which are those close to the equator, have darker plumages. While previous studies have tested ecogeographical rules irrespective of the nature of pigmentation promoting colour, in the **chapter I** we innovatively considered the two types of melanin, eumelanin and pheomelanin, as originally proposed by Rensch (1929), and recently advocated by Delhey (2017, 2019). Our analyses revealed that the proportion of both potential eu- and pheomelanin-based coloration also follows a geographical cline: the importance of pheomelanin colours was higher in equatorial areas and decreased both north and south, while the relative importance of eumelanin colours was negatively associated with latitude, with a pick near the equator. Interestingly, the two pigments seem to be differently influenced by environmental factors: eumelanin colours were positively associated to higher tree cover, whereas pheomelanin-based coloration increased with temperature and rainfall. The mechanisms underlying Gloger's rule are not easy to discern, but may include, among others, increased background matching for species living in relatively light or dark habitats, since vegetation structure is likely to vary according to geographical cline (Delhey 2017). Hence, the found association between pigmentation and tree cover suggests that a concealment function might be a potential mechanism explaining ecogeographical colour patterns in owls. Accordingly, there would be a stronger selection on eumelanin to achieve background matching, although cryptic patterns probably should simultaneously involve pheomelanin and eumelanin, a question that cannot be addressed in this thesis. All together, these results stressed that several alternative selective forces may act on colour patterns in an ecogeographical scale when different melanin types are involved.

On the other hand, melanin-based colour polymorphism in owls (**chapter II**) did not vary in relation to latitude, but rather it is driven by ecological factors on a smaller population scale.

Comparative analyses investigating which environmental predictors could promote the evolution of colour polymorphism underlined that owl species inhabiting habitats with more stable luminal conditions, as revealed by the degree of vegetation cover, are less prone to display polymorphic plumage. By contrast, owl species living under less stable light conditions were more likely colour polymorphic. Furthermore, coevolution analyses also revealed that colour polymorphism likely evolved in concert with vegetation cover in owls. Therefore, these findings globally suggest that light conditions in terms of habitat structure have a pivotal importance in the selection of plumage patterns in a worldwide framework (**chapter I**) and at lower niche scale (**chapter II**). It also underlines that the main adaptive function of plumage coloration in owls could be camouflage.

The camouflage hypothesis could also explain interspecific variability in iris coloration (**chapter III**), as dark irises might be beneficial in nocturnality, likely because they would allow dark-eyed species to approach their prey without being noticed. All together these findings provide support for the idea that avoiding detection from undesired visual receptors, a fundamental component of predator-prey interactions (Ortolani 1999, Pembury Smith and Ruxton 2020), may play an important role in crypsis in different phenotypic traits in both global (**chapter I**) and niche scale (**chapter II, III**). Furthermore, background matching might also serve as protection from intra-guild predation, since our results showed that polymorphism was more frequent among small-sized species occupying lower trophic levels (**chapter II**).

Nonetheless, it could be argued that diversity of colour patterns in owls could potentially be explained by several alternative mechanisms. Spatial colour variation may be due to physical-physiological reasons because large amounts of melanin would improve resistance to keratin-degrading microorganisms as well as reduce mechanical abrasion in feathers, which would be advantageous in densely vegetated, wetter and warmer environments. In addition, light-to-dark colour variation along climatic gradients, as well as the link between luminal niches and colour polymorphism, could be simply a side effect of selection on other traits determined by a shared

pathway of melanin synthesis with physiological processes differently selected in changing environmental conditions (Ducrest et al. 2008). Regardless of the mechanism behind, however, it can be inferred that global warming may represent a challenge for melanic species, since the increase of both temperature and rainfall may influence melanin-specific life-history strategies (Emaresi et al. 2014), and the proness to infection by parasite (Côte et al. 2018). Finally, activity rhythm may favour differentially dark or bright eyes for reasons other than camouflage such as improved vision of bright-eyed species during twilight and/or daytime. Otherwise, dark-eyed species would not invest in potentially costly pigment, whereas more diurnal species might rely on vividly coloured iris for communication purposes because of a higher use of visual channel. Following this line of reasoning, one of the main goals of the thesis was to study intraspecific variation in iris colour in owls and assessing the premise it may function as an indicator of individual quality. As study models, we considered Little Owl (*Athene noctua*) and Scops Owl (*Otus scops*) (**chapter IV**).

Individual variation in iris colour was quantified in a continuous scale using photographs. The colour of the iris showed to change with age in both species, and adults displayed more saturated yellow iris than owlets. In Scops Owls, I did not found any significant results, both in young and adult. Although variation in iris yellowness among owlets in Scops Owl nests was large, a key requirement for parent-offspring communication, it was uninformative of owlet condition. This might be explained by a high extra-pair paternity rate in the study population, a possibility worth exploring in future studies. In the Little Owl, owlets raised in the same nest showed low variation in iris yellowness, which was not related to owlet body condition. Therefore, it seems highly unlikely that adult Little Owls might use iris yellowness as an indicator of the young's condition or rely on it to adjust their parental effort. However, it was found that owlets raised later in the breeding season displayed more-yellow irises, suggesting that iris colour might be an indicator of parent quality in this species. In addition, further analyses revealed that nest-associated iris yellowness was inversely related to breeding success, which in turn tended to decrease as the season progressed. This suggests

that high values of iris yellowness might stem from a higher brood reduction in late nests. One possibility is that negative selection against owlets in the poorest condition (i.e. those with low yellowness) at the end of the breeding season resulted in an increase in average nest-associated yellowness. Otherwise, due to smaller brood size, owlets in later clutches could receive more food thus promoting higher yellowness. Supporting this premise, late nests had more frequent feedings than earlier ones, but diet delivered in the nests did not change seasonally. However, it cannot be discarded the possibility that iris yellowness reflects aspects of owlet quality that were not considered in our research.

On the other hand, Little Owl females with more yellow irises had higher nest success. Several potential mechanisms may explain this pattern. Firstly, covariation may be due to condition-dependence of expression of iris coloration in a signalling scenario selected through sexual processes, a possibility that could not be analysed in depth due to small sample size and the impossibility to include males in analyses. Secondly, iris colour variation might also be the result of quality-related differences in the rearrangement of internal iris structures or rather a by-product of other intrinsic measures of female quality pleiotropically regulated by genes underlying pigment expression. Finally, covariation may be promoted by age-related variation of iris coloration and fitness measures. Unfortunately, the low sample size precluded any sound analysis about age-related variation in adults.

Little Owl has a broader activity rhythm compared to crepuscular habits of Scops Owl (König and Weick 2008). This difference in activity rhythm, together with the high territoriality of the Little Owl (Holt 2019), may suggest a greater importance of visual communication based on chromatic signals in this species, potentially involving iris colour in territory defense as a deterrent of conspecifics.

Summing up, this thesis provides strong support for the idea that detectability in different light conditions, determined by both differences in activity rhythm and vegetation cover, may be a key

driver of inter- and intraspecific colour variation in different traits in owls. Plumage attributes (i.e. melanin-based coloration and colour polymorphism) and iris colour are adaptive traits likely maintained by the selective advantage of camouflage under different light regimes or in terms of physiological adaptation to climatic and/or environmental conditions via different mechanisms. Finally, this research gives a first glimpse at the role of iris colour in communication in different social contexts in owls. Evidently, further experimental work is needed to better understand causes promoting evolution of colour variation and disentangle the role of colour in signalling individual quality in owls.

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CONCLUSIONS

1. Plumage patterns in owls varied spatially according to wide environmental gradients. Plumage lightness and potential eumelanin and pheomelanin colours followed a latitudinal cline, as predicted by the simple version of Gloger's rule. Owls have darker phenotypes in regions with higher temperature and tree cover. The relative importance of eumelanin colours was higher in species inhabiting areas with a denser tree cover, while pheomelanin colour increased with rainfall, suggesting that several alternative selective forces may simultaneously act on eumelanin and pheomelanin plumage coloration.
2. The found association between pigmentation and tree cover in an ecogeographical scale suggests that melanin-based plumage colorations in owls may serve a camouflage function. Eumelanin would be under a stronger selection to achieve a good background matching, while pheomelanin would be most likely involved in physiological processes.
3. Colour polymorphism and activity rhythm evolved in concert in owls and the transition from monomorphism to polymorphism was more frequent in diurnal and crepuscular species than in nocturnal species. In addition, polymorphism was more likely to evolve in "intermediate" habitats regarding vegetation cover, where species experience more heterogeneous luminal conditions.
4. Changes in owl coloration were triggered by a previous change in the luminal niche of species, supporting the hypothesis that a change in the luminal niche preceded the evolution of colour polymorphism. Detectability in different light conditions may be a key predictor of colour polymorphism, which would be primarily driven by disruptive selection and maintained through selective advantage of morphs in terms of camouflage and/or physiological benefits.
5. Iris colour also coevolved with activity rhythm in owls. The most plausible evolutionary path that led to the higher proportion of dark irises in nocturnal owls seemed to be a transition from

diurnality to nocturnality followed by a change from light toward dark irises. However, analyses did not provide definitive evidence and the camouflage hypothesis was only partially supported.

6. Iris colour change with age in Little and Scops Owls, and adults consistently showed more intense yellow irises than owlets. The possibility that owlet iris colour was involved in parent-offspring communication seemingly is low, because of both the low variability between owlets and the low coupling with quality indicators in the two species. Iris colour, however, might potentially function as a signal of quality in adults Little Owls, as it related with nest success.
7. Variation in luminal conditions proved to be a key driver for the evolution of plumage and iris colour in owls, which suggests a fundamental adaptive role of camouflage in owls.

AGRADECIMIENTOS

Llegados a este punto, a menudo escribir unas líneas más se vuelve una hazaña insuperable, ya que las ganas de teclear el último punto y cerrar definida y rápidamente el asunto se hacen fuerte. Sin embargo, creo que los agradecimientos sean una ocasión importante para dar a conocer la historia personal del individuo y las fuerzas (muy poco adaptativas) detrás de tanto esfuerzo, cosas que raramente encuentran un espacio propio. Por tanto, hay que dedicarles el tiempo que merecen.

Quien escoge la ciencia como profesión sabe que no escoge el rumbo más fácil, sobre todo en sus comienzos. Todo investigador (o aspirante investigador) se siente tentado de rendirse ante la adversidad. Para no dejarse derrumbar por las dificultades son necesarias tres P (no, no tienen nada que ver con la probabilidad, si bien juega su papel): Paciencia, Pasión y Perseverancia. Etimológicamente paciencia y pasión son casi sinónimos, ya que ambas palabras derivan del mismo verbo latín que significa sufrir. Y no es un caso. Toda cosa que tenga valor requiere un sacrificio que, pero, no es destinado a extinguirse en breve en grandes llamas como sugiere la moderna interpretación de la palabra pasión. Al revés, es un esfuerzo guiado por la luz firme de la dedicación constante y que se basa en la capacidad de aceptar que los grandes logros requieren tiempo. Por esta razón la perseverancia es una componente fundamental del éxito. Es la fuerza de seguir en el camino sin desistir, con la convicción que la lucha en lo que creemos nos llevará a grandes logros. Juntas, nos convierten en individuos resilientes, permitiéndonos ver oportunidades donde los demás no ven nada, ver puertas donde los demás ven muros.

Llevar a cabo esta tesis no ha sido fácil. Ha costado sudor y lágrimas (literalmente). Y no habla solo de búhos, habla de un objetivo, un sueño, perseguido largamente. Trata de evolución, pero no solo en los estrígidos, sino también en la persona que está escribiendo. Al final, creo que terminamos por parecernos a lo que estudiamos. He tenido que adaptar mi ritmo de actividad para compaginar trabajo y tesis, viviendo tanto por la noche como por el día. Me he vuelto polimórfica para poder

adaptarme a las circunstancias, siendo profesora de italiano, recepcionista, camarera, y, en algunos momentos, todas estas cosas contemporáneamente. He notado una variación en el color con base melánica de mi pelo que podría indicar una disminución de la calidad individual. No tenía ni una cana cuando empecé la tesis y en poco tiempo he visto rayos de luna (se me conceda una definición más poética de las canas) aparecer sobre mi cabeza. Seguramente sea estrés oxidativo debido a la tesis. Y ¿qué decir de los ojos? No he registrado variaciones en el color del iris, pero una cosa sí la he notado. Mis ojos no son los que eran antes. Mejor dicho, su forma de mirar no es la misma, ahora ven el mundo de una forma diferente. Este intenso y extenso período formativo me ha ayudado a ampliar mis horizontes, además de haber sido una posibilidad única para desafiarme a mí misma.

De hecho, entre las numerosas (lo dicen mis amigas) virtudes que tengo, la paciencia no es la primera que se me ocurre, por lo que estuve a punto de dejarlo todo más de una vez. Pero yo, que tengo cierta propensión al desastre, soy evolutivamente preparada para resistir a los retos de la vida y cuento con una fuerte determinación, que en más de una ocasión ha compensado mi incapacidad de saber esperar, si bien todo me ha parecido tremendamente fatigoso de llevar. En este sentido, la experiencia del doctorado ha sido fundamental, no solo para avanzar profesionalmente, sino también para mejorarme a nivel personal. He aprendido que confiar en el tiempo es una dote importante, y que el éxito se construye cada día, sin prisa, pero sin parar. Las tres P solas no pueden estar, si una falta nuestros andares son cojo. Y de nada nos vale mirar lejos, hacia el futuro, si acabamos por perder de vista lo que está cerca, nuestro presente.

Por contra, mi pasión nunca ha vacilado. Se ha mantenido inoxidable, inalterada desde que era niña, tanto que a los 8 años miré a la cara mi madre con toda la seriedad posible para una niña de esa edad y le compartí mi iluminación: “Mamma, cuando sea grande quiero estudiar a los animales”. Mis padres al principio creían que era una idea pasajera, de esas ideas caprichosas que se les ocurren a los niños. Pero pronto se dieron cuenta que no lo era. Aunque siempre me ha gustado todo tipo de animal, mi preferencia para las aves se hizo evidente en seguida. Con gran desesperación de mi padre, empecé

a criar, primero canarios, y luego especies exóticas, hasta que reuní unos 100 pajarillos de más de 15 especies diferentes que no quería ver en jaulas (según mi visión les afectaba demasiado al comportamiento que era lo que quería "estudiar" y también porque me daba pena y era una manera de "limpiarme" la consciencia). Obligué mi padre a construir una enorme pajarera que ocupaba parte del jardín, dejando dentro plantas para recrear un ambiente natural. La pajarera se dividió en dos partes porque para mí era impensable mezclar especies africanas con especies australianas. El rigor científico antes de todo. Dado que algunas especies no podían aguantar las rígidas temperaturas invernales de mi tierra natal, convencí mi padre a dejarme una parte del cuarto que él utilizaba para guardar sus herramientas y la conecté con la pajarera de modo que, dejando abierta la ventana, mis queridos pajarillos salían y entraban a su antojo. A la llegada del frío, esperaba a que todos se recogieran para cerrar la ventana y calentar el cuarto. Cuarto en el que yo había recreado una pequeña selva. Mi padre me hizo prometer que cuando encontrara mi primer trabajo, le restituiría todo el dinero que gastaba en calefacción cada invierno. No sé si debería decirlo, pero han pasado años y no ha visto ni un duro.

Después de la universidad no quería seguir en el ámbito académico porque lo veía muy "agonístico", mientras yo estaba más interesada en la observación, disfrutando del campo. Así que empecé a trabajar como técnico y, durante muchos años, mi trabajo fue una fuente de grandes satisfacciones para mí. Pero estoy convencida que científico uno nace y una calidad importante de un científico es la curiosidad, y yo me hacía muchas preguntas. Me interrogaba sobre el porqué de determinados comportamientos o de las preferencias de algunas especies para ciertas características del hábitat. Y empecé a echar mucho de menos la parte de investigación. Recogía datos, pero no podía usarlos. De repente, me acordé de las horas que pasaba delante de mi pajarera, apuntando comportamientos, cantos, número de huevos, fechas de eclosión, y decidí que ser un técnico ya no era suficiente. Quería ocuparme también de los análisis de esos datos. Sin embargo, mi formación era incompleta y necesitaba hacer un doctorado para adquirir la competencia necesaria. Y aquí estoy.

Han pasado algo como 28 años desde que decidí que quería “estudiar a los animales” y he atravesado 5 años de profundo cambio, profesional y personal. Y muchas son las personas que han participado, más o menos activamente, en el proceso.

Ya. Porque la elaboración de una tesis doctoral es un recorrido largo y empinado que podría pensarse muy solitario, pues es del autor la tarea de llegar a la cumbre de la montaña (bueno, llegar a un altiplano, con una vista preciosa, por cierto, para darse cuenta que la cumbre aún está lejos, pero esta es otra cuestión). Sin embargo, después de haber atravesado todo el trayecto, uno se da cuenta que este camino comienza incluso antes de que empecemos (y terminemos) un doctorado y nunca se resuelve en soledad y que, como en muchas otras ocasiones de la vida, nuestros éxitos nunca son solo nuestros. Cuando llega el momento de cosechar, mucha gente debería compartir con nosotros el fruto de tanto trabajo. En mi caso, hubo muchísimas personas a mi lado que me acompañaron a lo largo de esta compleja y fascinante aventura, y que se han visto implicadas, de una forma u otra, y a las que he de agradecer.

No debería sorprender que, en primer lugar, quiera darle las gracias a mi familia, que, con muchísima paciencia y amor, soporta mis continuos cambios de rumbo (y domicilio) y me ha proporcionado siempre un apoyo incondicionado. Mi madre Ada, mi padre Giovanni y mi hermana Clara son mis faros en la niebla. Cuando me pierdo por el mundo sé hacia donde he de mirar. También he de agradecer a mi abuela Rita que me llama “vagabunda” y siempre me recuerda que reza mucho todos los días para que todo me vaya bien.

Además, es evidente que este trabajo no hubiese sido posible sin la guía atenta y meticulosa de mi director, Jesús, y, por un cierto periodo, sin la supervisión de Desi. Gracias por haberme dado la posibilidad de realizar una tesis como esta, de gran calidad científica y sobre un tema tan apasionante. Gracias por apostar en mí a pesar de mi situación, por escuchar y ordenar pacientemente mis enmarañadas ideas, dando finalmente una forma a mi entusiasmo caótico. Gracias por vuestros

consejos, que siempre han sido de gran utilidad (aunque a veces parece que no escucho), y por vuestras enseñanzas. Sin duda, habéis sido un ejemplo a seguir para mi carrera. ¡Mil gracias por todo!

¿Y qué haríamos sin nuestros amigos? ¿Sin alguien a quien contar nuestras miserias y que nos ayude a llevar mejor las adversidades? Por esta razón estos agradecimientos no tendrían sentido si no aparecieran mis amigas en Italia, que han sido un soporte muy fuerte en momentos de angustia y desasosiego. Porque da igual el lugar del mundo en el que uno se encuentre, y las veces (muchas) que los amigos tienen que lamentar nuestra ausencia. Aun así, ellos siguen junto a nosotros. Por tanto, quiero agradecer con todo mi corazón a Sere, Lucia (La Minky), Claudia, Ila, Sara, Simo, Ale Contu, Ele, Ale Vergio, que me aguantan desde hace muchos años. Grazie ragazze!

Y no he de agradecer solo a los amigos "antiguos". No tener una beca probablemente ha sido una suerte. Todo pasa por algo. Aparte que ¿cuándo me vuelve a pasar de hacerle de interprete a Sofia Loren o de ser la traductora de guiones y ver inesperadamente mi nombre en los créditos de una peli? Una beca me hubiera permitido dedicarme solo a la tesis, pero tal vez no tendría tantas historias para contar. Así tengo para escribir un libro. No tener una beca ha sido una suerte porque me ha dado la insustituible oportunidad de conocer a mi capitana Marian, que para mí es como una hermana, y descubrir mi rincón de la paz, San José. He pasado 3 años maravillosos trabajando con ella y con Mar, que también es una persona esplendida, y sé que, siempre que vuelva, habré un sitio y unas personas que puedo llamar "casa" y "familia". Gracias chicas por las risas, las cenas, los partidos con el Risk, los abrazos y por vuestra sincera e inspiradora alegría.

Estos 5 años no han sido solo dolor y sufrimiento, sino que han sido acompañado de muchos momentos amenos y a veces de desenfreno puro (echaré de menos ciertas noches en las Cuatro Calles de Almería). Y por estos momentos felices tengo que agradecer unos grupos de personas realmente majas que me han acogido calurosamente desde que llegué y que se han convertido en amistades muy bonitas. Siempre me han involucrado en las actividades que se organizaban, además de ayudarme en

las situaciones incomodas en las que me encontré en estos años. ¡Muchas gracias Chumbo Team por haber creado el mejor y más estimulante ambiente durante toda mi permanencia! Ha sido un placer compartir tiempo, cocina, ideas, vídeos de tesis, y también alguna desgracia más que otra, con vosotros. Unos agradecimientos especiales van para mis compañeros de "La compañía del autillo" que me han ayudado en todo momento y con los cuales me he encontrado muy a gusto trabajando. ¡Gracias Mónica, Juan y Ángel! También he de dedicar un agradecimiento especial a Teresa, por estar a mi lado, por su valioso aliento que ha despertado más de una vez la confianza en mí misma, ayudándome en decisiones cruciales, y a Miguel, por nuestras riquísimas conversaciones, y por su sensibilidad y amistad, que quedan bien guardada en mi corazón. Muchas gracias al grupo "Portu of Spain", sin el cual mi vida en Almería no hubiera sido la misma. Más tranquila quizá (tal vez hubiese terminado antes los artículos), pero enormemente aburrida. Gracias a José Antonio (Mori), Gustavo, Paula, Antonio, y a todos los demás, por soportar el columpio de mi estado emocional y haberme hecho sentir parte de un grupo. Os echaré muchísimo de menos. Gracias a Lili, mi colombiana favorita, por ser una confidente extraordinaria y haberme devuelto la vida. Ella sabe a qué me refiero. Gracias a Ángela, por haber sido la primera en mostrarme los tesoros de esa tierra asombrosa que es la provincia de Almería, por escucharme y reconfortarme en más de una ocasión, ayudándome a salir del pantano.

Mi sincera gratitud va también a la EEZA, que me ha acogido y me ha dado la posibilidad de utilizar las instalaciones para poder desarrollar mi tesis, proporcionándome una ayuda fundamental en la logística, y a todas las personas que componen el equipo humano, que me han tratado siempre con gran simpatía y cordialidad. Entre ellas quiero destacar Almudena y Jorge, que me han involucrado en su brillante proyecto de educación ambiental, y Paco, por sus valiosos consejos. Por último, pero no menos importante, me gustaría dedicar un pensamiento especial a Marcela y Miriam que han cuidado de mí y de los demás doctorandos con mucho cariño y a las que mando un fuerte abrazo.

Sorry, but now I have to suddenly switch the language to warmly thank the group of the Novia University, which has been a very good company during the last months of my PhD, in a difficult moment like that of the pandemic. I am especially in debt with Chiara for her careful and valuable revision of the English.

Es indudable que me estoy olvidando de muchas personas, pero espero que ellas no se sientan minusvaloradas por mi olvido y que reconozcan igualmente sus méritos, que estoy segura han sido muchos. A fin de cuenta, la formalidad que envuelve este texto hace perder un poquito el valor de mis agradecimientos ya que, según mi opinión, las gracias es preferible darlas en persona y mejor con gestos que con palabras. Sin embargo, si no lo he hecho, animo a estas personas a reclamarme lo que es justo.

Estos largos agradecimientos no tienen una finalidad auto-celebrativa. Mi intención es compartir mi experiencia para que sea útil a otras personas. A veces, saber que otros han recorrido los mismos senderos los hace menos oscuros y aterradores. En particular quería compartir una reflexión que creo sea fundamental cuando uno tiene que decidir si afrontar un nuevo camino. ¿Qué es lo que perdemos si decidimos no escuchar nuestras vocaciones, si decidimos quedarnos en la zona de confort por medio al fracaso, a la opinión de los demás? La respuesta solo la conoce la persona que se encuentra al bivio. Yo, mi respuesta ya la encontré.

"Para trascender en la vida, para ser de verdad, hay que ampliar

horizontes, hay que cambiar de lugar"

María Zambrano

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