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PRETTY FLY (FOR A WHITE OWL): EVOLUTIONARY PROCESSES SHAPING COLOR AND MC1R IN BARN OWLS (TYTO ALBA)

Ducret Valérie

Ducret Valérie, 2017, PRETTY FLY (FOR A WHITE OWL): EVOLUTIONARY PROCESSES SHAPING COLOR AND MC1R IN BARN OWLS (TYTO ALBA)

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Faculté de biologie
et de médecine

Département d'Écologie et d'Évolution

PRETTY FLY (FOR A WHITE OWL): EVOLUTIONARY PROCESSES SHAPING COLOR AND *MC1R* IN BARN OWLS (*TYTO ALBA*)

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de Biologie et de Médecine
de l'Université de Lausanne

par

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EVOLUTIONARY PROCESSES SHAPING COLOR
AND *MC1R* IN BARN OWLS (*TYTO ALBA*)**

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pour le Doyen
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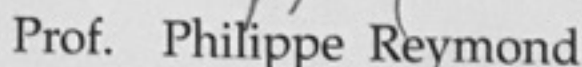

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Summary

Unraveling the distinct evolutionary mechanisms affecting phenotypic and genetic diversity has been a major focus in evolutionary and conservation biology because it is a necessary step to understand how populations can potentially adapt and evolve. In this context, coloration in the animal kingdom has provided remarkable illustrations of the strong effect of natural selection and adaptation to divergent environments. To understand the type of evolutionary processes affecting color variation, it is important to also study its underlying genetic basis. A recent series of papers has shown that a single gene, the melanocortin-1 receptor (*MC1R*), is responsible for color polymorphisms in a variety of vertebrates. The numerous phenotype-*MC1R* genotype associations in different species, renders the study of this gene particularly relevant to understand the respective roles of selection and demographic processes in shaping color variation in vertebrates.

In this PhD, we were able to make important contributions to the understanding of color and *MC1R* evolution under the contexts of sexual dimorphism, sexual genetic conflict, clinal variation, natural selection and the interplay of selection and dispersal. We considered numerous approaches from landscape genetics to population genetics and survival analysis, using the barn owl (*Tyto alba*) as a model species. In the first chapter of my thesis, we decrypted the effect of *MC1R* variants on pheomelanin- and eumelanin-based colorations in Swiss barn owls and we tested the hypothesis that this gene could be accountable for sexual dimorphism. We highlighted that *MC1R* has a strong effect on the rufous color variation, explaining 33% of the color trait variance. In addition, this gene contributed to sexual dimorphism of both pheomelanin- and eumelanin-based traits and suggests an advantage of the *rufous* and *white* allele in females and males, respectively. Also, we observed in the second chapter that males and females differentially inherit the *MC1R* variants, which support sexual conflict at this gene. Indeed, the *rufous* allele was recurrently less often transmitted from father to sons. Future investigation regarding inversion or pleiotropic effect of color-related genes will be necessary to better understand how this

mechanism may have evolved. To understand the evolution of sexual dimorphism for the rufous coloration, we investigated in chapter 3 the effect of selection and dispersal on *MC1R* in breeding barn owls in Switzerland. Using capture-recapture data, we observed a non-random gene flow of *MC1R* variants through a prominent rate of females' immigration, suggesting a possible advantage for dispersal in females when carrying a *rufous* allele. However, we did not highlight sex-specific natural selection on *MC1R*. Particularly, we did not detect a fitness advantage for survival or reproductive success in adult males when carrying *MC1R*$_{\text{WHITE}}$ allele, despite a heterozygote deficit at *MC1R*. The difficulty to highlight sex-specific natural selection can come from a weak selection and/or that different selective agents operate on color in males and females. Because the three-melanin based trait are highly genetically correlated, multivariate approaches combined with quadratic regression would give a better picture of how the rufous coloration evolved in each sex given this genetic constraint. Finally, in chapter 4 we investigated whether in Europe clinal variation in the rufous color and frequency of *MC1R* alleles is associated with climatic and landscape variables using a large dataset of GIS-based information. Despite a clear effect of geographic distance between samples, we report significant relationship with climatic variables, directly or indirectly linked to isothermality. Yet, the exact relationship between the rufous coloration and isothermality is not yet clear and future studies should confront the effect of several climatic variables on different color clines observed in *Tytonidae* worldwide using this individual-based approaches to better understand this relationship between climate and color.

Résumé

Décrire les mécanismes évolutifs qui affecte la diversité phénotypique et génétique a longtemps été l'objet de la discipline « biologie évolutive » et de la « biologie de la conservation » car cela permet de comprendre comment les populations évoluent et s'adaptent. Dans ce contexte, la coloration au sein du règne animal a fourni de remarquables exemples du fort effet de la sélection naturelle et de l'adaptation dans des environnements distincts. Pour comprendre ces processus évolutifs, il est important d'étudier en parallèle la base génétique sous-jacente de la coloration. Chez un beaucoup de vertébrés, le *MC1R* est un gène responsable des polymorphismes de couleur. Les nombreuses associations entre phénotypes et génotypes du *MC1R* chez différentes espèces rendent l'étude de ce gène particulièrement pertinente pour comprendre les rôles respectifs de la sélection naturelle et des processus démographiques dans l'établissement et la maintenance de la variation de couleur chez les vertébrés.

Lors de cette thèse de doctorat, j'ai pu apporter une contribution importante quant à l'évolution de la couleur et du *MC1R* dans le contexte du dimorphisme sexuel, du conflit génétique sexuel, de cline de couleur, de la sélection naturelle et de l'interaction entre sélection naturelle et dispersion. En étudiant la chouette effraie (*Tyto alba*), j'ai utilisé de nombreuses approches allant de la génétique des populations à l'analyse de survie. Dans le premier chapitre de ma thèse, l'effet de la variation au *MC1R* sur la variation de trois traits mélaniques a été décrypté chez les chouettes effraies suisses et nous avons testé l'hypothèse selon laquelle ce gène pouvait impacter le dimorphisme sexuel. Nous avons estimé que la variation au *MC1R* explique 33% de la variance de ce trait et que ce gène contribue au dimorphisme sexuel des trois traits de couleur, suggérant un avantage de l'allèle *roux* et *blanc* chez les femelles et les mâles, respectivement. En outre, nous avons observé dans le deuxième chapitre que l'allèle *roux* est moins souvent transmis de père en fils, ce qui soutiens l'hypothèse d'un conflit sexuel sur ce gène. Des futures recherches sur l'arrangement chromosomique ou les effets pleiotropes des gènes liés à la couleur seront nécessaires

pour mieux comprendre comment ce mécanisme a pu évoluer. Dans le chapitre 3, nous avons étudié l'effet de la sélection et de la dispersion sur les fluctuations de la fréquence des deux allèles du *MC1R*. En utilisant les données de capture-recapture, nous avons observé un flux génique non aléatoire des génotypes *MC1R* par le biais d'une immigration massive de femelles, particulièrement celles portant l'allèle *roux*, ce qui suggère un avantage sélectif chez ces femmes lors de leur dispersion dans notre population. Cependant, nous n'avons pas mis en évidence une sélection naturelle du *MC1R* qui serait différente entre sexe. En particulier, nous n'avons pas détecté un avantage pour la survie ou le succès reproducteur chez les mâles adultes lorsqu'ils portaient l'allèle *blanc*, malgré l'observation d'un déficit en hétérozygote au *MC1R*. La difficulté de mettre en évidence un effet différentiel de la sélection naturelle entre mâles et femelles peut provenir d'un faible effet sélectif et/ou due à différents agents sélectifs opérant sur la couleur des mâles et des femelles. Étant donné que les trois traits mélaniques sont très corrélés génétiquement, des approches multivariées associées à des régressions quadratiques donneraient une meilleure image de la façon dont la coloration rousse a évolué au sein de chaque sexe, compte tenu de ces contraintes génétiques. Enfin, dans le chapitre 4 nous avons cherché à expliquer le cline de couleur rousse et du *MC1R* en Europe par des facteurs climatiques et de paysages en utilisant des données SIG. Malgré un effet de la distance géographique entre les individus, nous avons pu mettre en évidence l'effet de variables climatiques, liées directement ou indirectement à l'isothermalité. Cependant, la relation entre la coloration rousse et l'isothermalité n'est pas entièrement élucidée et des études futures devraient confronter l'effet de plusieurs variables climatiques sur différents clines de couleur observés dans la famille des Effraies (*Tytonidae*) dans le monde pour mieux comprendre cette relation.

General introduction

Phenotypic and genotypic diversity in natural populations are important aspects that allow adaptation in the face of environmental changes, such as climate warming, habitat destruction or the increase of invasive species (Reed & Frankham 2003). Indeed, natural selection cannot operate unless there are phenotypic differences between individuals, as described by Charles Darwin about 150 years ago. Today, evolutionary biologists aim to understand how natural populations respond to environmental changes, knowing that this response depends on the interplay of a set of evolutionary and demographic processes. For example, the potential of adaptation can be constrained by the effect of dispersal, which can introduce maladaptive alleles into locally adapted populations (Slatkin 1987; Hu & Li 2003). Unraveling the distinct evolutionary mechanisms affecting phenotypic and genetic diversity has been a major focus in evolutionary and conservation biology because it is a necessary step to understand how populations can potentially adapt and evolve. In this context, this thesis aimed to describe the interplay of neutral and deterministic forces in shaping phenotypic and genotypic variation that are essential for adaptation.

Coloration: functions and adaptation

Animal or plant coloration has long been a suitable model to elucidate diverse questions related to evolution. In 1853, Gregor Mendel used the color pattern of garden peas (*Pisum sativum*) to study the inheritance of phenotypic variation, and his law of segregation has been widely used thereafter. In 1871, Charles Darwin used the plumage coloration of peacocks' tails to demonstrate his theory of sexual selection. In 1911, the artist and naturalist Abbott Thayer tried to demonstrate that animal coloration is only an adaptation for camouflage, and got strongly criticized by the politician Theodore Roosevelt who saw in his work "wild absurdities" and "preposterous misrepresentations" (Hendrick 1995). This passionate debate has caused a wave of interest in discovering the function of coloration.

Furthermore, the various functions of coloration in the animal kingdom have provided remarkable illustrations of the strong effect of natural selection and adaptation to divergent environments (Nachman *et al.* 2003; Hoekstra *et al.* 2004; Rosenblum *et al.* 2010). The first well-known evidence of adaptation comes from the camouflage of black and white morphs of

the peppered moth (*Biston betularia*) population in England during the 1850s (Cook *et al.* 2012). Since, we have other evidence that predator avoidance through specific coloration has a direct impact on individual fitness and adaptation of populations, such as in mice (e.g. *Chaetodipus intermedius*, *Peromyscus polionotus*) (Nachman *et al.* 2003; Hoekstra *et al.* 2004), octopus (Hanlon *et al.* 1999) or lizards (Rosenblum *et al.* 2010). Colors and pigments can have other adaptive functions such as photoprotection in humans (Jablonski & Chaplin 2000), thermoregulation in insects (Solensky & Larkin 2003; Forsman *et al.* 2002) or stress response and immunity in birds (Roulin *et al.* 2001; Almasi *et al.* 2010; Männiste & Hõrak 2014) through plausible pleiotropic effects of the melanocortin system (Ducrest *et al.* 2008). In addition to an adaptive function related to natural selection, coloration can also evolve under sexual selection. For example, melanistic and carotenoid coloration are often used for social signaling such as mate choice (Hanlon *et al.* 1999; Krüger *et al.* 2001; Ritland *et al.* 2001) and sexual competition (Senar 2006). Thus, natural selection and sexual selection often interact in complex ways to influence secondary sexual traits, particularly in color system (Stuart-Fox & Ord 2004), and this conducted to a broad range of theoretical and empirical studies to understand the evolution of sexual dimorphism.

Sexual conflict and the evolution of sexual dimorphism

Since the 1960s, evolutionary biologists started to widely accept the concept of sexual conflict, which arises when a mutation increases fitness in one sex but decreases it in the other sex (i.e. Sexually Antagonistic selection (SA); Rice 1984). When the same set of genes in males and females encodes the trait that experiences sexually antagonistic selection, a tension within the genome is inevitable (Arnqvist & Rowe 2005; Bonduriansky & Chenoweth 2009; Dean *et al.* 2012; Rice & Gavrilets 2014). This tension arises because parents pass on a given allele to the sex that benefits from it, but also to the other sex, which is disadvantaged. Selection may thus favor mechanisms that modulate the expression of the genes in a sex-specific way (Ellegren & Parsch 2007) and lead to the evolution of sexual dimorphism – or dichromatism when the trait of interest is coloration. Other less-known mechanisms can also resolve sexual conflict by preventing each sex to inherit a variant that will decrease its own fitness. For example, females of *Anolis* lizards in the Greater Antilles produce sons or

daughters more often when mated with large or small males, respectively (Calsbeek & Bonneaud 2008). Manipulation of sex ratio has also been observed in barn owls (*Tyto alba*), as male-like females and female-like males produced, respectively, more sons and daughters, which confers them a selective advantage (Roulin *et al.* 2010).

Although such conflict arises through opposing direction of selection on males and females characters, sexual conflict has been mainly studied in the context of sexual selection because males can develop striking color ornament or impressive weaponry to increase their reproductive rates by attracting females, yet decreasing females' fitness when expressing such traits (Foerster *et al.* 2007; Cox & Calsbeek 2009). In this context, Simpson and colleagues (2015) demonstrated that female wood warblers have lost the colorful ornamentation used for social signaling by males, because of increased predatory costs during migration for colorful females. In this context, sex-biased dispersal is often suggested for the evolution of sexual dimorphism. Indeed, dispersal is recurrently related to morphological, behavioral or ecological traits, known as dispersal syndromes (Cote *et al.* 2010; Ronce & Clobert 2012; Chakarov *et al.* 2013). Also, when dispersal is sex-biased, males and females can have different fitness optimum for those traits. This should conduct to the evolution of sex-specific expression in order for males and females to ultimately reach their phenotypic optima. Yet, there is still a lack of knowledge and empirical studies on the relationship between the evolution of sex-biased dispersal and the evolution of sexual dimorphism to understand how both processes affect local adaptation and fitness in a sex-specific manner.

Interplay of multiple evolutionary processes

Dispersal strongly affects gene flow, but it is not the only demographic processes affecting phenotypic and genetic diversity and the potential for adaptation of populations. Although the emergence and maintenance of color variation often reflects the effect of divergent environments, some empirical studies have found a diminished role of environment and a greater role of neutral processes. Indeed, demographic processes such as geographic isolation, rapid population expansion or small population size, can impact phenotypic variation and balance the role of selection (Haavie *et al.* 2000; Landry & Bernatchez 2001; Campos *et al.* 2006). For example in the red-backed fairy-wren (*Malurus melanocephalus*) subspecies,

demographic processes (drift-migration balance), coupled with divergent sexual selection, better explained plumage hue variation than environmental variation (Baldassarre *et al.* 2013). With the availability of a broad range of population genetic analyses, it is possible to decrypt neutral genetic and phenotypic structure that can result from contemporary drift-migration balance or from past evolutionary history (Merilä & Crnokrak 2001). Therefore, information related to neutral processes is necessary to acknowledge that adaptive processes better explain variation for a trait or a gene of interest. Following this step, the increased predictive power of recent advances in landscape genetics, Geographical Information System (GIS) technologies, and spatial statistics, can permit to rigorously identify the environmental variation that shapes intra-specific adaptive phenotypic or genetic diversity (Thomassen *et al.* 2010).

Computation and simulations are nowadays widely used and particularly useful because they allow making accurate estimations or predictions in any biological system. A good example is the work of Hedrick and Ritland (2012) on color polymorphism of the British Columbia population of bears (*Ursus americanus*). In this population, the white recessive phenotype is coded by a non-synonymous mutation at the melanocortin-1 receptor gene (*MC1R*). Using classical population genetic models and simulations, they highlighted that genetic drift must have been important in increasing the initial frequency of the *w* allele, coding for the white phenotype, to a frequency high enough that recessive homozygotes are maintained by natural selection. Also, they stated that the gene flow of the *W* allele, coding for the black phenotype, from other populations, should be small enough to not reduce the frequency of the *w* allele on two of the British Columbia islands. However, they observed that assortative mating has to be particularly high to generate the heterozygote deficit at *MC1R* observed in Ritland *et al.* (2001). However, a lack or incorrect information on the biological system, such as the frequency of the each *MC1R* variant in males and females, can lead to wrongly estimate parameters such as assortative mating.

Genetic basis: the melanocortin-1 receptor gene (*MC1R*)

To understand the type of evolutionary change affecting phenotypic variation, it is important to also study the underlying molecular mechanisms that lead to such variation. With the rise of new molecular techniques, evolutionary genetics allow genetic variation to be directly studied in natural populations. We have entered an exciting new era where for the first time it has become possible to identify the genes responsible for color variation, and these genes often play a similar role in multiple species. Indeed, a recent series of papers has shown that a single gene, the melanocortin-1 receptor (*MC1R*), is responsible for color polymorphisms in a variety of mammals, including humans, but also in domestic animals, such as cattle, pigs, goats or sheep (Fig. 1; Mundy 2005). In addition, numerous non-synonymous mutations at the *MC1R* gene have been found in a wide range of bird species, for example the chicken (*Gallus gallus*), the bananaquit (*Coereba flaveola*), the arctic skuas (*Stercorarius parasiticus*), the ruff (*Philomachus pugnax*) and the gyrfalcon *(Falco rusticolus)*. The numerous phenotype-*MC1R* genotype associations in different species, renders the study of this gene particularly relevant to understand the respective roles of selection and demographic processes in shaping color variation in the animal kingdom (Hoekstra 2006).

The *MC1R* codes for a seven transmembrane G-protein coupled receptor that is located on the surface of melanocytes. This receptor is responsible for the production of two types of pigment: the yellow-rufous pigment called pheomelanin, and the black pigment called eumelanin. The binding of its primary ligand, the ∝-melanocyte stimulating hormone (∝-MSH), activates the MC1R, which switches from the production of pheomelanin to the production of eumelanin. However, the *MC1R* antagonist (*ASIP*), coding for the agouti protein, can competitively bind the MC1R, thus preventing its ligand to activate the receptor. A recent study by San-Jose *et al.* (2017; Appendix) underlined a more complex and fundamental role of MC1R on the melanocortin system. Not only the expression of MC1R regulate the expression of melanogenic-related genes, downstream of MC1R, but also interact with melanocortin genes, upstream of MC1R, such as *ASIP* and also *PCSK2* responsible for the maturation of ∝-MSH.

Figure 1: MC1R sequence variants associated with plumage or hair color change in birds and mammals published in Mundy (2005).

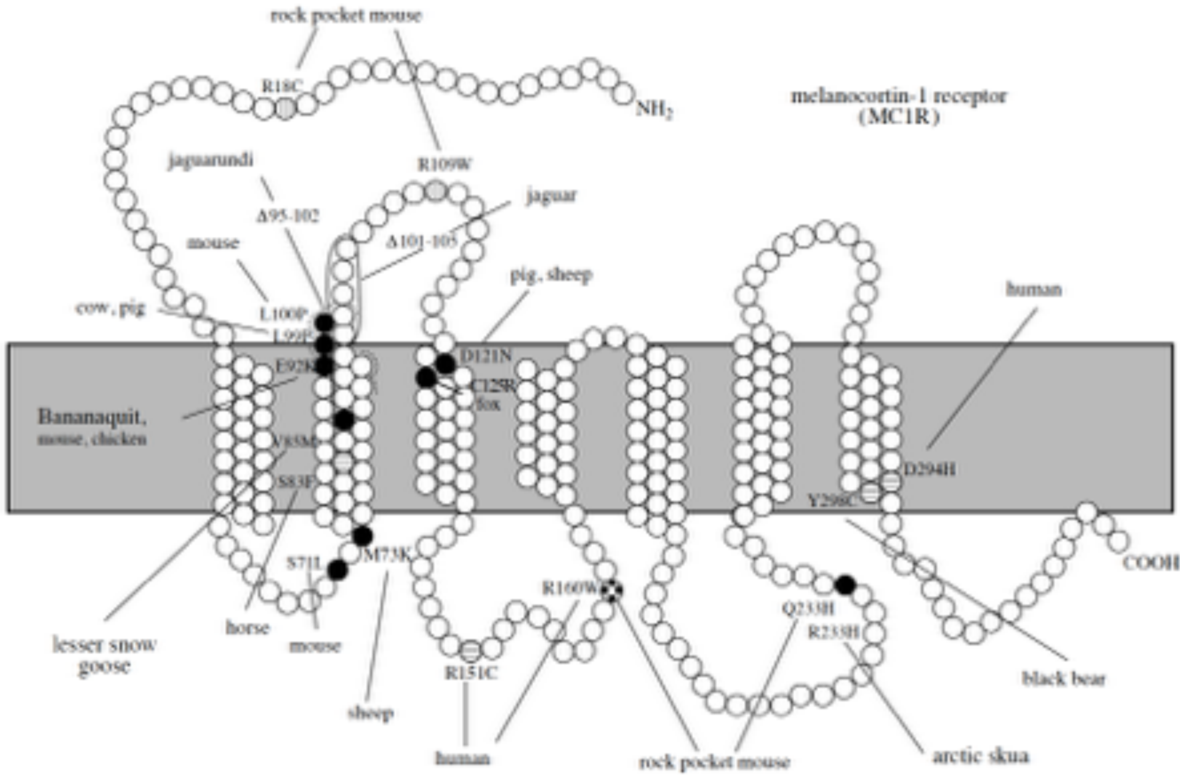
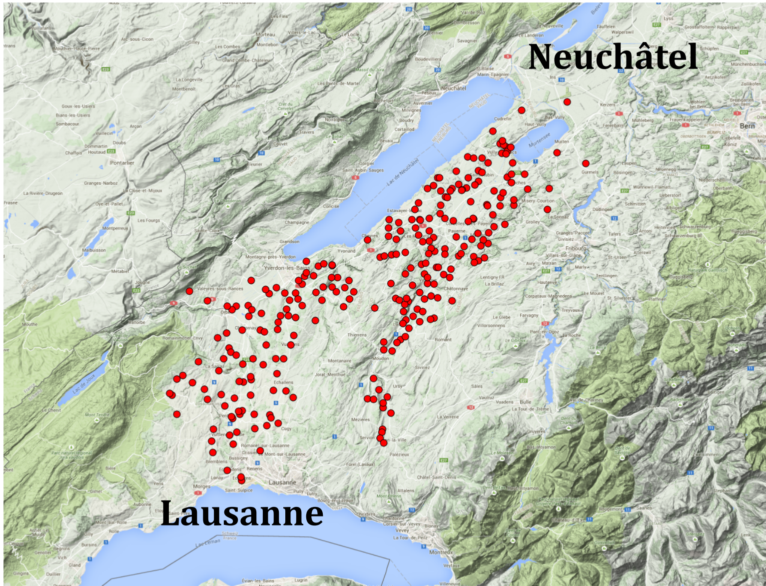


Figure 2: Position of about 250 artificial nest boxes for barn owls (red dots) controlled each year for nest in western Switzerland (46°49N/06°56E).



General aims

The general aims of my PhD were to answer diverse questions related to the evolution of pheomelanin-based color variation under the contexts of sexual dimorphism, sexual genetic conflict, clinal variation, natural selection and the interplay of selection and dispersal. Therefore, I considered numerous approaches from landscape genetics to population genetics and survival analysis, comprising simulations and computation. As a model species, I studied the barn owl because this species present several interesting features that make it particularly suitable to answer those aims. Indeed, the barn owl presents variation within- and between-sexes in its pheomelanin-based coloration. In Europe, the pheomelanin-based coloration varies clinally and was demonstrated to result from local adaptation of color variants. In addition, a potential candidate gene, the *MC1R*, has been highlighted previously to explain color variation in this species. Finally, Prof. Alexandre Roulin followed intensely for more than 20 years the Swiss population of barn owls breeding in artificial nests in an area covering 1070 km^2 (Fig. 2). Consequently, we possess an incredible amount of phenotypic and genotypic data as well as reproductive parameters that are valuable to understand the effect of natural, sexual or neutral processes shaping color and *MC1R* variation.

Therefore, I genotyped *MC1R* of about 3700 nestlings and 800 adults using an allelic discrimination (AD) assay, where fluorescent markers of the mutant and wild-type probes permitted to highlight the presence of each *MC1R* variants by quantitative amplification (San-Jose *et al.* 2015; Chapter 1). All adults and 1400 nestlings were also genotyped for 10 neutral markers to decrypt the neutral genetic variation in the Swiss population. I also contributed to genotype at 22 neutral markers the 109 new European barn owls analyzed in Chapter 4.

In the first chapter of my thesis, we decrypted the effect of *MC1R* variants on pheomelanin- and eumelanin-based colorations in the Swiss barn owls and we tested the hypothesis that a gene responsible for color variation could be accountable for sexual dimorphism. Despite inter-specific studies, how *MC1R* is related to within-species sexual dimorphism, and thereby to sex-specific selection, has not yet been investigated. As we found sex-specific expression of *MC1R* that could suggest sexual genetic conflict at this gene, we aimed in the second chapter to test if males and females differentially inherit the *MC1R* variants and if this pattern could be explained by assortative mating associated with sex ratio deviation. Distortion of allelic inheritance in natural populations has been rarely addressed despite the fundamental role of such pattern in resolving sexual genetic conflict. In the third chapter of my thesis, we investigated if *MC1R* genotypes deviate from Hardy-Weinberg equilibrium in adult males and adult female of the Swiss population, which could be caused by the effect of (sex-specific) selection and/or (sex-biased) dispersal. Although, most studies on dispersal tested its effect on either phenotypic or neutral genetic variation, we were still lacking empirical test of the effect of dispersal and gene flow on potential adaptive genetic variation. Finally, previous studies supported the effect of divergent selection rather than neutral processes to explain color and *MC1R* clinal variation in Europe. However, those methods lack the possibility to highlight the selective agent behind adaptive variation and therefore do not provide information on how or if population would be able to adapt in the face of environmental change. Therefore, we aimed in the fourth and last chapter to explain the variation in pheomelanic-based coloration and *MC1R* variation by climatic and landscape factors using a large dataset of GIS-based information of European barn owls.

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Effect of the *MC1R* gene on sexual dimorphism in melanin-based colorations

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Abstract

Variants of the melanocortin-1 receptor (*MC1R*) gene result in abrupt, naturally selected colour morphs. These genetic variants may differentially affect sexual dimorphism if one morph is naturally selected in the two sexes but another morph is naturally or sexually selected only in one of the two sexes (e.g. to confer camouflage in reproductive females or confer mating advantage in males). Therefore, the balance between natural and sexual selections can differ between *MC1R* variants, as suggest studies showing interspecific correlations between sexual dimorphism and the rate of nonsynonymous *vs*. synonymous amino acid substitutions at the *MC1R*. Surprisingly, how *MC1R* is related to within-species sexual dimorphism, and thereby to sex-specific selection, has not yet been investigated. We tackled this issue in the barn owl (*Tyto alba*), a species showing pronounced variation in the degree of reddish pheomelanin-based coloration and in the number and size of black feather spots. We found that a valine (V)-to-isoleucine (I) substitution at position 126 explains up to 30% of the variation in the three melanin-based colour traits and in feather melanin content. Interestingly, *MC1R* genotypes also differed in the degree of sexual colour dimorphism, with individuals homozygous for the II *MC1R* variant being 2 times redder and 2.5 times less sexually dimorphic than homozygous individuals for the VV *MC1R* variant. These findings support that *MC1R* interacts with the expression of sexual dimorphism and suggest that a gene with major phenotypic effects and weakly influenced by variation in body condition can participate in sex-specific selection processes.

Keywords: adaptive coloration, barn owl, genetic basis of coloration, natural selection pigmentation, sexual selection

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Introduction

The melanocortin-1 receptor (MC1R) is a classical example of a close match between genotype and phenotype. This receptor is involved in the biochemical cascade leading to the production of melanin pigments, and it is frequently associated with intra- and interspecific variation of pigmentation in wild (Theron *et al.* 2001; Rosenblum *et al.* 2004; Baião & Parker 2012; reviewed in Roulin & Ducrest 2013) and domestic animals (reviewed in Linderholm & Larson 2013). In wild animals, missense mutations at different sites of the *MC1R* gene result in abrupt colour changes that lead to the occurrence of alternative colour morphs within or between populations (Mundy 2005; Uy *et al.* 2009; Dessinioti *et al.* 2011; Nowacka-Woszuk *et al.* 2013). New mutations can be naturally selected particularly in response to selection for colour background matching and, thereby, in response to predator–prey relationships (Kaufman 1974; Hoekstra *et al.* 2004, 2006). This process seems to occur in different taxa (mammals; Nachman *et al.* 2003; birds; Cibois *et al.* 2012; and reptiles; Rosenblum *et al.* 2004), supporting the hypothesis that alternative colour morphs might have evolved in a convergent

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manner through mutations at the *MC1R* gene and potentially as a result of strong variation in natural selective pressures (Manceau *et al.* 2010).

Comparisons between species indicate that the evolution of *MC1R* may not only depend on natural selection but also on sexual selection (Nadeau *et al.* 2007), which further supports that colour evolution through *MC1R* may finally depend on the balance between these two selective forces. Nadeau *et al.* (2007) showed that the rate of nonsynonymous *vs*. synonymous amino acid substitutions (dN/dS) at the *MC1R* positively correlates with the degree of sexual dimorphism in melanin-based colour traits of galliforms. However, the mechanism through which *MC1R* could affect sexual dimorphism remains unknown, particularly because the potential link between *MC1R* and sexual selection has been largely overlooked for several reasons. First, the major effects of *MC1R* on the expression of colour morphs are not or scarcely sensitive to environmental variation (i.e. *MC1R*-related variation in colour unlikely functions as a sexually selected condition-dependent signal; Cotton *et al.* 2004). Second, the occurrence of assortative mating with respect to colour morphs suggests that no *MC1R* variant is expected to have a higher reproductive advantage (Mundy *et al.* 2004). Third, a system of discrete colour morphs encoded by *MC1R* has often been shown to play a major role in camouflage, photoprotection (Jablonski & Chaplin 2010) and, probably, thermoregulation (Clusella Trullas *et al.* 2007) and therefore, natural selection may have a more important role than sexual selection in the evolution of variation at the *MC1R* gene. Finally, although variation at the *MC1R* has been observed to underlie colour polymorphism in sexually dimorphic species (Doucet *et al.* 2004), most of the species studied until now show no sex differences in coloration and relatively simple, discrete colour variation.

However, because of its fundamental role in melanin synthesis, we predict that certain mutations at the *MC1R* may entail correlated changes in the extent to which colour differs between males and females. From a proximate point of view, a mutation that, for instance, induces an increase in *MC1R* activity may produce dark coloured traits where melanin concentration is closer to saturation (as for instance in black morphs of artic skuas, *Stercorarius parasiticus*; Mundy *et al.* 2004). If sexual dimorphism is based on factors inducing a higher or a lower melanin synthesis only in one sex, these factors may have a less evident effect when jointly expressed with a more active *MC1R* (i.e. both sexes are already close to saturation in melanin content) than with a less active *MC1R* variant. From an ultimate point of view, if *MC1R* affects the degree of sexual dimorphism, *MC1R* variants allowing for larger sexual dimorphism could be selected because a dark or pale coloration is sexually selected in one sex and/or because natural selection is stronger in one sex (for instance, for cryptic coloration in females). In contrast, if natural selection to be cryptic is similar in both sexes, *MC1R* variants inducing similar adaptive coloration will be positively selected in both sexes. When natural and sexual selection forces are more or less balanced, intralocus sexual conflict at the *MC1R* may occur given that a given variant will be positively selected in one sex (e.g. a variant allowing for noncryptic colour in the sexually selected sex) and an alternative variant in the other sex (e.g. a variant allowing for cryptic colours in the sex that takes care of the offspring).

Understanding the role of *MC1R* in the expression of sexual dimorphism is key to understand potential conflicts arising between natural and sexual selections during the evolution of melanin-based colour traits. Here, we investigated whether *MC1R* is polymorphic in the barn owl (*Tyto alba*) and whether this polymorphism is associated with pheomelanin-based coloration (varying from white to dark reddish) and with the number and size of black eumelanic spots located on the tip of the ventral feathers (Fig. 1A). Although each sex can express any phenotype, females have on average a redder pheomelanic plumage with more and larger black spots than males (Roulin 2003; Dreiss & Roulin 2010). The reddish pheomelanic coloration seems to have evolved in response to local selective pressures (Antoniazza *et al.* 2010, 2014), maybe as an adaptation to different physical habitats and/or to prey on different rodent species (Roulin 2004; Charter *et al.* 2012; Dreiss *et al.* 2012). Eumelanic black spots are sexually antagonistically selected, with females and males being selected to display large and small spots, respectively (Roulin 1999; Roulin *et al.* 2010; Roulin & Ducrest 2011).

We first examined whether *MC1R* is associated with pheomelanin and eumelanin feather contents and with the three melanin-based colour traits. We measured each plumage colour trait on different body parts: the breast, belly, flank and underside of the wings, given that there exists substantial variation among these body parts (Table 1), and therefore, they could be differently associated with *MC1R* and sex. We then specifically tested whether alleles at the *MC1R* are differentially related to the degree of offspring sexual dimorphism measured as the difference in plumage coloration between male and female nestlings of the same genotype. In the barn owl, the degree of sexual dimorphism changes with age because males and females show different patterns of plumage maturation (Dreiss & Roulin 2010). In both sexes, reddish plumage coloration becomes lighter with age, but males lose spots and females exhibit larger spots with age. Thus, we also

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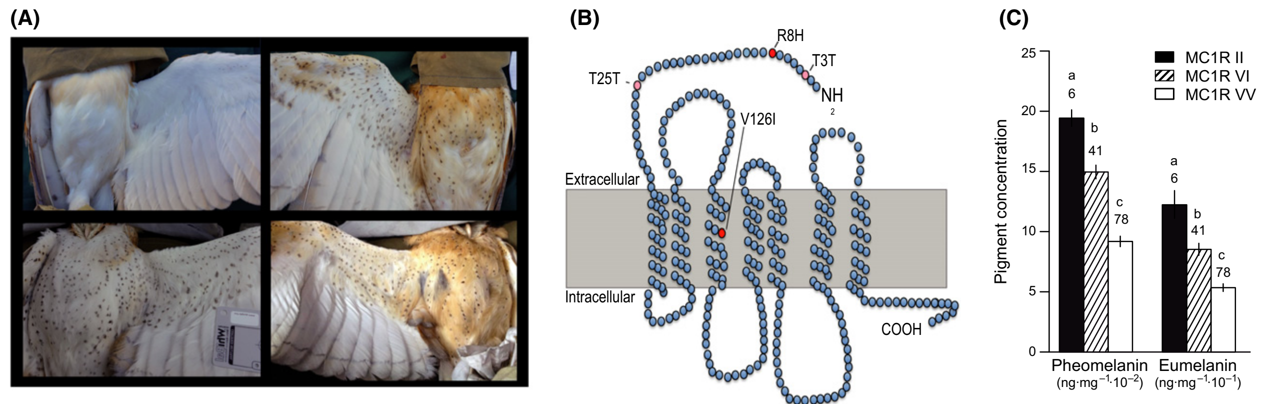

Fig. 1 Variation in melanin-based plumage traits, location of *MC1R* variants in the protein and effects of *MC1R* on melanin feather content in the barn owl. (A) Variation in the reddish pheomelanic coloration and in the number and size of eumelanic spots across and within nestling barn owls. (B) Two-dimensional model of the MC1R protein of the barn owl with polymorphic sites highlighted in red (nonsynonymous substitution) and light red (synonymous substitution). (C) Differences between *MC1R* genotypes for the mutation V126I in feather deposition of pheomelanin and eumelanin. For each pigment, mean (± SE) are reported, letters (a, b, c) indicate significant differences among *MC1R* genotypes, and numbers above bars indicate sample size.

analysed whether *MC1R* alters age-related changes in melanin-based traits and whether such changes induce variation in the degree of plumage sexual dimorphism.

Material and methods

Colour measurements and assessment of melanin pigments

The study was performed in western Switzerland in a population of wild barn owls breeding in nest boxes. Between 1996 and 2013, we collected blood and feather samples and measured melanin-based plumage traits on 2803 nestlings close to the fledging age (*c.a.* 50 days; for further details on sample size see Table S1 and S2, Supporting information). Nestlings were sired by 367 different males and 434 females (579 different pairs), and their sex was identified using molecular markers (Py *et al.* 2006). Because melanin-based traits are differentially expressed on the ventral body parts (Table 1), we measured plumage traits on the breast, belly and flank and on the underside of the wings. The pheomelanic reddish coloration, which is homogeneous on each body part, was scored using eight-colour chips ranging from -8 (white) to -1 (dark reddish), a method that highly correlates with objective spectrophotometric measurements ($r = -0.78$, $P < 0.0001$, $N = 1107$; Dreiss & Roulin 2010). The eumelanic black spots were counted within a 60 × 40 mm frame, and their diameter was measured to the nearest 0.1 mm. Measurement of all plumage traits are highly repeatable (for further details see Roulin 2004). A total of 783 adults (335 males and 448 females, Table S3, Supporting information) for which we have repeated measures over several breeding seasons were used to investigate the effect of *MC1R* on age-related changes in plumage traits. Some individuals ($n = 417$) were ringed as adults, and their age was estimated based on their moulting pattern (see Dreiss & Roulin 2010); however, statistical analyses (not shown) were qualitatively the same when only individuals of known exact age (i.e. ringed as nestlings) were used.

We analysed the amount of pheomelanin and eumelanin pigments in feathers in a subset of 125 nestlings (58 males and 73 females) from 43 nests using the same protocol as described in Roulin *et al.* (2013) for the barn owl (see also; Wakamatsu *et al.* 2002; Ito & Wakamatsu 2011).

MC1R sequencing

Genomic DNA was extracted from blood or dried feathers using DNeasy Blood Tissue or QiAmp DNA Micro kits (Qiagen, Hombrechtikon, Switzerland). Primers MC1R_44Fw and MC1R_944Rev designed based on *Gallus gallus* sequence (for sequences and protocols, see Table S4 and Appendix S1, Supporting information) amplified 900 bp of the *MC1R* coding sequence under the following conditions: 25 ng of genomic DNA, 250 nM of MC1R_43Fw and MC1R_944Rev, 200 μM dNTPs, 1× Qiagen buffer, 1× Q solution, 0.5 U of Taq polymerase (Qiagen, Hombrechtikon, Switzerland) at 95 °C for 5 min, followed by 34 cycles at 94 °C for 30 s, 59 °C for 30 s and 72 °C for 1 min and final elongation at 72 °C for 10 min in 50 μL. The amplicons of 23 individuals of the extreme colour morphs (dark reddish and heavily spotted vs. white and immaculate) were purified with MinElute PCR purification kit (Qiagen, Hombrechtikon, Switzerland), TA-cloned in pGEMT

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Table 1 Observed mean (± SE) plumage trait values in nestling barn owls of different *MC1R* genotypes. Values are given for each *MC1R* genotypes for the mutation V126I (II, VI and VV), each body part and each plumage trait

	MC1R genotype II				*MC1R* genotype VI				*MC1R* genotype VV			
	Breast	Belly	Flank	Wing	Breast	Belly	Flank	Wing	Breast	Belly	Flank	Wing
Reddish colour												
Male nestlings	2.64 ± 0.08	3.40 ± 0.09	2.74 ± 0.08	3.62 ± 0.11	3.12 ± 0.04	4.56 ± 0.07	3.51 ± 0.05	4.99 ± 0.07	5.15 ± 0.04	7.70 ± 0.02	5.90 ± 0.04	7.68 ± 0.02
Female nestlings	2.19 ± 0.08	2.76 ± 0.09	2.18 ± 0.08	2.88 ± 0.10	2.58 ± 0.03	3.43 ± 0.04	2.80 ± 0.04	3.53 ± 0.05	3.65 ± 0.02	6.18 ± 0.04	4.43 ± 0.03	5.98 ± 0.04
Number of black spots												
Male nestlings	60.96 ± 3.06	23.60 ± 2.53	51.04 ± 2.41	28.64 ± 2.27	61.33 ± 1.44	24.20 ± 1.04	53.54 ± 1.15	36.07 ± 0.92	50.28 ± 1.11	16.83 ± 0.63	52.31 ± 0.75	29.71 ± 0.53
Female nestlings	67.65 ± 3.22	25.48 ± 2.86	57.57 ± 2.92	35.40 ± 2.46	65.08 ± 1.16	29.09 ± 1.01	58.37 ± 1.05	41.34 ± 0.80	68.73 ± 0.90	36.16 ± 0.77	66.19 ± 0.64	51.25 ± 0.53
Spot diameter (mm)												
Male nestlings	1.20 ± 0.04	1.13 ± 0.07	1.44 ± 0.05	0.96 ± 0.04	1.10 ± 0.02	1.16 ± 0.03	1.39 ± 0.02	1.13 ± 0.02	0.94 ± 0.01	0.91 ± 0.02	1.40 ± 0.02	1.29 ± 0.02
Female nestlings	1.38 ± 0.04	1.25 ± 0.05	1.63 ± 0.05	1.12 ± 0.03	1.31 ± 0.02	1.31 ± 0.02	1.57 ± 0.02	1.24 ± 0.02	1.23 ± 0.01	1.44 ± 0.02	1.74 ± 0.01	1.59 ± 0.01

(Promega, Duebendorf, Switzerland) and plasmids sequenced in a 3130XL Genetic Analyzer (Life Technologies, Zug, Switzerland) with a special protocol that is in 10 μL with 2 μL of Big Dye V 3.1, 2 μL of 5× Q solution (Qiagen, Hombrechtikon, Switzerland), 1 μL of 10 μM of Primer T7 or SP6, 2 μL of plasmid diluted to 100 ng/ul and amplification at 98 °C for 2 min, 35 cycles at 96 °C for 15 s, 55 °C 15 s and 60 °C for 3 min. Sequences were aligned in CodonCode Aligner 3.7.1.2 (CodonCode Corporations, Dedham, MA, USA). To complete the coding sequence (CDS) and obtain the upstream and downstream UTR of *MC1R* sequences, we used RACE and genome walking assays using GeneRacer kit (Life Technologies, Zug, Switzerland) and GenomeWalker universal kit (Clontech, Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France), respectively (see Appendix S1, Supporting information). We then directly sequenced (without cloning) the whole CDS using MC1R-34Fw and MC1R_969Rev (located at the 5′ of the start codon and the 3′ of the stop codon, respectively). When DNA quality was not good enough to get the whole CDS, we separately amplified the first and second half of the gene with two distinct PCRs: one amplicon of 606 bp with the specific primers MC1R-34Fw and MC1R_568Rev and one of 565 bp with MC1R_404Fw and MC1R_969Rev (3 min at 95 °C; 35 cycles 30 s at 95 °C, 1 min at 62 °C, 1 min at 72 °C; 10 min at 72 °C). Sequencing was performed as described previously. The ancestral–derived status of *MC1R* alleles was defined by comparison with the *MC1R* sequence of the tawny owl, *Strix aluco* (Access number: KF201577.1), and chicken, *Gallus gallus* (NM_001031462).

Using allelic discrimination, we genotyped all the individuals for the mutation V126I, the most frequent nonsynonymous mutation found at the *MC1R* gene of the barn owl (see Results). Probably due to the high GC content of *MC1R*, a pre-amplification PCR was necessary before performing the allelic discrimination assay. Each individual was genotyped twice using two independent PCR products (for further details, see Appendix S1 and Table S4, Supporting information).

Statistical procedure

We first investigated whether *MC1R* genotypes for the nonsynonymous mutation V126I (i.e. homozygotes VV and II and heterozygotes VI) differ in the amount of pheomelanin and eumelanin pigments deposited in breast feathers collected in fledglings. We fitted separated linear mixed models for pheomelanin and eumelanin concentrations including nest of origin as random factor and *MC1R* genotype and sex as fixed factors. We then investigated the effect of *MC1R* on the expression

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of the reddish coloration and the number and size of the black spots in fledglings. Each plumage trait was analysed as dependent variable in separate linear mixed models. We accounted for within-subject colour variation among body parts (breast, belly, flank and the underside of the wings) by fitting mixed models for longitudinal data with nestling identity as random effect (Pinheiro & Bates 2000). Models also included the random effect of year of birth and of maternal and paternal identities as well as *MC1R* genotype for the V126I mutation, sex, and body part (and all their interactions) as fixed factors.

To specifically investigate whether *MC1R* accounts for differences in the degree of offspring sexual dimorphism in different plumage traits, for each breeding pair we calculated mean plumage trait values of brothers and then of sisters who shared the same *MC1R* genotype. For each plumage trait and body part, genotype and family, sexual dimorphism was calculated as 'daughter value – son value' (i.e. positive values indicate female-biased melanization and negative values male-biased melanization). Values of sexual dimorphism were then standardized for the statistical analysis. Degree of sexual dimorphism was analysed using linear mixed models using *MC1R* genotype, body part, and their interaction (fixed factors) and maternal and paternal identities (random factors). Finally, we investigated whether colour plumage maturation (Dreiss & Roulin 2010) differs between *MC1R* genotypes. Using breeding individuals recaptured over consecutive years (Table S3, Supporting information), we fitted repeated-measures linear mixed models for each colour trait with individual identity and year as random variables and *MC1R* genotype, sex, body part, age (in years) and all their interactions as fixed variables. For this analysis, the sample size for II individuals was low (see Table S3, Supporting information) and only VI and VV individuals were considered. All the analyses were run in R v.3.0.2 (R Core Team, Vienna, Austria), all tests were two-tailed, and significance was set at $\alpha = 0.05$.

Results

Genetic variability at MC1R

We sequenced 1334 bp of the *MC1R*, which comprises 343 bp of the 5′ UTR, 945 bp of the exon that contains the whole coding sequence (CDS) and 46 bp of the 3′ UTR. The sequence is highly GC rich with a GC content of 69% (ENDMEMO, http://www.endmemo.com/bio/gc.php). We sequenced 1003 bp (MC1R-34Fw, MC1R_969Rev), 900 bp (43–944), 603 bp (-34–569) and 565 bp (404-969) of the CDS of 17, 23, 76 and 5 barn owls, respectively. We found two synonymous transitions c.9G>A (T3T) and c.75G>A (T25T), and two nonsynonymous transitions c.23G>A and c.376G>A with the following frequencies of the derived alleles 4.3%, 3.0%, 0.5% and 15.4%, respectively (Fig. 1B). The c.23G>A transition caused an arginine-to-histidine substitution at position 8 of *Gallus* sequence (NM_001031462) (R8H), and that would be located within the first outer loop of the MC1R protein. The most frequent nonsynonymous mutation (c.376G>A) corresponded to a valine-to-isoleucine substitution at position 126 (V126I) and would be located in the third transmembrane of the MC1R. Hereafter, the 'valine' allele is quoted V and the isoleucine allele, I.

MC1R genotypes and melanin feather concentration

Pheomelanin and eumelanin feather contents significantly differed between *MC1R* genotypes ($F_{2,79} = 105.91$, $P < 0.0001$ and $F_{2,79} = 43.06$, $P < 0.0001$, respectively), which explained 47.2 and 34.1% of the total variance in each pigment content, respectively (Table 2). VV nestlings deposited significantly less pheomelanin and eumelanin in their feathers than VI nestlings, and VI nestlings significantly less than II nestlings (Fig. 1C). Pheomelanin and eumelanin feather contents were lower in males (mean ± SE: 1796.38 ± 79.06 ng/mg and 74.37 ± 0.08 ng/mg, respectively) than in females (mean ± SE: 2089.79 ± 68.34 ng/mg and 95.63 ± 0.07; $F_{1,79} = 29.44$, $P < 0.0001$, $F_{1,79} = 27.06$, $P < 0.0001$, respectively). Nest of origin modelled as random effect accounted for 15.2% and 31.1% of the variance in pheomelanin and eumelanin feather contents, respectively.

Effect of MC1R-genotypes on melanin-based plumage traits

The impact of *MC1R* on all plumage traits was sex specific and differed between body parts (significant interactions between *MC1R*, sex and body parts in Table 3). As it can be seen in Fig. 2, the effect of *MC1R* was stronger on the pheomelanin-based reddish coloration than on the number and size of the black spots, which was further confirmed by statistical analysis comparing the relative impact of *MC1R* on the three plumage traits (see Appendix S2, Supporting information).

MC1R explained 33.7% of the total variance of the reddish coloration (Table 2). In the two sexes and for all body parts, II nestlings were significantly but slightly darker reddish than VI nestlings, whereas VV nestlings were clearly lighter coloured than the other two *MC1R* genotypes (see contrasts in Fig. 2A). This effect was stronger in males than in females (Fig. 2A). *Post hoc* contrasts showed that, for all body parts, differences in reddish coloration between II and VV nestlings

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Table 2 Variation in melanin-based plumage traits in nestling barn owls explained by the *MC1R* gene. Shown is the percentage of variance explained by *MC1R* genotypes for the mutation V126I relative to the total variance of the trait (i.e. the four body parts of males and females combined in the same analysis) and relative to the variance within each sex and each body part. Nestling plumage dimorphism refers to the difference in melanin-based plumage traits between male and female siblings. Explained variance for adult coloration (estimated at mean adult age in our sample, i.e. 2 years old) was calculated from models accounting for age variation (see Methods)

		Breast		Belly		Flank		Wing	
Trait	% Of total variance	% Of male variance	% Of female variance	% Of male variance	% Of female variance	% Of male variance	% Of female variance	% Of male variance	% Of female variance
Melanin pigment feather content									
Pheomelanin	47.17	44.60	60.01	—	—	—	—	—	—
Eumelanin	34.11	35.27	49.79	—	—	—	—	—	—
Nestling plumage traits									
Reddish coloration	33.71	40.00	34.55	76.22	54.74	55.96	45.85	71.27	49.59
Number of spots	0.15	4.11	0.04	5.24	0.88	0.57	1.47	4.73	5.88
Spot diameter	0.05	5.72	2.22	5.78	0.15	0.54	0.76	2.25	13.35
Nestling plumage sexual dimorphism									
Reddish coloration	8.69	19.17	—	3.64	—	15.62	—	2.48	—
Number of spots	8.50	5.35	—	10.32	—	5.36	—	15.38	—
Spot diameter	2.10	1.61	—	1.24	—	4.24	—	2.09	—
Adult plumage traits									
Reddish coloration	22.72	53.34	10.32	77.75	30.49	61.22	16.00	79.08	27.88
Number of spots	0.54	1.35	1.39	0.50	0.06	2.45	0.42	0.33	0.92
Spot diameter	0.62	3.79	0.09	0.37	0.94	2.34	0.27	0.20	0.50

Table 3 Effect of *MC1R*-genotypes on reddish coloration, number and size of black spots in nestling barn owls. Linear mixed models to test whether *MC1R* has differential effect on males and females, and on the four different body parts (breast, belly, flank and underside of the wings)

	Reddish colour	Number of black spots	Spot diameter
Nestling identity	39.97%	29.30%	36.70%
Maternal identity	9.51%	13.49%	14.51%
Paternal identity	13.32%	18.54%	20.88%
Year	2.06%	5.04%	7.41%
MC1R	$F_{2,2676} = 1788.39$***	$F_{2,2622} = 4.96$**	$F_{2,2675} = 0.54$
Sex	$F_{1,2591} = 364.95$***	$F_{1,2386} = 60.96$***	$F_{1,2449} = 84.68$***
MC1R x Sex	$F_{2,2589} = 47.45$***	$F_{2,2399} = 47.12$***	$F_{2,2462} = 22.41$***
Body part	$F_{3,8328} = 1274.89$**	$F_{3,7162} = 1185.94$***	$F_{3,6894} = 445.64$***
MC1R x Body part	$F_{6,8331} = 333.20$***	$F_{6,7206} = 18.07$***	$F_{6,6938} = 168.09$***
Sex x Body part	$F_{3,8328} = 23.39$***	$F_{3,7225} = 1.15$	$F_{3,6963} = 4.31$**
MC1R x Sex x Body part	$F_{6,8331} = 19.82$***	$F_{6,7236} = 3.23$**	$F_{6,6971} = 19.68$***

We indicate the percentage of variance explained by the random variables (nestling, maternal and paternal identities as well as year). The symbols ** and ****P*-values below 0.01 and 0.001, respectively.

and between VI and VV nestlings were significantly larger in males than in females (all $t_{2589} > 2.58$, all $P < 0.015$). Differences in reddish coloration between II and VI nestlings were also larger in males than in females but only on the underside of the wings and on the belly (all $t_{2589} > 2.54$, all $P < 0.015$) but not on the flank or on the breast (all $t_{2589} < 0.77$, all $P > 0.47$; Fig. 2A).

With respect to the number of black spots, *MC1R* explained 0.2% of the total variance. This small percentage is in part due to the fact that the effect of *MC1R* differed between sexes and body parts (Table 3, Fig. 2B). When taken this into account, *MC1R* explained between 0.04% to 5.9% of the variance that was specific to each sex and body part (Table 2). *MC1R* sometimes showed even opposite effects in males compared to females. For

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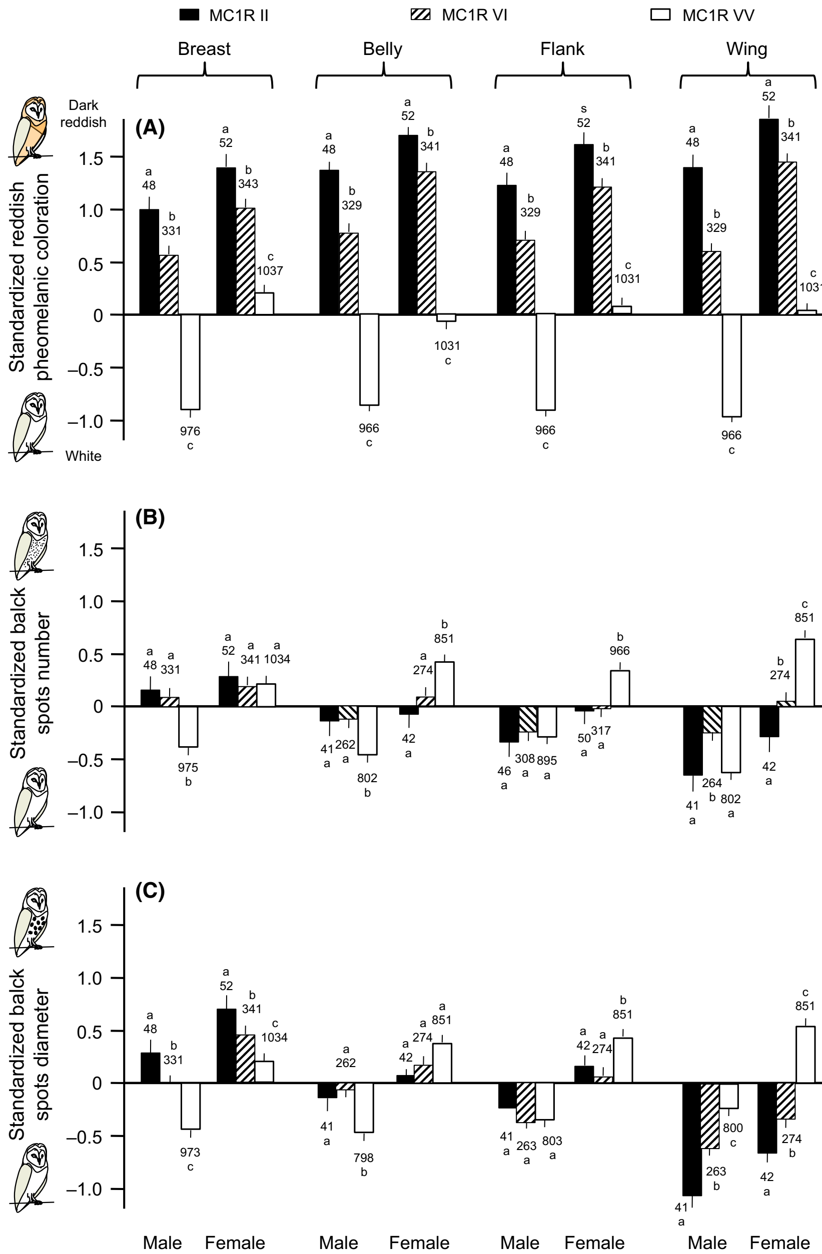

Fig. 2 Effect of *MC1R* on three melanin-based plumage traits in nestling barn owls. For each plumage trait (A. reddish pheomelanic coloration, B. number of black spots, C. diameter of black spots) and body part, we standardized values ([value - mean]/standard deviation) by pooling males and females. Reported are mean (± SE) predicted values obtained from linear mixed models including maternal and paternal identities as random variables and sex, *MC1R* and the interaction 'sex x *MC1R*' as dependent variables. Letters (a, b, c) indicate for each plumage trait and sex whether predicted means of the four body parts are different from each other in individuals sharing the same *MC1R* genotype; when two means have the same letter, it indicates that they are not significantly different from each other. Numbers above bars indicate sample size.

instance, on the belly, VV male nestlings displayed significantly fewer spots than II and VI males, whereas VV female nestlings showed significantly more spots than II and VI females (Fig. 2B). On the breast, significant differences among *MC1R* genotypes were found in males but not in females (VV males showed fewer spots than the other genotypes), whereas the opposite pattern was found on the flank (VV females showed more spots than the other genotypes and no differences existed in males; Fig. 2B). Furthermore, *MC1R* had a heterosis effect on the underside of the wings, because in males (but not in females), homozygous II and VV displayed fewer spots than heterozygous VI (Fig. 2B).

MC1R explained 0.05% of the total variance in spot diameter although *MC1R* explained between 0.2% and 13.4% of the variance that was specific to each sex and body part (Table 2). The effect of *MC1R* differed between body parts in interaction with sex (Table 3). On the breast, II nestlings displayed larger spots than VI nestlings that displayed larger spots than VV nestlings, an effect that was more pronounced in males than in females (t_{2462} = 2.61, P = 0.009; Fig. 2C). On the underside of the wings, the effects of *MC1R* reversed: VV nestlings displayed larger black spots than VI nestlings (particularly in females; t_{2462} = 4.53, P < 0.001), and II nestlings exhibited smaller black spots than VI

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(Fig. 2C). On the belly, *MC1R* genotypes differed in the size of the black spots only in males (VV males showed smaller spots than the other genotypes), whereas on the flank, *MC1R* genotypes differed only in females (VV females showed larger spots than the other two genotypes; Fig. 2C).

Effect of MC1R genotypes on nestling sexual dimorphism

The degree of sexual dimorphism in nestlings differed significantly between *MC1R* genotypes and body parts (Table 4). For all body parts, sexual dimorphism was more pronounced in VV than in VI and II genotypes with respect to reddish coloration (all contrasts t_{1400} > 2.17, all P < 0.031), spot diameter (all contrasts t_{1244} > 2.09, all P < 0.037) and number of spots (all contrasts t_{1194} > 2.84, all P < 0.005; Fig. 3). Sexual dimorphism between II and VI nestlings was only significantly different for the reddish coloration of the underside parts of the wings (t_{1400} > 2.45, all P = 0.014) but not for the reddish coloration of the other body parts or for the number and size of the black spots (all contrasts P > 0.068).

Table 4 Effect of *MC1R* genotypes on sexual dimorphism in reddish coloration, number and size of black spots in nestling barn owls. Results from linear mixed models testing whether *MC1R* has differential effect between males and females, and between the four different body parts (breast, belly, flank and underside of the wings)

	Sexual dimorphism in nestlings		
	Reddish colour	Number of black spots	Spot diameter
Paternal identity	20.12%	15.91%	30.73%
Maternal identity	24.22%	40.09%	40.81%
MC1R	$F_{2,1664}$ = 60.24***	$F_{2,1611}$ = 63.35***	$F_{2,1601}$ = 42.68***
Body part	$F_{3.1400}$ = 2.24	$F_{3.1246}$ = 5.77***	$F_{3.1194}$ = 1.63
MC1R x Body part	$F_{6,1400}$ = 15.74***	$F_{6,1244}$ = 4.11***	$F_{6,1192}$ = 6.83***

We report the percentage of variance explained by the random variables (paternal and maternal identities). ****P*-values are smaller than 0.001.

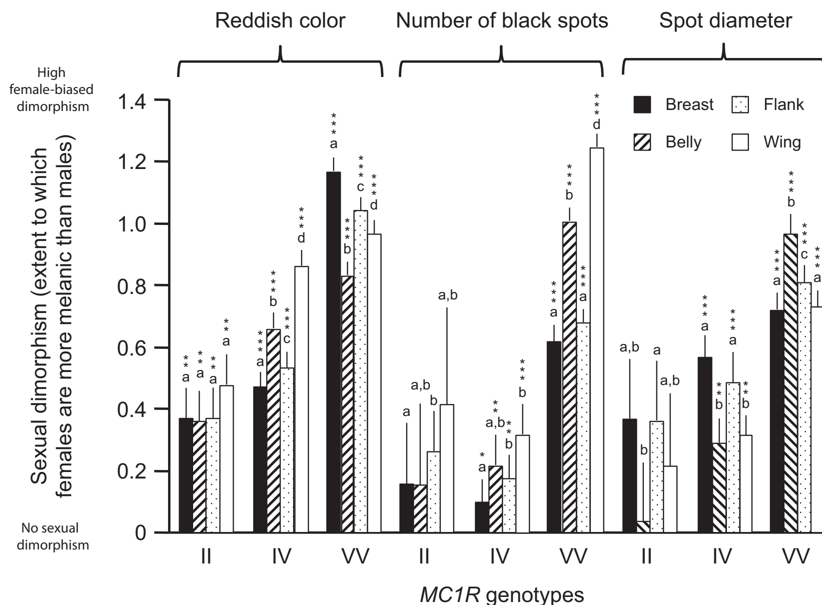

Fig. 3 Effect of *MC1R* genotypes on sexual dimorphism of three melanin-based plumage traits in nestling barn owls. For each colour trait and body part, we calculated sexual dimorphism as the difference between mean values of sons and daughters with the same *MC1R* genotype and use the standardized values for the statistical analyses. Means ± SE are reported. For each genotype and plumage trait, small letters indicate whether mean nestling sexual dimorphism is significantly different between body parts using paired *t*-test (two body parts with the same letter have similar means, whereas sexual dimorphism of two body parts having different letters have different means). Stars above letters indicate whether nestling sexual dimorphism is significantly different from zero using sign test (* for P < 0.05, ** P < 0.01 and *** P < 0.001).

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In homozygous II nestlings, sexual dimorphism did not differ significantly between plumage traits (comparing dimorphism between reddish coloration and number of spots, between reddish coloration and spot diameter and between number of spots and spot diameter; paired *t*-tests: *P*-values > 0.30) although only the reddish coloration was significantly sexually dimorphic (contrasts in Fig. 3). In VI nestlings, sexual dimorphism in reddish coloration was significantly stronger than in the number of spots (t_{135} = 4.79, P < 0.0001) but of similar magnitude as sexual dimorphism in spot size (t_{135} = 1.31, P = 0.19) and sexual dimorphism was more marked in the size than in the number of black spots (t_{135} = 6.78, P < 0.0001). Finally, in VV nestlings, sexual dimorphism was stronger in reddish coloration than in the number and size of black spots (t_{325} = 10.33, P < 0.0001, t_{325} = 8.04, P < 0.0001, respectively) and sexual dimorphism was significantly more pronounced in spot size than in spot number (t_{325} = 2.24, P = 0.026).

Effect of MC1R genotypes on adult sexual dimorphism

Age-related changes in all plumage traits were significantly dependent on the *MC1R* genotype in interaction with sex, *MC1R* and body part (Table 5; Fig. 4). Reddish coloration became lighter with age in all genotypes, body parts and sexes (all t_{4850} > 3.70, all P < 0.001). Males and females differed in the rate at which reddish colour became lighter with age (i.e. the degree of sexual dimorphism changed with age), and such differences were largely dependent on genotype and body part. In VV breeding birds, colour changed more intensely in females than in males (i.e. steeper slopes for the relationship between age and reddish colour in Fig. 4A) for all body parts (all t_{4850} > 2.71, all P < 0.007) except for the breast, where males and females changed with the same rate (t_{4850} = 1.11, P = 0.26). In contrast, in VI adults, male reddish colour changed more intensely than female colour on the belly and flanks (all t_{4850} > 3.55, all P < 0.001) but not on the breast or the underside parts of the wings (all t_{4850} < 1.65, all P > 0.09).

The number of spots significantly decreased with age in all male body parts and for all genotypes (all t_{4621} > 3.58, all P < 0.001), except for the underside parts of the wing in VI males, where no significant change was detected (t_{4621} > 1.82, P = 0.068). In females, it significantly decreased in all body parts of VV adults (all t_{4621} > 3.70, all P < 0.001), whereas in VI females, it significantly increased with age on the wings and flanks (all t_{4621} > 2.28, all P < 0.023) and no significant change occurred on the breast and belly (all t_{4850} < 1.34, all P > 0.18). Further contrasts showed that, in VV adults, the degree of sexual dimorphism increased with age given that number of spots decreased more pronouncedly in males than in females for all body parts (all t_{4621} > 2.66, all P < 0.008) except on the underside parts of the wings (t_{4850} = 0.87, P = 0.38). In VI adults, sexual dimorphism is less pronounced (see also Fig. 3) and only on the underside part of the wings, it was

Table 5 Effect of *MC1R* on age-related changes in reddish coloration, number and size of black spots in adult barn owls. Results from linear mixed models testing the relationship between *MC1R*, sex and body part (breast, belly, flank and underside of the wings) on age-related changes in plumage traits.

	Reddish colour	Number of black spots	Spot diameter
Individual identity	17.20%	43.50%	45.71%
Year	1.61%	1.75%	8.04%
MC1R	$F_{1,776.4}$ = 1195.98***	$F_{1,769.2}$ = 6.71**	$F_{1,795.6}$ = 0.08
Sex	$F_{1,774.4}$ = 533.51***	$F_{1,767.4}$ = 128.44***	$F_{1,794.4}$ = 155.93***
MC1R x Sex	$F_{1,774.5}$ = 3.29	$F_{1,767.4}$ = 37.89***	$F_{1,794.5}$ = 10.23**
Body part	$F_{3,4850}$ = 2922.39***	$F_{3,4628}$ = 1331.28***	$F_{3,4194}$ = 1344.24***
MC1R × Body part	$F_{3,4850}$ = 43.04***	$F_{3,4628}$ = 41.17***	$F_{3,4200}$ = 101.87***
Sex x Body part	$F_{3,4850}$ = 53.83***	$F_{3,4628}$ = 57.23***	$F_{3,4200}$ = 5.43***
MC1R × Sex × Body part	$F_{3,4850}$ = 174.73***	$F_{3,4628}$ = 7.27***	$F_{3,4200}$ = 2.76*
Age	$F_{1,1560}$ = 1238.58***	$F_{1,5239}$ = 140.52***	$F_{1,1735}$ = 1.20
MC1R × Age	$F_{1,5591}$ = 0.55	$F_{1,5239}$ = 42.61***	$F_{1,4601}$ = 18.18***
Sex × Age	$F_{1,5582}$ = 39.04***	$F_{1,5232}$ = 26.50***	$F_{1,4588}$ = 45.29***
MC1R × Sex × Age	$F_{1,5586}$ = 78.65***	$F_{1,5232}$ = 4.16*	$F_{1,4594}$ = 7.71**
Body part × Age	$F_{3,4850}$ = 57.25***	$F_{3,4621}$ = 20.02***	$F_{3,4181}$ = 9.31***
MC1R × Body part × Age	$F_{3,4850}$ = 77.48***	$F_{3,4621}$ = 5.44***	$F_{3,4184}$ = 0.64
Sex × Body part × Age	$F_{3,4850}$ = 34.26***	$F_{3,4621}$ = 3.02*	$F_{3,4183}$ = 0.18
MC1R × Sex × Body part × Age	$F_{3,4850}$ = 22.54***	$F_{3,4621}$ = 1.58	$F_{3,4183}$ = 3.31*

We report the percentage of variance explained by the random variables (individual identity and year). *,** and ****P*-values below 0.05, 0.01 and 0.001, respectively.

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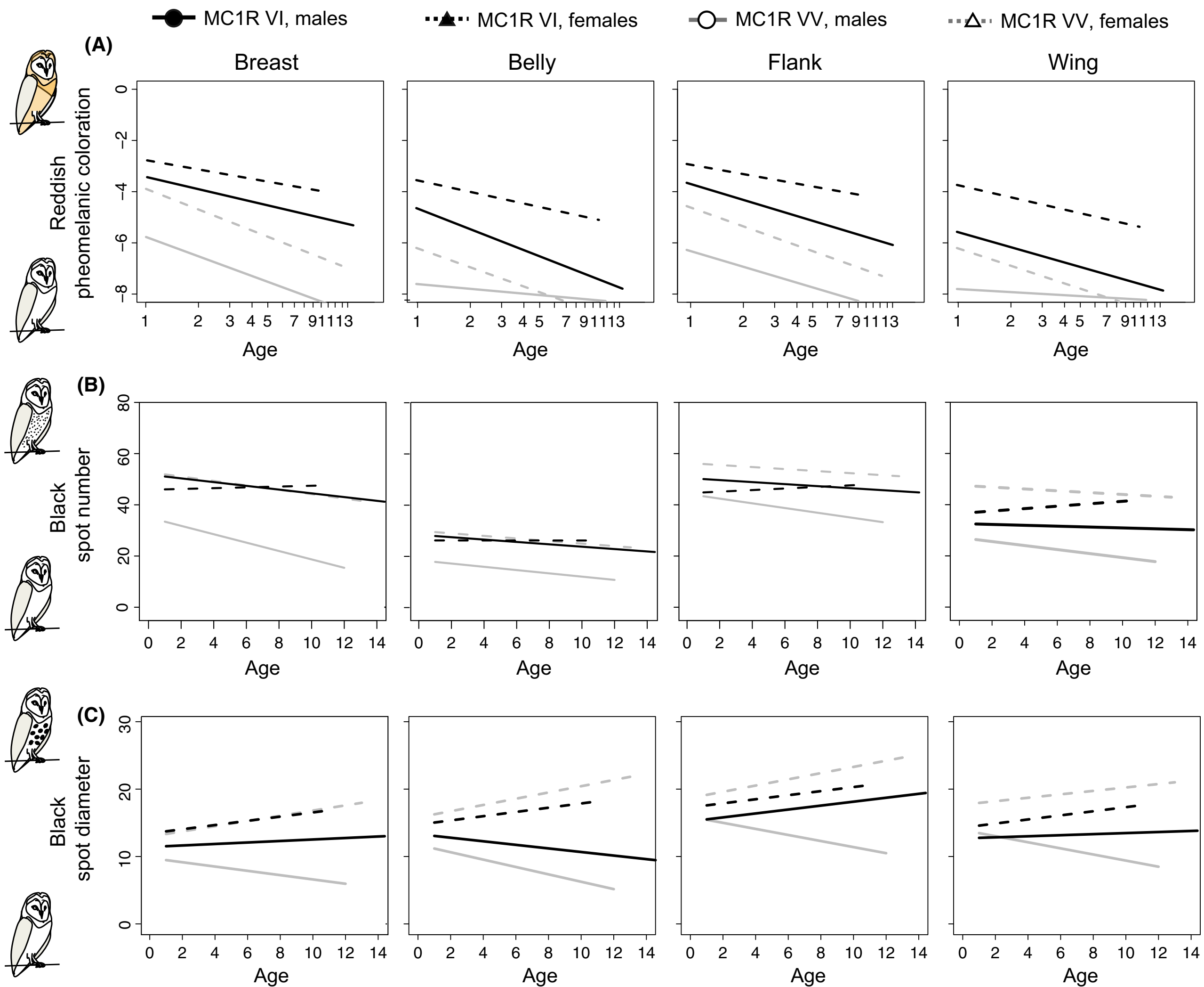

Fig. 4 Effect of *MC1R*-genotypes on age-related changes in three melanin-based plumage traits in adult barn owls. Shown are the predicted regression lines of age on reddish pheomelanin coloration (A), black spot number (B) and black spot diameter (C). For pheomelanic coloration (A), age is plotted with a logarithmic scale. Thick lines indicate slopes that were significantly different from zero.

observed a marked significant increase in sexual dimorphism with age ($t_{4850} = 4.23$, $P < 0.001$).

Spot diameter decreased with age in all body parts of VV males (all $t_{4183} > 3.18$, all $P < 0.002$). In VI males, spot diameter decreased with age on the belly and increased on the flanks (all $t_{4183} > 1.99$, all $P < 0.046$), whereas spot diameter remained unchanged on the wings and on the breast (all $t_{4183} < 0.81$, all $P > 0.42$). In VV females, spot diameter significantly increased in most body parts (all $t_{4183} > 2.20$, all $P < 0.027$) except on the belly ($t_{4183} = 0.29$, $P = 0.78$), whereas it remained unchanged in VI females (all $t_{4183} < 1.85$, all $P > 0.06$). Sexual dimorphism in spot diameter increased with age on all body parts of VV adults (all $t_{4183} > 4.34$, all $P < 0.001$), whereas in VI adults, sexual dimorphism increased on the belly ($t_{4183} = 2.82$, all $P = 0.005$) but remained constant on the other body parts (all $t_{4183} > 1.07$, all $P > 0.06$).

Discussion

Our study shows that polymorphism at the *MC1R* gene is associated with variation in pheomelanin- and eumelanin-based plumage traits as well as with feather pheomelanin and eumelanin contents in the barn owl. More importantly, our results indicate that *MC1R* genotypes differ in the degree of nestling sexual dimorphism and in age-related changes in the degree of adult sexual dimorphism. These findings are consistent with the hypothesis that even if natural selection is the major force promoting the evolution of MC1R-related variation in coloration (Kronforst *et al*. 2012), this gene may

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also play a role when selection on coloration is sex specific by allowing for colour variation between sexes.

Polymorphism at the MC1R gene relates to colour variation in the barn owl

In Swiss barn owls, the *MC1R* sequence presents one relatively frequent nonsynonymous mutation at the position 126 (V126I). Recently, we have also confirmed the presence of this mutation (as well as its association with plumage coloration) in 21 other barn owl populations across Europe (R. Burri, S. Antoniazza, A. Gaigher, A. L. Ducrest, C. Simon, The European Barn owl Network, L. Fumagalli, J. Goudet, A. Roulin, unpublished data). The same mutation with similar effects on the phenotype has been reported in other bird species, which supports the existence of convergence at both genetic and phenotypic levels (Manceau *et al.* 2010). As observed here in the barn owl, the mutation V126I is present in the Gyrfalcon (*Falco rusticolus*) and in the domestic duck (*Anas platyrhynchos*), where this valine–isoleucine substitution is also associated with darker plumage colorations (Johnson *et al.* 2012; Zhan *et al.* 2012; Yu *et al.* 2013). The same mutation has been also observed in chickens (*Gallus gallus*), although no clear association with plumage coloration has been reported, probably because of the masking effect of closely linked mutations at the MC1R (Kerje *et al.* 2003; Dávila *et al.* 2014). The V126I mutation found here in the barn owl is located in the third transmembrane domain of the MC1R (Fig. 1B), which (together with the second domain) plays a key role in MC1R activation (García-Borrón *et al.* 2005). Actually, in humans, mutations at this location (e.g. M128T) induce a partial loss of function of the MC1R (MC1R exhibits a lower affinity to bind alpha-MSH and low coupling activity to cAMP; Pérez Oliva *et al.* 2009). These findings suggest that the V126I mutation found here could have a functional impact on the MC1R although, obviously, biochemical analyses are still needed, particularly because of the expected conservative changes (the two amino acids share physicochemical properties). We found a second nonsynonymous mutation at position 8 (R8H), which has been previously detected in the artic skua in association with plumage coloration (Janssen & Mundy 2013). The H-allele (associated with pale coloration in skuas) occurred at very low frequency (0.5%) in our studied population and in other European populations (R. Burri, S. Antoniazza, A. Gaigher, A. L. Ducrest, C. Simon, The European Barn owl Network, L. Fumagalli, J. Goudet, A. Roulin, unpublished data), although whether it could be at higher frequencies at other world populations deserves further attention (Roulin *et al.* 2009).

In the barn owl, the mutation V126I is strongly associated with plumage traits and, particularly, with the pheomelanin-based plumage. MC1R explained around the 33% of the variance in the reddish plumage coloration (~40% of the genetic variation; Roulin & Jensen 2015) and 47% of the variance in feather pheomelanin content (Table 2), which indicates that other genes involved in coloration are yet to be discovered in this species in contrast to other species where MC1R accounts for all variation in coloration (e.g. Gangoso *et al.* 2011). The *MC1R* gene accounts for a similar amount of variance in other species where adaptive melanin-based colour variation exists (e.g. in the beach mouse, *Peromyscus polionotus*; Hoekstra *et al.* 2006). Previous studies also support that variation in the pheomelanin-based coloration in the barn owl could have evolved as an adaptation to local selective pressures (Antoniazza *et al.* 2010, 2014), which is also in line with previous findings showing that alternative colour morphs exploit different physical habitats (red individuals tend to occupy less forested habitats and white individuals open landscapes) and prey on different rodent species (Roulin *et al.* 2004; Charter *et al.* 2012; Dreiss *et al.* 2012). The *MC1R* gene could be therefore an important part of the genetic underlying basis of such adaptive process, although the question that remains to be tackled is the implication that other loci may have in interaction with *MC1R* and whether variation at the *MC1R* gene drove local adaptation across Europe by merely altering the reddish coloration or also by pleiotropically affecting other traits (Mogil *et al.* 2003; Gangoso *et al.* 2011).

Variation at the *MC1R* gene was less markedly associated with eumelanic traits (Fig. 2), explaining between 0.04 and 5.9% of the variance in the number of spots (between <1% and 9.5% of the genetic variance) and 0.2 and 13.4% of the variance in spot size (between <1% and 5% of the genetic variance; Roulin & Jensen 2015). In the breast, *MC1R* affects the production of eumelanin pigments and spot number and size in the similar sense as for reddish plumage coloration (I-allele leads to a higher expression of eumelanin and pheomelanin; Figs 1C and 2), rather than to a higher expression of pheomelanin at the expense of eumelanin as observed in other species (Hubbard *et al.* 2010). In the other body parts, MC1R differentially affects the expression of eumelanic plumage traits, suggesting that other genes than *MC1R* may influence the overexpression of eumelanin at the specific time points when these spots are produced. The additive or epistatic action of other genes might be responsible for the large variation observed in the effect of *MC1R* on different body parts. While the effects of *MC1R* seem to be always incompletely dominant for the reddish plumage coloration, we observed in the number of spots the existence of dominance effects (heterozygous VI and homo-

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zygous II were rather similarly coloured, whereas homozygous VV was clearly lighter coloured), opposite effects in males compared to females (on the belly, homozygous VV displays fewer spots in males but more in females compared to other genotypes) and effects only on heterozygous (heterosis) (on the wings, heterozygous males displayed more spots than homozygous II and VV males).

With respect to spot diameter, the impact of MC1R was exactly the opposite on different body parts, with the V-allele inducing larger black spots on the underside of the wings but smaller spots on the breast. Variation in plumage traits is pronounced not only between individuals but also within individuals. Thus, our results show that it is indeed the case with, for example for reddish coloration, the effect being less strong on the breast than on the belly, flank and wing, being stronger on the belly than on the flank and wing and being stronger on the wing than on the flank (Fig. 2). Similar variation in the strength of MC1R effects across body parts has been previously reported (e.g. Hoekstra *et al.* 2006), but, to our knowledge, variation in the direction of *MC1R* effects has never been reported in other species. This supports that *MC1R* can have an intricate effect on the expression of different plumage traits on different body parts, which suggests the existence of epistatic or additive effects between *MC1R* and other melanogenic genes.

Polymorphism at the MC1R gene and sexual dimorphism

We observed that the different genotypes at the *MC1R* gene differ in the degree of sexual dimorphism. In fledglings, we observed that homozygous VV individuals are more sexually dimorphic in all plumage traits than in the other genotypes (Fig. 3). Our results therefore suggest that the *MC1R* interacts in a nonadditive manner with the factors that determine colour variation between sexes in the barn owl. Otherwise, no significant effects of *MC1R* on the degree of sexual dimorphism would have been observed, which would have supported an additive effect (i.e. the *MC1R* gene affects coloration but with the same effect size on each sex). Nonadditive effects can result from epistatic effects (e.g. the phenotypic effects of the genes determining differences between sexes and age classes depend on the genotype at the *MC1R*) or from *MC1R* genotypes differing in their sensitivity to environmental conditions. The fact that colour traits are highly heritable in the barn owl and only very weakly sensitive to the environment (Roulin & Dijkstra 2003; Roulin *et al.* 2010) supports the existence of epistatic effects between the *MC1R* and genes inducing sexual dimorphism in coloration, although further studies are still needed to fully discard the existence of genotype-by-environment interactions.

By affecting sex-related colour variation, the way that the *MC1R* gene can drive the evolution of coloration grows in complexity. For instance, as observed here in the barn owl, *MC1R* affects the degree of sexual dimorphism of breast spots, a trait that has been shown to be under sexually antagonistic selection (large breast spots are favoured in yearling females but deselected in yearling males; Roulin *et al.* 2010). Homozygous females for the allele I exhibit larger spots and VV males exhibit smaller spots than other genotypes (Fig. 2C), suggesting that the I-allele and V-allele could be advantageous in females and males, respectively, and, moreover, that the *MC1R* could be responsible for the unsolved sexual conflict. However, we also observed that the V-allele allows for larger differences between sexes in breast spot size, supporting that this allele could still have a slightly higher advantage as it allows producing more sexually dimorphic offspring. Under this scenario, we would expect the V-allele to be more successful than the I-allele under sexual selection (or sex-specific natural selection). However, other factors should still be considered, particularly at the light of the multiple phenotypic effects of the alternative *MC1R* alleles shown by our study. Thus, as suggested above, the I-*MC1R* and V-*MC1R* variants may be subjected to local selection because of their effects on the reddish plumage coloration and, thus, the net selection on *MC1R* cannot be simply understood by its impact on spot size or in any single colour trait (the three plumage traits are genetically correlated; Roulin & Jensen 2015).

Moreover, we showed that *MC1R* genotypes also exhibit different patterns of colour maturation, affecting the degree of sexual dimorphism at different ages (Fig. 4). For some traits, for instance the diameter of breast black spots, sexual dimorphism increased with age in VV breeding birds but remained constant in VI individuals (Fig. 4C), reinforcing the pattern observed in nestlings (Fig. 3). Interestingly, *MC1R* age-related colour changes also led to opposite effects on sexual dimorphism in nestlings and in adults. For instance, differences between males and females in the reddish coloration of the belly (larger in VV than in II nestlings; Fig. 3) tend to disappear with age in VV adults but to increase in II adults (Fig. 4A). Therefore, net selection on *MC1R* has to be understood in a life history context, considering at what moment of the life, cycle selection is acting on coloration and the potential changes in the direction of selection that may occur across an individual lifetime. Although age-related changes in coloration are widespread, studies investigating selection in relation to coloration at different ages are generally lacking (although see Saino *et al.* 2013) and, to our knowledge,

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no study investigated whether selection on *MC1R* varies across an individual's lifetime.

The *MC1R* gene is a remarkable example to understand the genetic basis of convergent evolution on melanin-based traits, particularly in response to strong natural selection, for instance, for background matching (Manceau *et al.* 2010). Here, we investigate the effects of *MC1R* on plumage colour traits of the barn owl but also its impact on sexual dimorphism, which is ubiquitous in animal populations. We showed that the *MC1R* gene explains a substantial part of variation in plumage traits in the barn owl but, moreover, that it has nonadditive effects on the degree of sexual dimorphism. These findings support that the evolution of colour variation through the *MC1R* gene is likely subjected to the interplay between multiple selective forces. Future studies are therefore needed to understand how often such forces conflict between each other and, for instance, whether pre-existing selection for sex-related colour variation hinders the evolution of adaptive colour variation through the *MC1R* gene. Such conflict is likely to occur given that often selection favours a concealed sex (usually females) and a more conspicuous sex (usually males).

Our study also provides answers to previous studies evidencing that the *MC1R* is somehow involved in the evolution of sexual dimorphism. Nadeau *et al.* (2007) showed that bird clades that evolved a more marked sexually dimorphic melanin-based coloration present a higher rate of amino acid changes (dN/dS) at the *MC1R* but not at other melanogenesis-related genes such as tyrosinase (*TYR*), tyrosinase-related protein-1 (*TYRP1*) and DOPA-chrome tautomerase (*DCT*). As observed here for the V-allele, some variants of *MC1R* allow for larger differences between sexes, suggesting that increased sexually dimorphism can evolve through the accumulation of *MC1R* mutations of similar effects. Our study offers a more complex picture of the potential effects of *MC1R* in coloration and highlights the need to approach the study of *MC1R* considering the action of the multiple selective forces acting on coloration.

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A.R. conceived the study and obtained funding. A.R. and A.-L.D. designed the study. A.R. and P.B. conducted fieldwork; A.-L.D. and V.D. conducted all the genetic analysis; K.W. conducted feather pigment analyses; and A.R. and L.M.S.-J. conducted the statistical analyses. A.R. and L.M.S.-J. wrote the manuscript with important contributions of A.-L.D. and V.D. All authors read and provided input on the manuscript.

Data accessibility

MC1R sequences: GenBank Accession nos.: KR018388, KR018389, KR018390, KR018391, KR018392. Phenotypic and genotypic data: Dryad repository: doi:10.5061/dryad.202f5.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Sample size in nestling barn owls of different *MC1R*-genotypes.

Table S2. Number of male and female nestling barn owls sampled between 1996 and 2013.

Table S3. Sample size of individual barn owls recaptured over several years.

Table S4. Sequences of the Primers used in this study.

Appendix S1. Supplementary Material and Methods

Appendix S2. Supplementary Results.

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Supporting Information

Table S1. Sample size in nestling barn owls of different *MC1R* genotypes. Number of male and female nestling barn owls as well as the number of families for which we could measure sexual dimorphism between brothers and sisters. Sample sizes are given for each *MC1R* genotype at the V126I mutation (II, VI and VV), each body part, and each plumage trait (reddish coloration, the number and size of the black spots).

	MC1R genotype II				*MC1R* genotype VI				*MC1R* genotype VV			
	Breast	Belly	Flank	Wing	Breast	Belly	Flank	Wing	Breast	Belly	Flank	Wing
Reddish colour												
Male nestlings	49	49	49	49	333	331	331	331	981	971	971	971
Female nestlings	52	52	52	52	344	342	342	342	1043	1037	1037	1037
Sexual dimorphism	16	16	16	16	16	11	15	11	16	11	11	11
Number of black spots												
Male nestlings	49	42	47	42	333	263	309	265	980	806	899	806
Female nestlings	52	42	50	42	342	275	318	275	1040	857	972	857
Sexual dimorphism	136	136	136	136	136	108	127	110	136	109	109	109
Spot diameter												
Male nestlings	49	42	42	42	333	263	264	264	978	802	807	804
Female nestlings	52	42	42	42	342	275	275	275	1040	857	857	857
Sexual dimorphism	329	325	325	325	327	271	306	271	326	269	271	271

Table S2. Number of male and female nestling barn owls sampled between 1996 and 2013.

Year	Male nestlings	Female nestlings	Sum
1996	94	114	208
1997	48	61	109
1998	90	91	181
1999	65	66	131
2000	12	9	21
2001	85	96	181
2002	131	121	252
2003	88	64	152
2004	59	88	147
2005	90	85	175
2006	28	35	63
2007	148	120	268
2008	97	104	201
2009	21	32	53
2010	86	82	168
2011	66	91	157
2012	147	170	317
2013	9	10	19

Table S3. Number of barn owls for which we measured plumage traits over several years. Sample sizes are given for each *MC1R* genotype at the V126I mutation (II, VI and VV), each body part, sex and plumage trait. For the statistical analysis on age-related colour changes, II individuals were excluded given the low sample size.

	MC1R genotype II				*MC1R* genotype VI				*MC1R* genotype VV			
	Breast	Belly	Flank	Wing	Breast	Belly	Flank	Wing	Breast	Belly	Flank	Wing
					Reddish colour							
Adult males	10	10	10	10	91	91	91	91	234	234	234	234
Adult females	11	11	11	11	120	120	120	120	317	317	317	317
					Number of black spots							
Adult males	10	10	10	10	91	88	88	88	234	226	226	226
Adult females	11	11	11	11	120	111	111	111	317	300	300	300
					Spot diameter							
Adult males	10	10	10	10	91	76	76	76	234	199	200	200
Adult females	11	6	6	6	120	94	94	94	317	258	258	258

Table S4. Sequences of the Primers used in this study.

Primer Name	**Sequence (from 5' to 3')**
a) Primers designed on Gallus	
MC1R_43Fw	AACGCCAGTGAGGGCAACCA
MC1R_944Rev	TACCAGGAGCACAGCACCACCT
b) Genome walking primers	
MC1R_660fw	CATCCTCCTGGGCGTCTTCTTCATCT
MC1R_775fw	TCCACATCCTCATCATCTGCAACTCGG
c) Race primers	
MC1RTa_134Rev	AGGAAGAGCCCGTTGGGGATGT
MC1RTa_228Rev	CAGATGAAGTAGTACGTGGGCGAGTG
d) Sequencing specific primers	
MC1R_-34fw	GGGACCCCGGGGTTGAGGCG
MC1R_568rev	GGCAGAGGAGGATGGCGTTGTTGCG
MC1R_404fw	TCATCGCCGTGGACCGCTACATCACCA
MC1R_969rev	GCGTTAACCCGCGTCCCGCTGC
e) Allelic discrimination assay primers	
MC1R_198fw	CCTGCACTCGCCCACGTACTACTTC
MC1R_453Rev	GTGGTAGCGCAGGGCGTAGAAGAT
V126I_fw	CATGGACAACGTCATCGA
V126I_rev	GCGTAGAAGATGGTGATGTA
V126I_wt_Fam-BHQ1	TGCAGCTCCGTCGTGTCCTC
V126I_mut_ATTO550-BHQ2	TGCAGCTCCATCGTGTCCTC

Supplementary Materials and Methods

Genome walking were used to identify the 3' end of the *MC1R* coding sequence. According to the GenomeWalker universal kit (Clontech, Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France), the primers MC1R_660fw was used in combination with AP1, while primers MC1R_775fw was used in combination with AP2 primer from the universal kit during the initial and nested PCR, respectively. Cycling conditions and polymerase were those recommended by the kit.

For the Race, **t**otal RNAs were extracted from growing feather bases using RNeasy mini kit (Qiagen, Hombrechtikon, Switzerland) and 2.5 μg of total RNA was reverse transcribed with oligodT primer and Superscript III and proceeded to the RACE assay following the GeneRacer kit protocol (Life Technologies, Zug, Switzerland) and cloned and sequenced as described in Materials and Methods. 500 nM MC1RTa228Rev was used in combination with 500 nM GeneRacer-5' to the first amplification of cDNA prepared as described in the kit with 200μM dNTPs, 1x Kapa buffer A, 1x Kapa Enhancer, 1U Kapa Robust 2G (Labgene Scientific, Châtel-St-Denis, Switzerland) in 50 μl with a first denaturation at 95°C for 5 min, a touchdown cycling with 95°C 25 sec, a decrease of 1°C at each cycle for 10 cycles for the annealing starting at 70°C 30 sec and 72°C 1 min, followed by 32 cycles with 95°C 25sec, 60°C 30 sec, 72°C 1 min and a final elongation at 72°C for 10 min. The nested PCR was then conducted in 50 μl with 1/50 of the product of the first PCR with 500 nM GeneRacer-5'-nested, 500 nM MC1RTa134Rev and the Kapa Robust 2G (Labgene) as above with the following conditions: 95°C 5min, 35 cycles at 95°C 25 sec, 59°C 30 sec, 72°C 1min and 72°C 10 min. The PCR products were then gel purified on 1% agarose in 1xTBE with the Minelute Gel extraction kit (Qiagen, Hombrechtikon, Switzerland), cloned and sequenced as described previously.

For the allelic discrimination (AD) assay, pre-amplification PCRs were performed using exactly 20 ng of DNA sample, 1x Q-solution (Qiagen, Hombrechtikon, Switzerland), 200µM MC1R_-198fw and MC1R_453rev primers, 0.2 U of Taq (Qiagen, Hombrechtikon, Switzerland) into a final volume of 20µl with the following cycle conditons: 95 °C for 5 min, 34 cycles at 94 °C for 30 sec, 63 °C for 30 sec, and 72 °C for 30 sec, and final extension at 72°C for 10 min. As initial DNA concentration is critical for AD assays, relative quantity of the PCR products were compared using a 2% agarose gel and adjusted among each other and then diluted 100 times before the AD assay. AD assays were run in an ABI 7500 qPCR machine (Life Technologies, Zug, Switzerland). Each qPCR plate contained three positive controls (corresponding to each genotype) and at least two negative controls. The qPCR MasterMix Plus Low ROX (Eurogentec, Liège, Belgium) was used with an annealing temperature of 57°C for 60 sec in a final volume of 24 µL with 300 nM of V126I_fw and V126I_rev, 100 nM of V126I_wt_Fam-BHQ1 (Microsynth, Balgach, Switzerland), 250 nM V126I_mut_ATTO550-BHQ2 (Microsynth, Balgach, Switzerland) and 2 µl of diluted DNA.

Supplementary results:

Relative impact of *MC1R* on plumage traits in the barn owl

To statistically test the relative impact of *MC1R* on the three plumage traits, we performed linear mixed models on standardized plumage trait values for each body part and using nestling, maternal and paternal identities as random factors and sex, *MC1R,* type of plumage trait (reddish coloration, number of spots, spot diameter), and all their interaction as independent variables. On the breast, the impact of *MC1R* was stronger on reddish coloration than number of spots (interaction *MC1R* × plumage trait: $F_{2,2793} = 309.82$, $P < 0.0001$), on

reddish coloration than spot diameter (interaction *MC1R* × plumage trait: $F_{2,2788} = 228.18$, $P < 0.0001$) and on spot diameter than number of spots (interaction *MC1R* × plumage trait: $F_{2,2788} = 14.50$, $P < 0.0001$). We obtained similar results for the belly, flank, and the underside of the wings (all *P*-values < 0.04) except that, on the flank, the impact of *MC1R* on the number and size of spots was not significantly different (interaction: $F_{2,2458} = 1.88$, $P = 0.15$).

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Sex-specific allelic transmission bias suggests sexual conflict at *MC1R*

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Abstract

Sexual conflict arises when selection in one sex causes the displacement of the other sex from its phenotypic optimum, leading to an inevitable tension within the genome – called intralocus sexual conflict. Although the autosomal melanocortin-1-receptor gene (*MC1R*) can generate colour variation in sexually dichromatic species, most previous studies have not considered the possibility that *MC1R* may be subject to sexual conflict. In the barn owl (*Tyto alba*), the allele $MC1R_{WHITE}$ is associated with whitish plumage coloration, typical of males, and the allele $MC1R_{RUFOUS}$ is associated with dark rufous coloration, typical of females, although each sex can express any phenotype. Because each colour variant is adapted to specific environmental conditions, the allele $MC1R_{WHITE}$ may be more strongly selected in males and the allele $MC1R_{RUFOUS}$ in females. We therefore investigated whether *MC1R* genotypes are in excess or deficit in male and female fledglings compared with the expected Hardy–Weinberg proportions. Our results show an overall deficit of 7.5% in the proportion of heterozygotes in males and of 12.9% in females. In males, interannual variation in assortative pairing with respect to *MC1R* explained the year-specific deviations from Hardy–Weinberg proportions, whereas in females, the deficit was better explained by the interannual variation in the probability of inheriting the $MC1R_{WHITE}$ or $MC1R_{RUFOUS}$ allele. Additionally, we observed that sons inherit the $MC1R_{RUFOUS}$ allele from their fathers on average slightly less often than expected under the first Mendelian law. Transmission ratio distortion may be adaptive in this sexually dichromatic species if males and females are, respectively, selected to display white and rufous plumages.

Keywords: assortative pairing, colour polymorphism, heterozygote deficit, melanocortin-1 receptor, pheomelanin-based coloration, transmission ratio distortion

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Introduction

Males and females share most of their genomes; consequently, selection exerted on a trait in one sex can cause the other sex to move from its phenotypic optimum if the second sex is selected in the opposite direction. For example, in dichromatic species, males may be sexually selected to exhibit conspicuous colour patterns and females naturally selected to express a drabber version of this colour trait to improve camouflage (Siefferman & Hill 2003; Simpson *et al.* 2015). When a phenotype selected in opposite directions in males and females (i.e. sexually antagonistic selection) is encoded by the same set of genes in males and females, a tension within the genome is inevitable – called intralocus sexual conflict (Arnqvist & Rowe 2005; Bonduriansky & Chenoweth 2009; Dean *et al.* 2012; Rice & Gavrilets 2014). This tension arises because parents pass on a given allele to the sex that accrues fitness benefits from it but also to the other sex, which is disadvantaged. Selection may thus favour mechanisms that prevent members of one sex from inheriting (Calsbeek & Bonneaud 2008) or expressing (Ellegren & Parsch 2007) alleles that are detrimental to that sex but beneficial to the other sex.

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To address this conflict, we can examine whether there is departure in the proportion of different genotypes from Hardy–Weinberg (HW) equilibrium in male and female offspring for the gene suspected to be under sex-specific selection. Such a departure may be due to assortative pairing, the process by which individuals with similar genotypes or phenotypes mate more frequently than expected at random. This pairing pattern maximizes the likelihood that the offspring inherit beneficial parental alleles or phenotypes but not in a sex-specific manner. For this reason, this mechanism may have a weak impact on the resolution of intralocus sexual conflict unless the sex ratio is biased towards sons when the allele or phenotype most likely to be inherited is beneficial only to males (and inversely for daughters) (Roulin *et al.* 2010). Indeed, the inheritance of maladaptive or incompatible alleles can lead to mortality at an early developmental stage (Pryke & Griffith 2009), possibly in a sex-specific manner (Qvarnstrom & Bailey 2009). Such a phenomenon is detected if there is a significant departure from 1:1 Mendelian ratios of allele inheritance. This 'transmission ratio distortion' (TRD) (for a review, see Huang *et al.* 2013) is detected when some parental alleles are transmitted to the next generation at lower or higher frequency than expected under random segregation of alleles. This distortion could similarly affect the two sexes if selective pressure to inherit the beneficial allele is exerted similarly on the two sexes; as a corollary, distortion may differ between the two sexes if selective pressure to inherit adaptive alleles is sex specific. For example, the so-called meiotic drive (Sandler & Novitski 1957) facilitates the transmission of one allele at the expense of the other alleles at the same locus to increase its own representation among gametes. Such a mechanism could easily spread if it confers an increase in fitness to the carrier (Johns *et al.* 2005; Wilkinson *et al.* 2006; Gell & Reenan 2013; Zanders & Malik 2015).

Although intralocus sexual conflict was until recently mainly considered in captive organisms, new evidence from wild populations of nonmodel species is expected to provide comparative data and to highlight which mechanisms may be responsible for the evolution of sexually dimorphic traits (Bonduriansky & Chenoweth 2009). One appropriate system is the barn owl (*Tyto alba*), a cosmopolitan nocturnal bird of prey for which in Europe, mutations at the *MC1R* locus explain up to 30% of the variation in the sexually dichromatic rufous coloration (at position 126, the ancestral valine $MC1R_{WHITE}$ allele (V) encodes white coloration, and the derived valine-to-isoleucine $MC1R_{RUFOUS}$ allele (I) encodes rufous coloration; San-Jose *et al.* 2015; Burri *et al.* 2016). This gene is responsible for melanin-based colour polymorphism in several species of birds and mammals (Majerus & Mundy 2003; Fontanesi *et al.* 2010; Nunes *et al.* 2011; Hedrick & Ritland 2012; Roulin & Ducrest 2013; Abitbol *et al.* 2014; Ding *et al.* 2014; Lamichhaney *et al.* 2016). Although each sex can express any phenotype, males are on average whiter than females, which suggests that males are more strongly selected to display a pale plumage than females or that the two sexes are selected in opposite directions (i.e. males to be white and females to be rufous) (Roulin 2003; Dreiss & Roulin 2010). In this context, white males have a higher probability to be recruited in the local breeding population than their redder conspecific (Roulin & Altwegg 2007). Previous studies showed that the pheomelanin-based coloration is involved in predator–prey relationship (Roulin 2004; Charter *et al.* 2012) and a current experiment suggests that a white coloration could provide a selective advantage over a reddish coloration because their preys 'freeze' (i.e. remain motionless) under full-moon conditions and so are vulnerable for a longer period of time (L. M. San-Jose, C. Judes, A. Questiaux, B. Almasi, P. Beziers, A. Amar & A. Roulin, unpublished data). Moreover, local adaptation for hunting is expected to be stronger in males because they provide most of the prey items to their progeny and their partner until the three-first weeks of rearing. Regarding females, the rufous morph acquires a fitness advantage when breeding in open habitats possibly as a result of crypsis (Dreiss *et al.* 2012) and suggests that in some instances the $MC1R_{RUFOUS}$ allele is beneficial at least in females. We therefore predict differences in the direction of selection for colour between males and females; therefore, we might expect an intralocus sexual genetic conflict at *MC1R*.

In this study, we combined population genetic analyses and field observations from a long-term study of a Swiss barn owl population to test whether in daughters and sons, the frequencies of *MC1R* genotypes (i.e. VV, VI and II) depart from HW proportions because of genotype- and sex-specific mortalities, nonrandom segregation of alleles and/or nonrandom pairing. We genotyped individuals at 10 microsatellite markers to ensure that HW deviation in *MC1R* is not due to population structure or demography, as well as to confirm that extra-pair paternity and inbreeding do not bias our estimates. Subsequently, we tested whether HW deviation could be explained by three factors. First, assortative pairing with respect to *MC1R* can ensure that offspring will inherit a specific allele. However, because this pairing pattern affects sons and daughters similarly, we examined whether the offspring sex ratio differs between categories of breeding pairs with, for example, more sons being produced by $♂_{VV} \times ♀_{VV}$ pairs than $♂_{VI} \times ♀_{VI}$ or $♂_{II} \times ♀_{II}$ pairs. Second, a

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deviation in HW proportions in a population may be caused by fitness differences between breeding pairs that produce different proportions of heterozygous offspring. We therefore measured a number of fitness-related traits (clutch size, hatching success, fledging success and overall success of the clutch from laying to fledging) in relation to parental genotypes. Third, we tested for transmission ratio distortion and whether this can be explained by nestling mortality at different developmental stages (in eggs, during hatching and before fledging), processes that can be sex specific.

Materials and methods

Data collection

From 1998 to 2012, we followed a population of barn owls in western Switzerland (46°49′N/06°56′E) in an area covering 1070 km^2, where we fixed artificial nest boxes. We monitored 90–95% of the breeding population and marked with aluminium rings 90% of the breeding adults and all nestlings since 1988. We could therefore establish a reliable pedigree. In this population, 8% of the males and 13% of the females produce two annual clutches (Béziers & Roulin 2016), and 42% of the males and 27% of females bred in more than one year (males are more philopatric than females). For these reasons, several broods were sampled from the same breeding individuals (for males, individuals produced between one and nine broods during the study period with a mean of 2.25 broods, and for females, between one and 11 broods with a mean of 1.89 broods).

MC1R and microsatellite genotyping

For each nestling and adult, we collected a blood sample to extract DNA using the DNeasy Tissue and Blood kit or the Biosprint robot (Qiagen). Nestling sex was identified using sex-specific molecular markers (Py *et al.* 2006). We determined *MC1R* genotypes by allelic discrimination (see protocol in San-Jose *et al.* 2015) in 322 male and 443 female adults and in 1490 female and 1457 male fledglings that survived up to fledging, which occurs at *ca.* 55 days of age (of 4420 laid eggs, 324 did not hatch; of 4096 hatchlings, 1058 died before fledging; 91 fledglings could not be genotyped or sexed). The method of allelic discrimination is accurate and presented no difficulty in differentiating heterozygous from homozygous individuals. In each assay, we always ran positive controls for each genotype (II, VI and VV) and each sample was run in duplicate with two different PCRs. Because we could not genotype most of the nestlings that died, we analysed deviations in the proportions of the different genotypes (II, VI, VV) from HW proportions only on individuals that successfully fledged. Nevertheless, we obtained blood samples from 169 nestlings that died before fledging, which gave us the opportunity to test for survival differences between *MC1R* genotypes.

We assessed the neutral genetic diversity using 10 microsatellite loci in 755 male and 737 female nestlings sampled in a large number of families (n = 619) to avoid genotyping too many closely related individuals (an average of 2.11 siblings per family). All adults genotyped at *MC1R* were also genotyped for microsatellites. This gave us the opportunity to test for extra-paternity in the 1492 nestlings (for the methods, see Henry *et al.* 2013). Genotyping procedure for the 10 microsatellite markers (multiplex sets 3 and 4) is described in Burri *et al.* (2016).

Statistical procedure

HW proportions. HW equilibrium assumes random mating, which results in random union of male and female gametes to form zygotes. The expected frequency of heterozygotes (H_E) in the next generation after random mating and the departure from HW proportions (F_{IS}) are calculated as follows:

$$F_{IS} = 1 - \frac{H_O}{H_E} \qquad \text{(eqn 1)}$$

$$H_E = 2 * p * q \qquad \text{(eqn 2)}$$

where H_O is the observed frequency of heterozygotes, and p and q are the allelic frequencies of the alleles I and V in offspring. The measure of H_E is based on the assumption of equal allelic frequencies in male and female adults and of unchanged allelic frequencies from one generation to the next (i.e. p and q are similar in adults and nestlings). Although in our population, the frequencies of the I and V alleles in male and female adults are different, we precisely calculate the expected frequency of heterozygotes in the next generation as follows:

$$H_{E(t+1)} = p_{♀(t)} * q_{♂(t)} + p_{♂(t)} * q_{♀(t)} \qquad \text{(eqn 3)}$$

Finally, for each year, we calculated the F_{IS} values for male and female nestlings using eqns (1) and (3), with H_O being the observed proportion of heterozygotes in nestlings and H_E the expected proportion given the frequencies in their parents.

For microsatellites, we tested the departure from HW proportions (i.e. F_{IS} values) in nestlings using FSTAT v. 2.9.3 (Goudet 1995). F_{IS} values were calculated for each cohort, and a global F_{IS} value was derived by combining all cohorts (Weir & Cockerham 1984). We tested whether F_{IS} values differed from 0 by randomly

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permuting alleles (n = 10 000 permutations). Confidence intervals (95%) around F_{IS} estimates for microsatellites were calculated using GENETIX v4.05.2 (Belkhir *et al.* 1996-2004) by bootstrapping loci (n = 1000). Analysis using CERVUS (Kalinowski *et al.* 2007) and MICRO-CHECKER v.2.2.3 (Van Oosterhout *et al.* 2004) detected no null alleles in any microsatellite across samples.

Inbreeding and extra-pair paternity. To confirm that extra-pair paternity and inbreeding are not responsible for the deviation in HW proportions, we calculated an inbreeding coefficient using the kinship2 R package (Therneau *et al.* 2015) based on the pedigree containing ancestors recorded before 1998. We also analysed the rate of extra-pair paternity using CERVUS software and the paternity analysis function (Kalinowski *et al.* 2007) with an augmented data set compared with previous papers (211 offspring were analysed in Roulin *et al.* (2004) and 455 other nestlings in Henry *et al.* (2013); in this study, we analysed a total of 1492 offspring, including those already analysed in the two previous studies).

Null distribution of F_{IS} values under the hypothesis of random pairing at MC1R. We first tested for a global excess or deficit of heterozygotes at *MC1R* over the 15 years of study for male and female nestlings. To test for a deviation from HW proportions at *MC1R*, we generated the distributions of F_{IS} values under the null hypothesis of random mating. To this end, the pool of breeders' genotypes was randomized to simulate random pairing within each year from 1998 to 2012 (performed using R software v3.1.1; R core team, 2015; Supporting information). Because differential fertility between parental genotypes in 1 year can affect the proportion of each genotype among the offspring, we also permuted the number of genotyped nestlings between breeding pairs. For each new generated breeding pair, a number of offspring were attributed to each genotype following the model of random union of gametes (i.e. Mendelian inheritance) and depending on the fertility of the breeding pair. For example, a pair ♂$_{VI}$ × ♀$_{VV}$ should theoretically produce 50% VI offspring and 50% VV offspring. Additionally, in our model, this 50% ratio will vary depending on the number of offspring they produce (after randomization of the number of genotyped nestlings). We then calculated the observed (H_O) and expected (H_E) heterozygote frequency and the F_{IS} value for each permutation as in eqns (1) and (3). Finally, we averaged the expected F_{IS} values over the 15 years, and to account for annual variation in sample sizes, we weighted the annual F_{IS} values by the corresponding number of genotyped nestlings. This procedure was repeated 10 000 times to generate the null distribution of F_{IS} values and to examine whether the observed F_{IS} in male and female nestlings (i.e. mean F_{IS} values over the study period calculated from the observed frequency of heterozygotes and also weighted by the sample size) falls within the null distribution. If the observed values are outside the confidence intervals of the distributions obtained by permutations, they are considered to depart significantly from HW equilibrium.

Because a global deviation from HW proportions was detected, we applied a post hoc analysis to determine whether these deviations were present in each year and in both male and female nestlings. Permutations of breeders' genotypes were performed 10 000 times each year to generate the null distribution of F_{IS} values and to examine whether the observed F_{IS} value falls within the null distribution. We applied a sequential Bonferroni correction for multiple testing because we performed analyses on a data set of 15 years in both males and females (the threshold of significance is divided by 30, i.e. the number of years multiplied by two sexes, giving a threshold of significance of 0.00167 for the smallest P-value to 0.05 for the largest P-value; Sokal & Rohlf 1995). We report significant results both before and after applying the sequential Bonferroni correction.

Pairing with respect to MC1R to explain HW deviation. We tested whether pairing of adults with respect to *MC1R* is random using a chi-square test on breeding pairs that did successfully fledge offspring (i.e. the same breeders as those used for the F_{IS} test). However, the chi-square approximation was not accurate because some pairing types were rare (Table 1). Therefore, we generated the null distribution of global chi-square values based on all breeding pairs using 10 000 permutations of the *MC1R* genotypes between breeding pairs within each year (Supporting information). If the observed global chi-square value was outside the confidence intervals of the distributions obtained by permutations, we assumed that pairing with respect to *MC1R* genotypes was not random. Expected proportions were calculated using the chisq.test function in R.

Because pairing across years was significantly assortative, we applied a post hoc analysis to examine

Table 1 Assortative pairing with respect to *MC1R* genotypes in breeding barn owls from 1998 to 2012. The expected numbers of each type of breeding pairs expected under random mating were calculated using the chisq.test function in R and are indicated in parentheses

		Females		
		II	VI	VV
Males	II	2 (0)	5 (8)	21 (20)
	VI	7 (2)	58 (49)	106 (120)
	VV	1 (7)	134 (140)	358 (346)

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whether this pairing pattern was present in each year. For each year, we permuted the *MC1R* genotypes between breeding pairs (n = 10 000) to obtain a null distribution of chi-square values, and we examined whether the observed chi-square values fall within the null distribution. We applied a sequential Bonferroni correction (the threshold of significance is divided by 15, i.e., number of years, giving a threshold of significance of 0.0033 for the smallest P-value to 0.05 for the largest P-value; Sokal & Rohlf 1995), and we observed significance both before and after applying this correction.

To estimate the exact effect of nonrandom pairing on the overall deviation from HW proportion, we calculated the proportion of couples that paired assortatively according to *MC1R*-related plumage colour. Because II and VI individuals are similarly coloured, and hence differ markedly from VV individuals (San-Jose *et al.* 2015), we pooled II and VI individuals into the category 'Rufous' (R), and VV individuals are referred to as 'White' (W). We call P the proportion of individuals with phenotype R and Q = 1–P, the proportion with phenotype W. If individuals are pairing at random, we expect to find proportions P^2 of R*R couples, 2PQ of R*W and W*R couples and Q^2 of W*W couples. With a fraction 'x' of assortative pairing, the proportions of R*R and W*W couples are each increased by a fraction PQx, while the proportion of R*W and W*R couples decreases from 2PQ to 2PQ(1−x). To estimate the proportion of couples that assort according to *MC1R*-related plumage colour, we sought the value that minimizes the squared difference between the expected and observed number of the different types of breeding pairs. With the observed proportion of assortative pairing, we calculated the expect proportion for each genotype in offspring and its associated F_{IS}. To obtain a confidence interval for the expected F_{IS}, we generated random draws (n = 10 000) from the expected distributions of couples, with and without assortative pairing, and drew the distribution of expected F_{IS} values. The statistical power to detect assortative pairing was assessed by generating 10 000 samples from a multinomial distribution of the breeding pairs according to their observed frequencies and to the proportions of assortative pairing. A chi-square test was used to compare the 10 000 3 × 3 matrices of pairs of genotypes obtained this way to what should be expected under random pairing. The statistical power was estimated as the proportion of the 10 000 chi-square tests giving a P-value less than 0.05 (Supporting information).

To test whether the observed deficit of heterozygotes is larger than expected by assortative pairing alone, we calculated the proportion of randomizations that gave larger F_{IS} values than those observed in male and female nestlings. To test whether heterozygote deficiency in male and female nestlings responded similar to the assortative pairing proportion in their parents (which would be expected if heterozygote deficiency was due only to assortative mating), we carried out an ANCOVA with annual F_{IS} values as the response variable, sex as a factor and the proportion of assortative pairing as a covariate. A significant interaction between sex and proportion of assortative pairing is an indication that heterozygote deficiency in the two sexes is driven by different mechanisms.

Offspring sex ratio to explain HW deviation. We analysed the offspring sex ratio in relation to *MC1R* breeding pairs because the higher observed deficit of heterozygotes in females compared with males in the population may be due to a higher production of daughters in pairs producing mainly homozygous offspring. To test this hypothesis, we applied a generalized linear mixed model (GLMM) with binomial error to examine the probability of the offspring being male versus female in relation to parental *MC1R* genotype. As a factor, we created three categories of breeding pairs depending on the probability of producing heterozygous offspring: 50% (pairs VI*VI, II*VI and VI*VV), 100% of heterozygotes (II*VV) and pairs producing only homozygotes (II*II, VV*VV). As random factors, we added maternal and paternal identities as well as year.

Fitness differences to explain HW deviation. To examine whether another mechanism may be involved in the deficiency of heterozygotes, we tested whether breeding pairs producing 50% and 100% of heterozygotes showed a lower fitness than pairs producing only homozygotes. To this end, we applied GLMMs with Poisson error to investigate whether clutch size differs between these three categories of *MC1R* breeding pairs. We also tested for differences in hatching success (proportion of eggs that hatched), fledging success (proportion of hatchlings that fledged) and the overall success of the clutch (proportion of eggs that produced a fledgling) using GLMM with binomial error. As random factors, we added maternal and paternal identities as well as year. Parents rarely abandoned their clutches, and such instances were not considered in the analyses of hatching success.

Transmission ratio distortion to explain HW deviation. Genotype proportion deviations could result from nonrandom segregation of alleles during gamete production or from genetic incompatibility leading to differential mortality of genotypes. We performed a GLMM with binomial error to test whether the $MC1R_{RUFOUS}$ ('I') allele is found at a higher or lower frequency in male or female nestlings than expected at

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random given the genotypes and sexes of their parents (i.e. distortion of the sex-of offspring and sex-of parent-specific transmission ratio). To this end, we considered offspring of pairs ♂$_{VI}$ × ♀$_{VV}$ and ♂$_{VV}$ × ♀$_{VI}$ because only in those pairs can we identify from which parent the $MC1R_{RUFOUS}$ allele is inherited. The presence or absence of this allele in nestlings is the response variable, and the breeding pair type (♂$_{VI}$ × ♀$_{VV}$ or ♂$_{VV}$ × ♀$_{VI}$) and interaction with nestling sex ($n_{♂}$ = 493, $n_{♀}$ = 507) are the explanatory variables. A significant difference in the intercept indicates departure from expected ratio of 0.5 according to Mendelian segregation of alleles. Nest of origin and also year were entered as random factors. Because we found a deficiency in the transmission of the 'I' allele over 15 years, we performed a post hoc analysis for each year. We could not run a model with year as a fixed effect (to obtain an intercept for the Sex * Breeding pair type interaction each year) because the model did not converge correctly with three fixed effects and their interactions. However, the random factor 'year' did not explain any variance in the first model; thus, we performed 12 independent models (we could not run models for 2004, 2006 or 2009 due to low sample sizes). We applied a sequential Bonferroni correction and performed that correction for each single effect and for the interaction separately, as the factors are not independent within each model.

We tested whether heterozygote deficiency in male and female nestlings can be explained by deviation from random segregation of alleles. We carried out an ANCOVA with annual F_{IS} values as the response variable, with sex and *MC1R* breeding pair types (♂$_{VI}$ × ♀$_{VV}$ and ♂$_{VV}$ × ♀$_{VI}$) as fixed factors and annual deviation from random segregation of alleles as a covariate (calculated as the difference from 0.5, i.e. the expected proportion of heterozygote offspring in pairs VI*VV).

To examine whether nestling mortality could explain the lower or higher transmission of the $MC1R_{RUFOUS}$ allele in the breeding pairs ♂$_{VI}$ × ♀$_{VV}$ and ♂$_{VV}$ × ♀$_{VI}$, we tested for a difference in the count of each genotype in male and female nestlings that died before fledgling ($n_{♂}$ = 91 and $n_{♀}$ = 78 out of 439 hatchlings that died before fledging) using a chi-squared test. We also applied generalized linear mixed models (GLMMs) with Poisson error to test whether clutch size differed between the two types of breeding pairs in interaction with year. We also tested for a difference in hatching and fledging success each year using a GLMM with binomial error. We added maternal and paternal identities as random factors. Additionally, we tested for a sex ratio bias in interaction with year using a GLMM with binomial error and maternal and paternal identities as random factors.

Results

HW deviation in the proportions of nestling genotypes

In male and female nestlings, we observed totals of, respectively, 42 and 49 homozygotes for the $MC1R_{RUFOUS}$ allele (2.88% and 3.29%, respectively), 358 and 351 heterozygotes (24.57% and 23.56%) and 1057 and 1090 homozygotes for the $MC1R_{WHITE}$ allele (72.55% and 73.15%). By averaging the F_{IS} values over years and weighting them by the sample size, we found an overall deficit of heterozygous males and females (respectively, F_{IS} = 0.075, P = 0.037, F_{IS} = 0.129, P = 0.001). However, we found a large annual variation in the deviation from HW proportions when analysing each cohort and sex separately, with F_{IS} ranging from −0.577 to 0.681 in males and from −0.108 to 0.473 in females. We detected significant deficits of heterozygous females in 1998 (F_{IS} = 0.446, P = 0.0008), 2007 (F_{IS} = 0.318, P = 0.0145) and 2008 (F_{IS} = 0.259, P = 0.0466), deficits of heterozygous males in 2001 (F_{IS} = 0.279, P = 0.0149) and 2009 (F_{IS} = 0.681, P = 0.0027) and deficits in both male and female nestlings in 2002 (respectively F_{IS} = 0.242, P = 0.0265; F_{IS} = 0.266, P = 0.017) (Fig. 1, Table S2, Supporting information). After sequential Bonferroni correction for multiple testing, the deficits of heterozygotes in *MC1R* are significant only for females in 1998 and for males in 2009.

Demographic processes to explain HW deviation

The microsatellite markers showed no evidence of deviation from HW proportions when combining the 15 years of data (male nestlings: F_{IS} = 0.002, P = 0.36; female nestlings: F_{IS} = 0.005, P = 0.21) or when analysing each single year (Table S1, Supporting information). Of 830 different breeding pairs totalling 1176 reproductive attempts, we found only 10 pairs (1.20%; totalling 11 reproductive attempts, 0.94%) composed of highly related individuals (i.e. coefficient of relatedness ≥ 0.25): four 'mother × son' pairs producing in total 11 offspring, two 'father × daughter' pairs producing 12 offspring, two 'brother × sister' pairs producing eight offspring, one 'grandmother × grandson' pair producing two offspring and one 'aunt × nephew' pair producing three offspring. Paternity analysis using 10 microsatellite loci confirmed that extra-pair paternity is very low: 27 of 1403 nestlings (0.02%) were not sired by the male that was feeding them. The levels of inbreeding and extra-pair paternity are very low and thus cannot explain the deviation of *MC1R* from HW proportions.

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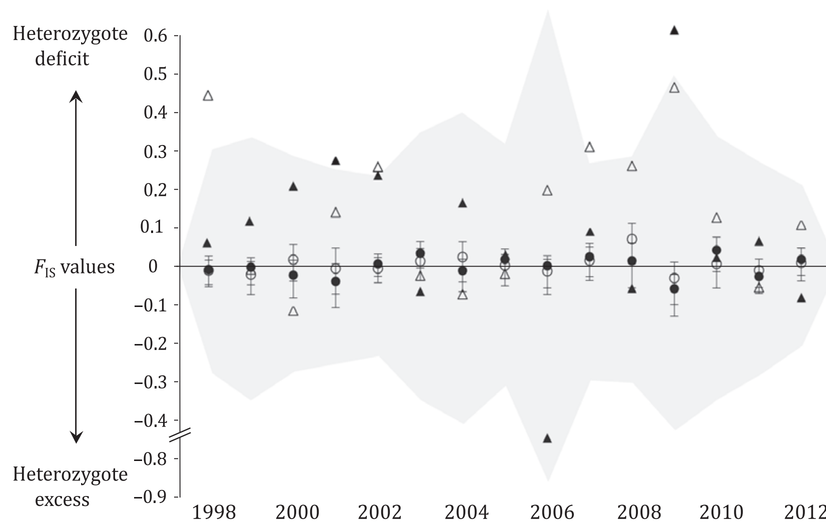

Fig. 1 Deviation in Hardy–Weinberg proportions of nestling genotypes (sample sizes are indicated in Tables S1 and S2) with respect to neutral genetic markers and *MC1R* in barn owls. F_{IS} values were calculated for *MC1R* in males (black triangles), females (open triangles) and for microsatellite loci in males (black circles) and females (open circles). Vertical bars correspond to confidence intervals (95%) around F_{IS} values for microsatellites after bootstrapping among loci (n = 1000). The grey area corresponds to 95% confidence intervals around the F_{IS} values for *MC1R* calculated from the null distribution of F_{IS} under Hardy–Weinberg equilibrium. Values outside the grey area are considered to deviate significantly from expected Hardy–Weinberg proportions.

Nonrandom pairing to explain HW deviation

A potential mechanism explaining a deficit of heterozygous nestlings at the *MC1R* is nonrandom pairing with respect to this gene. We found a proportion of assortative pairing of 8.7%, which is significantly different from random pairing (χ^2 = 25.18, P = 0.0012; Table 1). When evaluating each year separately, we detected significant positive assortative pairing in 1998 and 2002 (respectively, χ^2 = 12.29, P = 0.031 and χ^2 = 14.12, P = 0.007), which was, however, no longer significant after sequential Bonferroni correction (Table S3, Supporting information).

Although the proportion of assortative pairing (8.7%) was low, the statistical power to detect such a value was high (70.9%) because it is based on a large number of breeding pairs (n = 723). It is therefore not surprising that when analysing each year separately, the statistical power to detect a small value of assortative pairing was lower (ranging from 0% to 50.68%). Therefore, we analysed the effect of assortative pairing on the global heterozygote deficiency identified during the entire study period. Accordingly, this pairing pattern can account for 5% of the overall heterozygote deficiency ($F_{IS\ \mathrm{expected}}$ = 0.051, 95% CI = [−0.109; 0.111]), which is lower than the observed value in females (F_{IS} = 0.129; P = 0.011) but not in males (F_{IS} = 0.075; P = 0.11). In line with the prediction that assortative pairing alone cannot explain the higher heterozygote deficiency in female nestlings, we found no significant correlation between the annual proportion of assortative pairing and F_{IS} in females (β = 0.26, t = 0.88, P = 0.39) in contrast to males (β = 1.27, t = 4.31, P < 0.001; Sex * Assortative pairing, ANCOVA, $F_{1,26}$ = 5.89, P = 0.022; Fig. 2).

Sex ratio bias and fitness differences between MC1R breeding pairs to explain HW deviation

The deficit of heterozygotes in daughters could be due to sex ratio deviation if, for example, *MC1R* breeding pairs producing 100% homozygotes (II*II, VV*VV) produced more daughters than *MC1R* breeding pairs producing 50% heterozygotes (VI*VI, II*VI and VI*VV) and those producing 100% heterozygotes (II*VV). This was, however, not the case (GLMM binomial: χ^2 = 0.813, P = 0.67), and sex ratio never departed from 50% in these three types of pairs (mean % of males ± SE: 49.39 ± 1.27%, 49.81 ± 1.39% and 44.83 ± 5.36%, respectively). We also did not find evidence that the sex difference in the heterozygote deficit among fledglings could be explained by differences in fitness between the three types of *MC1R* breeding pairs. They did not differ in the number of eggs laid per clutch (mean ± SE, 6.25 ± 0.08, 6.43 ± 0.09 and 6.48 ± 0.36 eggs, respectively; GLMM Poisson: χ^2 = 0.746, P = 0.69), hatching success (93.15 ± 0.66%, 93.86 ± 0.64% and 91.08 ± 3.17%; GLMM with binomial error: χ^2 = 1.7, P = 0.43), fledging success (73.95 ± 1.46%, 72.27 ± 1.56% and 71.54 ± 5.41%; GLMM binomial error: χ^2 = 0.279, P = 0.87) or the

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percentage of eggs that produced a fledgling (68.56 ± 1.46%, 66.60 ± 1.56% and 64.9 ± 5.9%; GLMM binomial: χ^2 = 1.54, P = 0.47).

Transmission ratio distortion to explain HW deviation

Sex-specific deviation from HW could be the result of either bias transmission of alleles from parents or combined *MC1R*- and sex-specific mortality. To examine this possibility, we specifically tested for transmission ratio distortion of the $MC1R_{RUFOUS}$ allele. We considered the offspring of pairs ♂$_{VI}$ × ♀$_{VV}$ and ♂$_{VV}$ × ♀$_{VI}$ because we can identify which parent transmitted the $MC1R_{RUFOUS}$ allele. We found that the probability for nestlings to inherit this allele depends on their sex in interaction with the type of breeding pair (GLMM binomial, χ^2 = 7.67, P = 0.0054; Fig. 3): in ♂$_{VI}$ × ♀$_{VV}$ breeding pairs, the fathers had a higher probability to transmit the allele 'I' to their daughters (48.9%) than to their sons (40.2%), whereas in ♂$_{VV}$ × ♀$_{VI}$ breeding pairs,

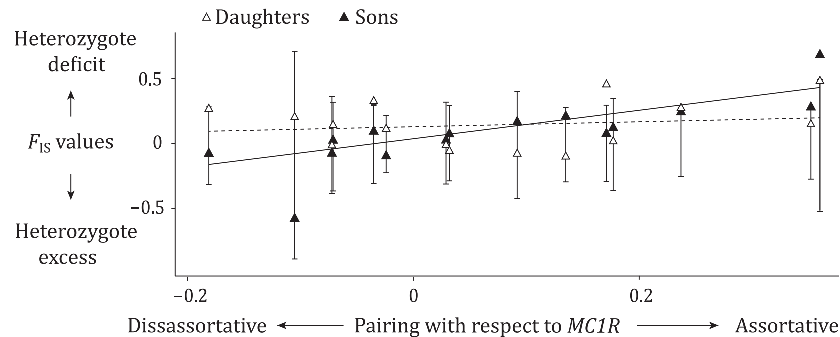

Fig. 2 Annual deviation from Hardy–Weinberg proportions at *MC1R* in male (closed symbols and solid line) and female barn owl nestlings (open symbols and dashed line) in relation to assortative pairing with respect to *MC1R*. Error bars correspond to 95% confidence intervals around the F_{IS} values for *MC1R*, calculated from the null distribution of F_{IS} under Hardy–Weinberg equilibrium (see Fig. 1). Values outside the 95% CI (and after sequential Bonferroni correction) are considered to deviate significantly from expected Hardy–Weinberg proportions.

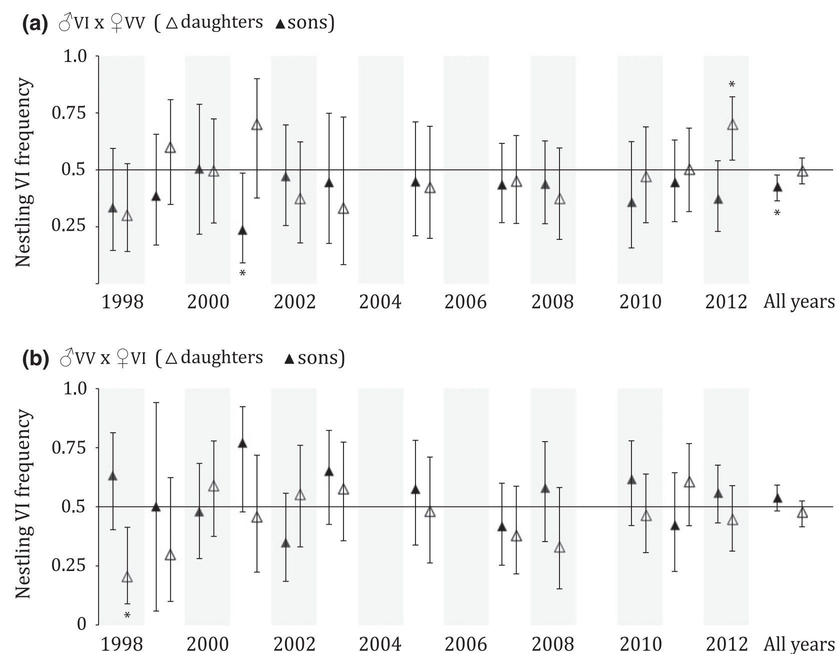

Fig. 3 Heterozygote (i.e. VI) frequencies in male (black triangles) and female (open triangles) nestling barn owls when their father (a) or mother (b) transmitted the mutation $MC1R_{RUFOUS}$ 'I'. The horizontal line for expected 0.5 probabilities under Mendelian inheritance is drawn for illustrative purposes. Statistically significant transmission ratio distortions are indicated by asterisks (P < 0.05; the sex effect in 1998 is rendered nonsignificant after sequential Bonferroni correction). Probability values correspond to estimates from the GLMMs (see results), and error bars correspond to the confidence intervals around the estimates.

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the mothers had a higher probability of transmitting the allele 'I' to their sons (54.8%) than their daughters (45.7%); the random effects 'year' and 'nest of origin' explained, respectively, less than 0.001% and 3% of the variance in the model. The percentage of VI male nestlings born from $♂_{VI} \times ♀_{VV}$ breeding pairs (40.3%) was significantly lower than the expected 50% under random segregation of alleles ($z = -2.84$, $P = 0.0045$), whereas the probability to inherit the 'I' allele from mother (54.8%) was not significantly higher than 50% ($z = 1.56$, $P = 0.12$). Regarding female nestlings, the probability to inherit the 'I' allele from father or mother was not significantly different from expectations (respectively, 48.9%, $P = 0.74$, 45.7%, $P = 0.16$).

When evaluating each year separately, 2 years each showed a significant transmission bias of $MC1R_{RUFOUS}$ allele after correction for multiple testing. In 2001, the transmission of the allele 'I' was again sex specific, but the direction of the relation was opposite between the types of breeding pairs (GLMM binomial, $\chi^2 = 8.12$, $P = 0.0044$). In $♂_{VI} \times ♀_{VV}$ breeding pairs, fathers transmitted the allele 'I' to their sons less often than expected at random (23.5% compared to random expectation of 50%; $z = -2.061$, $P = 0.039$; Fig. 3a), and sons inherited the allele 'I' less often than their sisters (23.5% vs. 70.0%; $P = 0.024$). This sex difference was not significant when mothers transmitted the allele 'I' in $♂_{VV} \times ♀_{VI}$ pairs ($P = 0.12$; Fig. 3b). Although in 2012, we did not observe any significant deviations from HW proportions ($F_{IS\ female} = 0.105$, $P = 0.18$; $F_{IS\ male} = -0.096$, $P = 0.81$; Fig. 1), we found an effect of nestling sex in interaction with the types of breeding pairs on the transmission of the allele 'I' ($\chi^2 = 9.16$, $P = 0.0025$). Fathers from $♂_{VI} \times ♀_{VV}$ breeding pairs transmitted the allele 'I' to their daughters more often than expected at random (70.6% vs. 50.0%; $z = 2.33$, $P = 0.02$), and their brothers inherited less often the allele 'I' than their sisters (38.7% vs. 70.6%; $P = 0.011$). The sex difference was not significant when mothers transmitted the allele ($P = 0.11$; Fig. 3b).

In 1998, the allele 'I' was transmitted to the daughters at a much lower rate than expected under random Mendelian inheritance (26.8% compared to random expectation of 50%; GLMM binomial, $z = -2.846$, $P = 0.0044$; Fig. 3b) and independently of the types of breeding pairs (Sex * Breeding pair: $\chi^2 = 2.04$, $P = 0.15$; *MC1R*: $\chi^2 = 0.62$, $P = 0.43$). The allele 'I' was also transmitted less often to daughters than to sons (26.8% vs. 51.5%; $\chi^2 = 4.76$, $P = 0.029$). However, the effect of sex becomes nonsignificant after sequential Bonferroni correction for multiple year testing. Although in 2006 and 2009, we observed deviations from HW proportions and we could not test the transmission of the $MC1R_{RUFOUS}$ allele in daughters and sons in the two types of breeding pairs ($♂_{VI} \times ♀_{VV}$ and $♂_{VV} \times ♀_{VI}$) because too few nestlings were genotyped in those pairs (13 and 15, respectively). Finally, we did not detect any significant transmission bias in the other years (Fig. 3).

The higher deficit of heterozygous females compared with males could be explained by higher deviations from random segregation of alleles in daughters in particular years. In line with this prediction, we found a significant relationship between annual F_{IS} in daughters and the annual deviation from random segregation of alleles in pairs in which mothers transmit the 'I' allele ($♂_{VV} \times ♀_{VI}$; ANCOVA, $\beta = -0.92$, $t = -2.72$, $P = 0.009$) in contrast to sons ($\beta = -0.27$, $t = -0.46$, $P = 0.65$) and to daughters and sons when fathers transmitted the 'I' allele ($♂_{VI} \times ♀_{VV}$; respectively $\beta = -0.44$, $t = -1.39$, $P = 0.17$ and $\beta = -0.18$, $t = -0.51$, $P = 0.61$). In years when mothers transmitted the 'I' allele to daughters at a lower frequency than expected under random segregation of alleles, the deficit in heterozygotes compared with HW equilibrium was more pronounced (Fig. 4). The slopes for males and females within and between

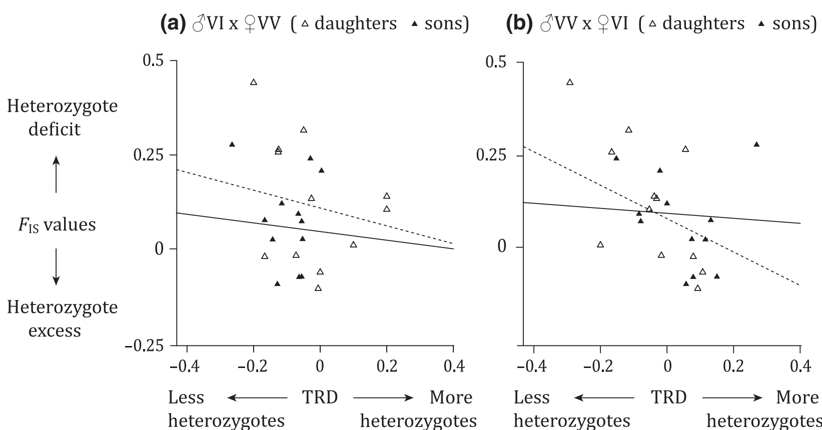

Fig. 4 Annual deviation from Hardy–Weinberg proportions at *MC1R* in male (closed symbols and solid line) and female barn owl nestlings (open symbols and dashed line) in relation to deviation from random segregation of alleles in VI × VV breeding pairs (a) when the father or (b) mother transmits the mutation $MC1R_{RUFOUS}$ 'I'. Values of transmission ratio distortion (TRD) correspond to deviation from the expected 0.5 ratios of heterozygotes in offspring, with negative values for deficit of heterozygotes and positive values for excess of heterozygotes.

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the two types of breeding pairs are, however, not significantly different ($F_{1,44} = 0.45$, $P = 0.50$).

MC1R- and sex-specific mortality to explain HW deviation

We found no significant differences in clutch size, hatching success, fledging success or clutch success between the $♂_{VI} \times ♀_{VV}$ and $♂_{VV} \times ♀_{VI}$ breeding pairs when combining all years (Tables S4 and S5, Supporting information). For the years showing allele transmission bias (i.e. 1998, 2001 and 2012; Fig. 3), we did not find any significant differences between the two types of breeding pairs in these reproductive parameters (Tables S4 and S5, Supporting information).

We found no evidence that sex-specific nestling mortality or sex ratio adjustment accounts for sex-specific TRD. First, the offspring sex ratio at fledging was similar in the two types of breeding pairs (pairs $♂_{VI} \times ♀_{VV} = 49.5\%$, and pairs $♂_{VV} \times ♀_{VI} = 49.1\%$, $P = 0.89$; Table S6, Supporting information). Second, there were no differences in the numbers of VV and VI male and female nestlings that died before fledging in the two types of breeding pairs ($♂_{VI} \times ♀_{VV}$: $n_{♂VI} = 21$, $n_{♂VV} = 25$, $n_{♀VI} = 22$, $n_{♀VV} = 22$; $♂_{VV} \times ♀_{VI}$: $n_{♂VI} = 22$, $n_{♂VV} = 23$, $n_{♀VI} = 18$, $n_{♀VV} = 16$; $\chi^2 = 0.44$, $P = 0.93$).

Discussion

Using a large data set, we report a weak but significant departure from HW equilibrium for the *MC1R* gene in fledgling barn owls. In nestling males, interannual variation in assortative pairing with respect to *MC1R* explained the year-specific deviations from HW proportions, whereas in nestling females, the deficit was better explained by the interannual variation in the probability of inheriting the $MC1R_{WHITE}$ or $MC1R_{RUFOUS}$ allele from either parent. As discussed below, our results reinforce the idea that *MC1R* is subject to sexual genetic conflict because on average, males inherit the allele that relates to the females' phenotype (i.e. rufous coloration) slightly less often than expected. However, the genetic conflict might not be complete because in some years, females inherit the allele that relates to the males' phenotype (i.e. white coloration) more often than expected at random.

Nonrandom pairing with respect to MC1R

Assortative pairing can account for strong deviation in the proportion of heterozygotes expected under HW equilibrium (Allendorf & Luikart 2007). This mechanism has recently been suggested to cause the observed deficit of heterozygotes at *MC1R* in the vermilion flycatcher (*Pyrocephalus rubinus*; in seven subpopulations, deficits ranged between 3% and 25%, with an average deficit of 12.9%; Schmitt 2015). In Swiss barn owls, 8.7% of the breeding individuals paired assortatively, which can explain most of the heterozygote deficit in fledgling males (5%, two-thirds of the 7.5% deficit) but less than half of the deficit in females (5% compared with the 12.9% deficit). When evaluating each year separately, we identified a significant positive relationship between the proportion of assortative pairing and heterozygote deficit in male nestlings but not in female nestlings. This suggests that assortative pairing accounts for the deficit of heterozygotes in males, whereas in females, other mechanisms are involved in the deficit of *MC1R* heterozygotes.

Studies from other European barn owl populations did not find any evidence of assortative pairing with respect to the degree of rufous coloration (France: Baudvin 1975; Hungary: Matics *et al.* 2002; Germany: Kniprath & Stier-Kniprath 2014). A plausible scenario is that assortative pairing is detectable mainly when pooling the data, as the frequency of each genotype varies between years. Accordingly, we found evidence of assortative pairing when pooling the data for the years 1998–2012, whereas in each year, there was little evidence that pairing departed from random. Thus, even if barn owls may not actively pair assortatively, (stochastic) demographic effects may induce nonrandom pairing with respect to *MC1R*, which ultimately contributes to deviation from the expected HW proportions. Assortative pairing cannot be the result of nonrandom spatial distribution of individuals with respect to their rufous coloration because we previously showed a relationship between coloration and the breeding habitat only in females (Dreiss *et al.* 2012).

Sex-specific transmission ratio distortion of MC1R alleles

Our results show a general pattern of sons inheriting the $MC1R_{RUFOUS}$ allele from their fathers – an allele that relates to females' plumage coloration – less often than expected at random. In some years, females inherit the $MC1R_{WHITE}$ or $MC1R_{RUFOUS}$ allele more often than expected at random from either their father or mother. The transmission ratio distortion of *MC1R* alleles seems to have evolved mainly to prevent males from inheriting the $MC1R_{RUFOUS}$ allele rather than to prevent females from inheriting the $MC1R_{WHITE}$ allele.

The recurrent selection for the $MC1R_{WHITE}$ allele in males can be expected because the genetic correlation between the degree of reddish coloration and two eumelanic traits (i.e. number and diameter of black feather spots) is 1.6 times higher in males than in

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females (Roulin & Jensen 2015). Thus, if positive selection is acting on white males, it should be reinforced because they would also express small black feather spots, a sexually antagonistic selected trait for which the expression is minimally regulated by *MC1R* (Roulin 1999; Roulin *et al.* 2010; San-Jose *et al.* 2015). The strong genetic correlations between the various melanin-based traits suggest that they have redundant functions; thus, selection at *MC1R* and other genes encoding plumage spottiness could function in opposite directions in males and females. We are currently investigating this issue by testing whether the transmission ratio distortion of *MC1R* alleles covaries with plumage spottiness.

The variability in the inheritance of $MC1R_{RUFOUS}$ and $MC1R_{WHITE}$ in females may indicate that the genetic conflict at *MC1R* is relatively weak in females if they can derive some fitness advantage when carrying the $MC1R_{WHITE}$ allele. Indeed, previous study showed that females with white plumage can produce more offspring than their rufous conspecifics when breeding in wooded habitats (Dreiss *et al.* 2012). Another possibility is that the dominance effect of the $MC1R_{RUFOUS}$ allele on the expression of rufous coloration hides the presence of the $MC1R_{WHITE}$ allele in the heterozygous state, allowing this allele to be maintained in the female population even if females are selected to have a rufous coloration.

Molecular mechanisms causing transmission ratio distortion

Transmission distortion may be explained by several mechanisms that are not mutually exclusive. First, we tested the possibility of sex-specific embryonic lethality increasing with the transmission of a given *MC1R* allele (de la Casa-Esperón *et al.* 2000; Eversley *et al.* 2010), but we did not find evidence of higher mortality at the different developmental stages (eggs, nestlings, fledglings) in relation to *MC1R* in offspring or parents. However, we may have lacked the power to detect such pattern if, for instance, unsuccessful eggs are removed from the nest and replaced with new, successful eggs.

An alternative hypothesis is meiotic drive, in which a selfish element biases Mendelian segregation by moving away from dead-end polar bodies into the functional egg during oogenesis (Johns *et al.* 2005; Wilkinson *et al.* 2006; Gell & Reenan 2013; Friberg & Rice 2014; Zanders & Malik 2015). However, this scenario is unlikely because the recurrent pattern of segregation distortion occurs in the parental male line (i.e. ♂$_{VI}$ × ♀$_{VV}$ breeding pairs). It can be further noted that in years when the $MC1R_{RUFOUS}$ allele was transmitted to one sex less often than expected at random, it was transmitted more often to the other sex, as in 2001 (23.5% in sons vs. 70.0% in daughters from ♂$_{VI}$ × ♀$_{VV}$ breeding pairs) and 2012 (38.7% ♂$_{VI}$ vs. 70.6% ♀$_{VI}$ ♂$_{VI}$ × ♀$_{VV}$ from breeding pairs). Meiotic drive alone cannot explain this pattern of sex-specific *MC1R*-allele transmission because, even if a form of meiotic drive would have occurred during spermatogenesis (e.g. sperm selection through motility; Holt & Van Look 2004), this mechanism would have implied an overall deficit of spermatozoids possessing the 'I' allele that would have affected both sons and daughters.

A third hypothesis is associated with egg-sperm recognition phenomena, but we are not aware of any studies that investigated this possibility regarding *MC1R*. For a long time, oocytes have been recognized as passive acceptors of sperm, whereas differential male success in fertilizing eggs results from an arms race between male gametes to reach eggs (Holt & Van Look 2004; Gasparini & Pilastro 2011). Still, some empirical studies found that eggs play a major role in fertilization through specific sperm-egg surface recognition using surface binding proteins (Palumbi 1999; Galindo *et al.* 2003). For instance, transmission of gene variants under strong selection, such as *MHC* genes, are known to be differently able to fertilize eggs carrying similar or dissimilar genotypes (Yeates *et al.* 2009; Lovlie *et al.* 2013). Therefore, a hypothesis to explain the sex-specific deficit of the 'I' allele when the father transmits it (in ♂$_{VI}$ × ♀$_{VV}$) is that spermatozoids possessing this allele are outcompeted by spermatozoids possessing the V allele to fertilize oocytes possessing the Z sex chromosome, whereas lower or no effects would be observed when oocytes possess the W sex chromosome (in birds, females are heterogametic ZW). This proposition remains merely a hypothesis and would require further molecular and cellular studies for confirmation.

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Data accessibility

MC1R and microsatellites data files, *MC1R* breeding pairs and their fitness parameters file, R scripts for the simulation of F_{IS}, of chi-square and of proportion of assortative pairing can be found on Dryad: doi: 10.5061/dryad.nm61g.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Observed F_{IS} values in male and female nestling barn owls for 10 microsatellite loci and number of genotyped nestlings from 1998 to 2012.

Table S2 Observed F_{IS} values in male and female nestling barn owls for *MC1R* and number of genotyped nestlings from 1998 to 2012.

Table S3 Assortative pairing with respect to *MC1R* in Swiss barn owls from 1998 to 2012. *P*-values from chi-square tests are obtained after using permutations of genotypes in breeding pair to test the null hypothesis of random pairing.

Table S4 Annual clutch sizes, hatching successes (proportion of eggs that hatched) and fledging successes (proportion of hatchlings that fledged) in two types of barn owl pairs (♂$_{VI}$ × ♀$_{VV}$ and ♂$_{VV}$ × ♀$_{VI}$). The *P*-values correspond to significant differences between the two types of breeding pairs.

Table S5 Annual clutches' success (proportion of eggs that hatched and hatchlings that fledged) in two types of barn owl pairs (♂$_{VI}$ × ♀$_{VV}$ and ♂$_{VV}$ × ♀$_{VI}$). The p-values correspond to significant differences between the two types of breeding pairs.

Table S6 Annual nestling sex ratios in two types of barn owl breeding pairs (♂$_{VI}$ × ♀$_{VV}$ and ♂$_{VV}$ × ♀$_{VI}$). The *P*-values correspond to significant differences from expected ratio of 0.5 in each type of breeding pairs (a value of 1 corresponds to 100% of males) or to significant sex ratio differences between breeding pairs.

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Supporting information

Table S1. Observed *Fis* values in male and female nestling barn owls for 10 microsatellite loci and number of genotyped nestlings from 1998 to 2012.

Year	Sample sizes		Observed *Fis* values in male nestlings	Observed *Fis* values in female nestlings
	Males	Females		
1998	72	76	-0.004	-0.018
1999	48	47	-0.02	-0.035
2000	45	51	-0.014	0.021
2001	40	41	-0.041	-0.003
2002	131	111	0.003	-0.011
2003	76	58	0.05	0.026
2004	39	42	-0.022	0.011
2005	44	38	0.024	0.023
2006	29	31	0.001	0.002
2007	46	41	0.021	0.026
2008	27	36	0.027	0.097
2009	24	33	-0.058	-0.032
2010	36	36	0.03	-0.006
2011	46	52	0.002	0.001
2012	52	44	-0.014	0.024
Total	755	737	0.002	0.005

Fis values are calculated after permutation of alleles (n = 10,000) using FSTAT v. 2.9.3 (Goudet, 1995).

Table S2. Observed *Fis* values in male and female nestling barn owls for *MC1R* and number of genotyped nestlings from 1998 to 2012.

Year	Number of nestlings with genotype II, VI, VV		Observed *Fis* values in male nestlings	Observed *Fis* values in female nestlings
	Males	Females		
1998	7,27,66	3,16,80	0.076	**0.446**
1999	1,15,46	3,18,45	0.121	0.009
2000	0,29,96	6,39,75	0.209	-0.108
2001	10,25,65	9,31,64	**0.279**	0.140
2002	4,33,111	5,29,100	**0.242**	**0.266**
2003	1,22,62	0,16,49	-0.076	-0.022
2004	0,11,57	1,20,74	0.166	-0.086
2005	2,28,84	2,29,82	0.025	-0.019
2006	0,5,30	0,3,38	-0.577	0.195
2007	3,31,117	1,20,109	0.093	**0.318**
2008	2,25,69	1,19,86	-0.077	**0.259**
2009	2,2,21	3,5,30	**0.681**	**0.473**
2010	4,23,62	1,19,63	0.024	0.134
2011	1,24,54	6,37,63	0.073	-0.064
2012	5,58,117	8,50,132	-0.096	0.105
Total	42,358,1057	49,351,1090	**0.075**	**0.129**

Calculation of *Fis* values is detailed in the material and methods section. Bold numbers correspond to statistically significant deviation from Hardy-Weinberg proportion ($P < 0.05$) and correspond to an excess (i.e. $Fis < 0$) or deficit (i.e. $Fis > 0$) of heterozygotes. Mean observed *Fis* values are weighted by the number of genotyped nestlings each year. Only values for females in 1998 and for males in 2009 are significant after sequential Bonferroni correction.

Table S3. Assortative pairing with respect to *MC1R* in Swiss barn owls from 1998 to 2012. P-values from chi-square tests are obtained after using permutations of genotypes in breeding pair to test the null hypothesis of random pairing.

Year	Number of II, VI, VV in adults		Observed chi-squared values	*P*
	Males	**Females**		
1998	2,12,31	2,12,31	12.29	**0.0307**
1999	1,10,21	1,7,24	3.28	0.489
2000	4,10,35	1,15,33	4.845	0.269
2001	3,16,26	1,16,28	7.37	0.12
2002	4,17,46	2,19,46	14.12	**0.007**
2003	1,6,29	0,12,24	0.52	1
2004	2,4,33	0,9,30	2.298	0.26
2005	2,11,36	0,14,35	1.138	0.54
2006	0,2,19	0,2,19	<0.001	1
2007	3,13,46	0,13,49	1.082	0.615
2008	2,15,42	0,14,45	3.72	0.162
2009	0,7,12	0,4,15	1.433	0.122
2010	1,9,30	0,14,26	2.613	0.23
2011	2,16,28	1,16,29	3.17	0.493
2012	1,23,59	2,30,51	3.169	0.121
Total	28,171,493	10,197,485	25.18	**0.0012**

Bold numbers correspond to statistically significant assortative pairing ($p<0.05$) after permutation of genotypes among breeding pairs and simulation of chi-square distribution. 1998 and 2002 values are no longer significant after sequential Bonferroni correction ($p>0.0033$).

Table S4. Annual clutch sizes, hatching successes (proportion of eggs that hatched) and fledging successes (proportion of hatchlings that fledged) in two types of barn owl pairs (♂$_{VI}$ x ♀$_{VV}$ and ♂$_{VV}$ x ♀$_{VI}$). The p-values correspond to significant differences between the two types of breeding pairs.

Year	Clutch sizes			Hatching successes			Fledging successes		
	♂ VI	♀ VI	***P***	♂ VI	♀ VI	***P***	♂ VI	♀ VI	***P***
1998	6.714	6.429	0.835	0.936	0.956	0.683	0.806	0.847	0.928
1999	6.5	4.333	0.205	0.872	1	0.992	0.863	0.775	0.31
2000	6.6	5.9	0.606	0.939	0.966	0.552	0.948	0.838	0.372
2001	6.667	6.167	0.733	0.95	0.946	0.936	0.77	0.8	0.777
2002	5.5	5.8	0.791	0.932	0.983	0.223	0.892	0.778	0.289
2003	6	5.9	0.945	0.958	0.915	0.501	0.964	0.897	0.387
2004	6.5	6.857	0.864	1	1	1	0.703	0.62	0.722
2005	6.571	5.8	0.582	0.978	0.966	0.702	0.838	0.889	0.855
2006	7	6.5	0.847	0.857	0.769	0.56	0.591	0.708	0.796
2007	6.636	7.667	0.39	0.918	0.899	0.691	0.588	0.742	0.25
2008	6	5.8	0.851	0.956	0.948	0.839	0.602	0.511	0.59
2009	6.75	6	0.794	0.778	1	0.995	0.704	0.163	0.136
2010	6	7.9	0.15	0.952	0.937	0.726	0.722	0.666	0.795
2011	6.8	6.2	0.764	0.897	0.952	0.254	0.406	0.721	<0.001
2012	7.533	6.9	0.422	0.965	0.964	0.972	0.641	0.624	0.814
Total	6.54	6.42	0.707	0.937	0.954	0.251	0.701	0.726	0.713

Table S5. Annual clutches' success (proportion of eggs that hatched and hatchlings that fledged) in two types of barn owl pairs (♂$_{VI}$ x ♀$_{VV}$ and ♂$_{VV}$ x ♀$_{VI}$). The p-values correspond to significant differences between the two types of breeding pairs.

Year	Clutches' success		
	♂ VI	♀ VI	***P***
1998	0.797	0.833	0.617
1999	0.796	0.813	0.886
2000	0.892	0.831	0.401
2001	0.769	0.787	0.83
2002	0.846	0.795	0.464
2003	0.923	0.843	0.318
2004	0.765	0.727	0.755
2005	0.812	0.866	0.441
2006	0.667	0.684	0.906
2007	0.682	0.742	0.354
2008	0.718	0.659	0.369
2009	0.675	0.545	0.429
2010	0.764	0.725	0.594
2011	0.651	0.753	0.159
2012	0.733	0.694	0.431
Total	0.764	0.762	0.914

Table S6. Annual nestling sex ratios in two types of barn owl breeding pairs (♂$_{VI}$ x ♀$_{VV}$ and ♂$_{VV}$ x ♀$_{VI}$). The p-values correspond to significant differences from expected ratio of 0.5 in each type of breeding pairs (a value of 1 corresponds to 100% of males) or to significant sex ratio differences between breeding pairs.

Year	♂ VI		♀ VI		**Breeding pair differences**
	Sex ratio	***P***	**Sex ratio**	***P***	***P***
1998	41.2	0.306	47.5	0.752	0.586
1999	46.4	0.706	16.7	0.0377	0.089
2000	35.7	0.136	51.1	0.882	0.201
2001	63	0.183	50	1	0.343
2002	51.5	0.862	55	0.528	0.767
2003	60	0.442	52.6	0.746	0.628
2004	11.1	0.0499	40	0.277	0.137
2005	52.8	0.739	53.2	0.662	0.87
2006	42.9	0.706	50	1	0.797
2007	62.2	0.105	53.1	0.668	0.37
2008	58	0.26	39.4	0.226	0.099
2009	42.9	0.594	0.1	0.97	0.97
2010	44.8	0.578	43.5	0.378	0.909
2011	42.3	0.435	41.5	0.277	0.946
2012	47.7	0.71	57	0.163	0.24
Total	0.495	0.815	0.491	0.648	0.887

Chapter 3

Female-biased dispersal and non-random gene flow of *MC1R* variants do not conduct to a migration load in barn owls

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Personal contribution: I designed the project, performed the lab work, analysed the data and wrote the manuscript.

Abstract

Disentangling the effect of selection and dispersal in shaping genetic variation in natural populations is crucial to understand the impact of gene flow on local adaptation. Gene flow effects are generally complex because dispersal is often not random regarding particular phenotypes and genotypes or can be sex-biased, which could influence fitness in a sex-specific manner. We used direct and indirect methods to estimate and characterize dispersal and gene flow in a wild population of barn owls breeding in Switzerland. The indirect approach using 10 microsatellite loci did not reveal a pattern of female-biased dispersal. Monte-Carlo simulations, however, revealed a low statistical power of genetic indexes in detecting sex-biased dispersal in case of high dispersal rate. However, direct methods using capture-recapture data permitted to detect a female-biased dispersal and non-random gene flow of *MC1R* variants, a gene responsible for 30% of the rufous coloration in this population. This non-random gene flow results in a heterozygote excess at *MC1R* observed in adult females only, whereas no sex difference in heterozygosity was observed at neutral markers. It suggests that *MC1R* could be selected within females to provide an advantage during dispersal, concordant with previous results showing a link between color and dispersal. Finally, we only find a weak decreased apparent survival or reproductive success for individuals carrying at least one copy of the $MC1R_{RUFOUS}$ allele, suggesting that the non-random gene flow of *MC1R* variants do not conduct to a strong migration load in this population.

Introduction

Unraveling the effect of natural selection and dispersal in shaping genetic and phenotypic variation is crucial to understand the impact of gene flow on local adaptation. Dispersal can counteract the effect of natural selection by introducing maladapted alleles into locally adapted populations (Slatkin 1987; Hu & Li 2003), an effect that depends on the strength of selection against immigrants and the alleles they carry (Lenormand 2002; Postma & van Noordwijk 2005). On the contrary, the absence of dispersal, and consequently gene flow, can limit local adaptation due to decreased genetic variation on which natural selection can work (Bourne *et al.* 2014). Dispersal can thus potentially facilitate the movement of adaptive variation among populations (Bolnick & Otto 2013; Portnoy *et al.* 2015) or even among closely related species that hybridize (Fraisse *et al.* 2014; Palmer & Kronforst 2015).

Gene flow plays a major role in evolutionary processes, but its effects are generally complex (Ciani & Capiluppi 2011; Edelaar & Bolnick 2012; Bolnick & Otto 2013). Dispersal is often not a random set of individuals that diffuse between populations but instead can be enriched for certain phenotypes and underlying genotypes, as shown in humans for the DRD4 gene, which is associated with novelty seeking and hyperactivity traits (Chen *et al.* 1999). Dispersal is also often sex-biased in vertebrates (Palo *et al.* 2004; Biek *et al.* 2006; Berg *et al.* 2009; Paquette *et al.* 2010; Trochet *et al.* 2016) meaning that dispersal and gene flow could differentially impact male and female fitness (Tarka *et al.* 2014; Camacho *et al.* 2013). Thus, describing gene flow based on particular genotypes or gender is important to understand how dispersal could favor or constrain the adaptive effects of natural selection within populations in a sex-specific manner (Edelaar & Bolnick 2012).

In natural populations, dispersal and gene flow can be estimated with direct methods that measure the movement of individuals through capture-recapture (Conrad *et al.* 1999; Baguette 2003; Schtickzelle *et al.* 2006) or GPS tracking methods (Cooke *et al.* 2004; Ropert-Coudert & Wilson 2005). However, the direct assessment of dispersal can be difficult or inaccurate in some organisms, such as in smaller-sized organisms, which preclude direct marking. In bigger-sized populations, a relatively small number of animals can be marked, which impede identification of dispersers. Indirect methods based on genetic data offer an alternative approach to estimate

dispersal and gene flow by comparing genotype frequencies between populations (Wright 1943; Wright 1951; Slatkin 1985) or between sexes (Goudet *et al.* 2002; Hansson *et al.* 2003). In many instances, those indirect methods can also be inaccurate in estimating gene flow or population divergence, for example when individual movements are asymmetric between populations (Boileau *et al.* 1992; Knutsen *et al.* 2011). This is why the combination of direct and indirect approaches is useful to underline the importance of dispersal and gene flow in shaping neutral genetic variation (Slatkin 1985), as shown in Odonata (Watts *et al.* 2007), Atlantic cod (Knutsen *et al.* 2011), alpine salamander (Helfer *et al.* 2012) and social weavers (van Dijk *et al.* 2015), or in shaping phenotypic and neutral genetic variation in blue tits (Garcia-Navas *et al.* 2014). However, we are still lacking more empirical studies that combine both methodologies to clarify the effect of dispersal and gene flow in shaping adaptive genetic variation because most studies performed so far only considered neutral genetic markers.

The European barn owl (*Tyto alba*) is an appropriate model system to study the effect of dispersal on neutral and adaptive genetic variation. The striking variation of its pheomelanin-based coloration is associated with a non-synonymous mutation at the melanocortin-1 receptor gene (*MC1R*) (San-Jose *et al.* 2015; Burri *et al.* 2016), a key element in the vertebrate melanin synthesis pathway (Bennett & Lamoreux 2003). The pheomelanin coloration and its underlying *MC1R* alleles vary clinally in Europe, from high frequency of the $MC1R_{WHITE}$ allele (V), which encodes for a white ventral plumage, in the Iberian Peninsula to a high frequency of the $MC1R_{RUFOUS}$ allele (I), which encodes for a rufous ventral coloration, in northeast Europe, while both mutations occur at intermediate frequencies in Central Europe (Antoniazza *et al.* 2010; Burri *et al.* 2016). At neutral markers, a low genetic differentiation between European populations highlights important gene flow at the level of the continent. Therefore, the maintenance of both genetic and phenotypic clines, despite strong gene flow, supports the presence of local adaptation (Antoniazza *et al.* 2010), as demonstrated through ABC simulations (Antoniazza *et al.* 2014). On a smaller spatial scale, there is also evidence for local adaptation in Switzerland, where coloration of females matches specific habitat types (Dreiss *et al.* 2012) and coloration is related to different prey-predator strategies (Roulin 2004; Charter *et al.* 2012). Moreover, the

MC1R$_{RUFOUS}$ mutation seems to be recurrently counter-selected in males at the juvenile stage (Ducret *et al.* 2016).

Here, we explore the effect of dispersal on the frequency of *MC1R* variants in breeding adults in a Swiss population of barn owls. First, we investigated if *MC1R* genotypes deviate from Hardy-Weinberg equilibrium in male and female breeders, which could be caused by the effect of (sex-specific) selection and/or (sex-biased) dispersal. To disentangle which of these two processes is the most likely cause of a deviation, we also analysed Hardy-Weinberg at 10 neutral genetic markers (microsatellite loci). Because the Swiss barn owl population is at an intermediate position along the European cline, we would expect gene flow to cause a deficit rather than excess of heterozygotes for both microsatellites and *MC1R*. This is due to the genetic admixture of differentiated populations from each extremity of the cline, also known as the Wahlund effect (Wahlund 1928). We analyzed capture-mark-recapture data and neutral genetic data, respectively direct and indirect methods, to characterize and estimate immigration rate of the *MC1R* genotypes and to test if dispersal is female-biased, as generally found in birds (Trochet *et al.* 2016). Lastly, we tested for sex-specific selection on *MC1R* genotypes in adults by analyzing reproductive success and survival.

Materials and methods

Data collection

The study was performed from 1998 to 2016 in western Switzerland where 1,040 wild barn owls were captured breeding in artificial nest boxes. Clutch size and number of hatchlings and fledglings were recorded for each nest. All nestlings and 90% of breeding adults are captured and marked with aluminium rings since 1988. The ringing method allowed us to differentiate adults born in our study area (philopatric) from those born outside of it (immigrant). Immigrants' age can be estimated based on the moulting pattern of the primary wing feathers (Dreiss & Roulin 2010).

We collected and stored blood samples at -80˚C until DNA extraction. DNA was extracted using the DNeasy Tissue and Blood kit or the Biosprint robot (Qiagen). We determined *MC1R* genotypes for all adults by allelic discrimination (see protocol in San-Jose *et al.* 2015). In each assay, we always ran positive controls for each

genotype (II, VI and VV; I and V correspond to the *white* and *rufous* allele respectively) and each sample was run in duplicate with two different PCRs. Only adults from 1998 to 2012 (N = 755) were genotyped for 10 polymorphic microsatellite loci in two sets of multiplex. Genotyping procedure and description of multiplex sets (named 3 and 4) are described in Burri *et al.* (2016). Thus, the genetic analyses were constrained to the period 1998-2012, with 316 males and 439 females analysed for Hardy-Weinberg proportions at both *MC1R* and microsatellites, for sex-biased dispersal and for assignment tests. Addition of the other 285 individuals sexed and genotyped at *MC1R* gave qualitatively similar results in terms of Hardy-Weinberg proportion at *MC1R* or sex-biased dispersal (Supporting information).

Genetic analyses

MC1R *proportions*

Deviation from Hardy–Weinberg equilibrium (F_{IS}) at *MC1R* was computed for each year using FSTAT v2.9.3 (Goudet 1995) and significant difference between males and females on the range of F_{IS} values was determined using Wilcoxon signed-rank test. We tested for differences in the proportion of *MC1R* genotypes between immigrant and philopatric males and females using a Fisher's exact test and computed the F_{IS} values per sex and dispersal status (i.e. resident vs. immigrant) with FSTAT.

Sex-biased dispersal

The difference in the proportion of immigrants between the two sexes was assessed by means of a Chi-square test using capture-mark-recapture data. In addition, we used microsatellite data to detect female-biased dispersal by quantifying the mean assignment index (*mAIc*) and variance of the assignment index (*vAIc*) separately for both sexes using the R package HIERFSTAT (Goudet 2005; R Core Team 2013) and significant differences were determined using a one-tailed permutation test (N = 1,000). Deviations from Hardy–Weinberg equilibrium (F_{IS}) per year of sampling were also computed using FSTAT and significant differences between males and females on the range of F_{IS} values were determined using the Wilcoxon signed-rank test. Sex-biased dispersal is expected to result in a lower *mAIc* and larger *vAIc* in the dispersing sex as well as positive F_{IS} values due to a stronger Wahlund effect among adults of the sex dispersing most (see Goudet *et al.* 2002).

Individual-based Monte Carlo simulations

The large amount of immigrants compared to residents (71%) in our study area and the fact that the tests were performed on a single population may decrease the sensibility of the *mAIc* and *vAIc* tests in detecting sex-biased dispersal. Also, we estimated the statistical power of *mAIc* and *vAIc* using individual-based Monte Carlo simulation from EASYPOP (Balloux 2001), with the dispersal rate of males and females estimated by capture-mark-recapture (respectively, $d_m = 0.62$ and $d_f = 0.78$) and with 10 simulated loci. As in Goudet *et al.* (2002), dispersal follows an island model; the chosen mutation rate is 0.001 with 25 allelic states and the KAM mutation model (see detailed description of the parameters in Goudet *et al.*, 2002). The simulation ran with 10 populations containing each 50 males and 50 females, which represent the average annual number of breeding males and females in our study area. To achieve mutation-migration-drift equilibrium, each of 99 replicates was run for 1,000 generations. We also simulated a higher sex-biased dispersal, once with a total dispersal rate similar with the previous simulation ($d_m = 0.5$ and $d_f = 0.1$, $d_t = 0.75$), and then with a lower total dispersal rate ($d_m = 0$ and $d_f = 0.5$, $d_t = 0.25$). Finally, we applied the *mAIc* and *vAIc* tests using HIERFSTAT to a single or all populations and for each replicate. Thus, we defined the statistical power of the two tests in detecting the female-biased dispersal as the number of times the tests were significant ($P \leq 0.05$) over the 99 replicates.

Fitness components

To estimate fitness, we measured the ability of the *MC1R* genotypes to (i) survive in our study area and (ii) produce viable offspring (i.e. fledglings). Because homozygotes for the $MC1R_{RUFOUS}$ were in very low frequency, we distinguished genotypes as carrying at least one or zero copy of the $MC1R_{RUFOUS}$ allele (i.e. II/VI vs. VV).

Survival probability

Annual capture-recapture data of adult barn owls that have been collected in our study area from 1998 to 2016 were analysed with Comarck-Jolly-Seber (CJS) models (Lebreton *et al.* 1992) using the program MARK (White & Burnham 1999). The 1,040 captured adults that were sexed, genotyped at *MC1R* (II/VI vs. VV) and with

dispersal status (either locally born or immigrant) constituted eight groups of individuals. We estimated apparent survival (φ), which is the probability to survive and to remain in the study area. Although we cannot distinguish between mortality and emigration, both processes lead to similar effect on the fitness of individuals at the local population level. We estimated the recapture probabilities (ρ), which is the probability to recapture a marked individual present in the study area. We started with a general model that assumed survival to vary over time in each of the eight groups independently from each other and that recapture probability varied over time in males and females independently from each other. In the first modeling step, we verified whether survival and recapture varied over time and whether temporal variation was additive to the group effects. In the second modeling step, we kept the recapture model at the most parsimonious structure and modeled the eight groups acting on survival. Specifically we fitted all possible models including single effect of sex, status, *MC1R* genotype, and their two-way and three-way interactions. At each step, we fitted several candidate models that were ranked based on Akaike Information Criterion corrected for small sample sizes (*AICc*: Burnham *et al.* 2011). We verified the goodness-of-fit of the most complex model with the program U-CARE (Choquet *et al.* 2009), and this test shows a good fit ($\chi^2 = 102.08$, df = 159, $P = 0.99$).

Reproductive success

We analysed the reproductive success as the difference in the number of eggs produced, fledging success and number of fledglings produced in each nest from 1998 to 2016 between sexes, dispersal status (immigrant vs. resident) and *MC1R* genotypes (II/VI vs. VV) using Generalized Linear Mixed Models (GLMMs with single effects and their two-way and three-way interactions). The number of eggs and fledglings produced were analyzed using Poisson errors, whereas fledging success was analyzed using binomial errors (successful fledglings vs. dead nestlings). In all models, the laying date was incorporated as covariate and standardized (scaled and centered) to permit correct model convergence. Adults' and sites' identities and year were incorporated as random factors to account for temporal and spatial pseudo-replication. To identify the best statistical models in terms of predicting reproductive success, we ranked competing models based on the *AICc* using the function 'dredge' of package

MuMIn (Barton 2016). The best models were kept based on a $\Delta AICc \leq 2$ and the model with the lowest *AICc* score was selected as the best-fitting model. Overdispersion was checked for all models using the function 'overdisp.glmer' of the package RVAideMemoire (Hervé 2016). The statistical analyses were conducted with the R software v3.2.4 (R Core Team 2013).

Results

Hardy-Weinberg proportion at microsatellites and *MC1R*

A total of 105 alleles were found across the 10 microsatellite loci. The number of alleles per locus ranged from 4 (locus Ta202) to 25 (locus Ta402). Randomisation of alleles within sampling years indicated that in males one marker presented significant positive F_{IS} (0.154) and two markers significant negative F_{IS} (-0.061 and -0.1) after Bonferroni correction ($P < 0.001$; Table S1). In females, one marker presented significant positive F_{IS} (0.062) after Bonferroni correction ($P < 0.001$; Table S1). We discovered the presence of null alleles for this marker (Ta212) and we removed it for all adults in the subsequent analyses. Also, females did not show significantly higher F_{IS} values (average F_{IS} = 0.011, range of F_{IS} values = -0.039 to 0.067) than males (average F_{IS} = -0.005, range of F_{IS} values = -0.061 to 0.047) (Wilcoxon sum rank test, W = 131, P = 0.23).

The pattern at *MC1R* is different compared to neutral markers. Adult females showed a significantly higher excess of heterozygotes (average F_{IS} = -0.069, F_{IS} year-range = -0.18 to 0.076) than males (average F_{IS} = 0.077, F_{IS} year-range = -0.1 to 0.319) (Wilcoxon rank sum test, W = 32, $P < 0.001$; Figure 1).

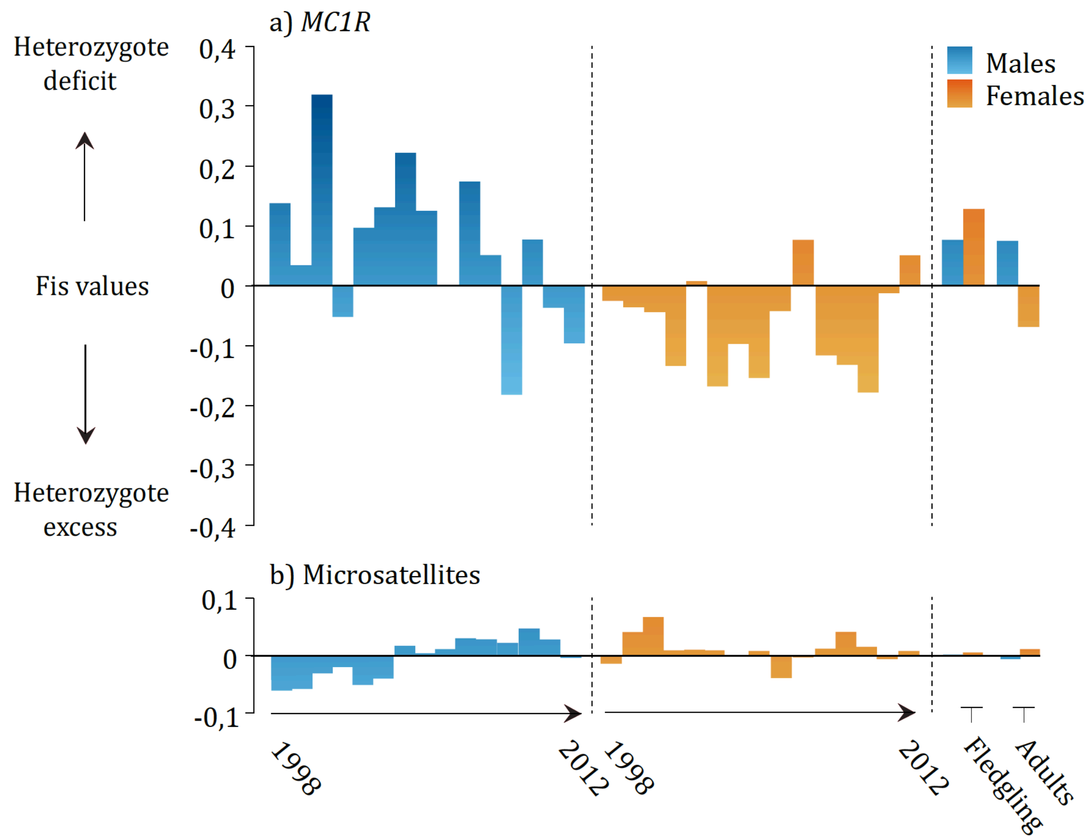

Figure 1. F_{IS} values computed for a) *MC1R* and b) microsatellites and averaged over loci per year of sampling and sex in breeding barn owls. Positive and negative values represent respectively deficit and excess of heterozygote at *MC1R*.

Dispersal and gene flow

Our marking-recapture data indicated that only 29% of the adults breeding in our study area were born inside the study area. Also, we observed a higher proportion of breeding males born inside the study area compared to females (38% vs. 22%; Chi-square test, $\chi^2 = 24.26$, $P < 0.001$; Table 1). Using microsatellite data, we observed a weak but significantly higher variance assignment index for females than for males (respectively $vAIc_f = 11.03$, $vAIc_m = 9$; permutation test, $P = 0.021$), but the mean assignment index (*mAIc*) was close to 0 in both sexes ($P = 0.48$).

In females, immigrants were more often heterozygous for the *MC1R* gene than residents (Fisher's exact test, $P = 0.042$), while in males we did not find such a

difference (P = 0.93; Table 1). Those differences in the level of heterozygosity between immigrants and residents within each sex were reflected in the F_{IS} values, although the deviations were not significant. Philopatric females showed an excess of homozygotes (F_{IS} = 0.197, P = 0.05) and immigrant females a slight excess of heterozygotes (F_{IS} = -0.084, P = 0.1), whereas we observed a slight excess, albeit non-significant, of homozygotes in both philopatric (F_{IS} = 0.058, P = 0.33) and immigrant males (F_{IS} = 0.040, P = 0.35).

Table 1. Proportion and percentages of *MC1R* genotypes in philopatric and immigrant male and female barn owls breeding in western Switzerland between 1998 to 2012 (II, homozygotes for the $MC1R_{RUFOUS}$ allele; VI, heterozygotes; VV, homozygotes for the $MC1R_{WHITE}$ allele).

		II	**VI**	**VV**	**Total**
Males	Philopatric	4 (3.3%)	31 (25.4%)	87 (71.3%)	122
	Immigrant	7 (3.6%)	54 (27.8%)	133 (68.6%)	194
Females	Philopatric	5 (5.2%)	21 (21.9%)	70 (72.9%)	96
	Immigrant	5 (1.5%)	101 (29.4%)	237 (69.1%)	343

Statistical power of *vAIc* and *mAIc* tests

The power analyses revealed that, when the dispersal rate is high, as observed in our Swiss population (d_m = 0.62 and d_f = 0.78), the *mAIc* and *vAIc* tests have a very low power to detect the female-biased dispersal with 2% and 6% of the replicates having a significant *vAIc* and *mAIc* test, respectively. The statistical power did not increase when, for a comparable average dispersal rate, the sex-biased dispersal increased (d_m = 0.5 and d_f = 1.0) with 6% and 7% of the replicates having a significant *vAIc* and *mAIc* tests, respectively. The power of the tests did not differ and was always very low if the tests were performed on a single or on all simulated populations. With a reduced average dispersal rate (0.25) but with the same strong sex-biased dispersal as previously (d_m = 0.0 and d_f = 0.5), the power of the two tests increased drastically with 50% and 92% of the replicates having a significant *vAIc* and *mAIc* tests respectively, and when the tests were performed on the data containing the 10 populations. If the tests were performed on a single population, the *vAIc* test became significant in only 9% of the cases, whereas the *mAIc* test was significant in 30% of

the cases. The lower statistical power of the *vAIc* compared to the *mAIc* when the dispersal rate is low was expected based on previous simulation (Goudet *et al.*, 2002).

Apparent survival in relation to MC1R

The model with time-specific recapture probabilities and time-dependent survival probabilities with an additive group effect was by far the most parsimonious model (Table S2). Although the model with an additive sex effect on recapture probability was close to the best model, it did not improve the prediction of survival regarding sex, dispersal status or *MC1R* (Table S3). By keeping the recapture probabilities and apparent survival as time-dependent, two models received much more support by the data than the other models (Table 2). They both included an additive effect of sex and dispersal status on apparent survival (Table 2). Females had significantly lower apparent survival than males (β = -0.526, 95% CI = -0.763– -0.288) and immigrants had significantly lower apparent survival than locally born individuals (β = -0.404, 95% CI = -0.652– -0.156). An additive effect of MC1R was observed in the second best model (ΔAICc = 1.40), where individuals with at least one copy of the $MC1R_{RUFOUS}$ allele tended to have a lower survival than homozygotes for the $MC1R_{WHITE}$ allele (β = -0.110, 95% CI = -0.367–0.146).

Table 2. Model selection results of apparent survival (φ) of adult barn owls breeding in western Switzerland in function of time (t: year), sex, dispersal status (immigrant vs. resident) and *MC1R* genotypes (II/VI vs. VV). Note that the model for recapture probability (ρ) was always time dependent (ρ(t)) and is not included in the model notation. The most complex model for survival (i.e. φ(sex + status + *MC1R* + sex * *MC1R* + sex * status + status * *MC1R* + sex * status * *MC1R* + t)) corresponds exactly to model φ(g + t) from the first modeling step (see Table S2).

Model	AICc†	ΔAICc§	*w*‡	K	Deviance
φ(sex + status + t)	2191.23	0.00	0.34	37	785.17
φ(sex + status + *MC1R* + t)	2192.63	1.40	0.17	38	784.46
φ(sex + status + sex * status + t)	2193.34	2.11	0.12	38	785.17
φ(sex + status + *MC1R* + sex * *MC1R* + t)	2193.80	2.57	0.09	39	783.51
φ(sex + status + *MC1R* + *MC1R* * status + t)	2194.27	3.04	0.07	39	783.98
φ(sex + status + *MC1R* + sex * status + t)	2194.74	3.51	0.06	39	784.46
φ(sex + status + *MC1R* + sex * *MC1R* + *MC1R* * status + t)	2195.14	3.91	0.05	40	782.73
φ(sex + status + *MC1R* + *MC1R* * status + sex * status + t)	2195.14	3.91	0.05	40	782.73
φ(sex + status + *MC1R* + sex * *MC1R* + sex * status + t)	2195.92	4.69	0.03	40	783.51
φ(sex + status + *MC1R* + sex * *MC1R* + sex * status + status * *MC1R* + sex * status * *MC1R* + t)	2199.05	7.83	0.01	42	782.41
φ(sex + t)	2199.40	8.17	0.01	36	795.45
φ(sex + *MC1R* + t)	2200.56	9.33	0.00	37	794.50
φ(sex + *MC1R* + sex * *MC1R* + t)	2201.60	10.37	0.00	38	793.43
φ(status + t)	2205.87	14.64	0.00	36	801.91
φ(*MC1R* + status + t)	2207.69	16.46	0.00	37	801.62
φ(*MC1R* + status + *MC1R* * status + t)	2209.22	17.99	0.00	38	801.05
φ(*MC1R* + t)	2225.46	34.23	0.00	36	821.51

†AIC value corrected for small simple sizes
§Difference in a model's AICc to the best-ranked model's AIC
‡Model weight: probability of the model given the data
K: Number of parameter estimated

Reproductive success in relation to *MC1R*

We did not find evidence that different MC1R genotypes produced a different number of eggs, had a different fledging success and number of fledglings (Table 3). Only in the third best model, we detected a tendency for an interaction between sex and MC1R on the number of fledglings (GLMM Poisson, $\chi^2 = 4.65$, P = 0.098), while the single effect of MC1R was not significant ($\chi^2 = 0.77$, P = 0.38).

Table 3. Effects of *MC1R* genotypes (II/VI vs. VV), sex, dispersal status and laying date on the reproductive success of adult barn owls. Shown are the best models based on AICc (ΔAICc ≤ 2).

Predictors	**AICc†**	**ΔAICc§**	***w*‡**	**K**
Number of eggs				
LD	8317.7	0.00	0.46	5
LD + status	8318.7	0.99	0.28	6
LD + *MC1R*	8318.9	1.21	0.25	6
Clutch size				
LD	8199.4	0.00	0.22	5
LD + status	8199.6	0.24	0.20	6
LD + *MC1R*	8200.4	0.98	0.14	6
LD + status + *MC1R*	8200.7	1.32	0.11	7
Number of fledglings				
LD	9114.7	0.00	0.26	5
LD + status + Sex * status	9115.4	0.63	0.19	8
LD + *MC1R* + Sex * *MC1R*	9115.4	0.65	0.19	8
LD + *MC1R*	9116.0	1.23	0.14	6
LD + status	9116.6	1.84	0.11	6
LD + status + *MC1R* + sex * status	9116.6	1.89	0.11	9

†AIC value corrected for small simple sizes
§Difference in a model's AICc to the best-ranked model's AIC
‡Model weight: probability of the model given the data.
K: Number of parameter estimated

Discussion

Disentangling the effect of selection and dispersal in natural populations is often challenging because both processes interplay in shaping genetic and phenotypic variation. Particularly, we barely understand the consequences of dispersal in shaping adaptive variation as most studies use neutral genetic variation to confound the effect of dispersal and selection (Mullen & Hoekstra 2008; Antoniazza *et al.* 2010). Here, we studied a barn owl (*Tyto alba*) population breeding in Switzerland and explored the effect of dispersal on the local frequency of *MC1R* variants, a key gene involved in vertebrate melanin synthesis pathway (Bennett & Lamoreux 2003) and suspected to be under local adaptation in Europe (Antoniazza *et al.* 2010, 2014). Previous studies showed that *MC1R* and neutral markers varies clinally in Europe due (Burri *et al.* 2016). Because the Swiss barn owl population is at an intermediate position along the European cline, we expected gene flow to cause a deficit of heterozygotes for both categories of markers, also known as the Wahlund effect (Wahlund 1928). However, we highlighted a female-biased dispersal associated with heterozygosity excess for *MC1R* in females. This excess of heterozygosity contrasts with the observed deficit of heterozygotes in locally born females and in males born inside or outside the study area but also with the deficit of heterozygotes observed at the fledging stage (Ducret *et al.* 2016). Those results were detectable with the long-term capture-mark-recapture data but not with indirect estimations using neutral genetic markers. Besides, sex-biased dispersal and non-random gene flow of *MC1R* variants could impact differently male and female fitness (Camacho *et al.* 2013; Tarka *et al.* 2014) but our results did not show an effect of *MC1R* variants on survival and reproductive success. This finding is of high importance because sex-biased dispersal associated with the immigration of particular *MC1R* genotypes should not conduct to a decrease of the population fitness (i.e. migration load,).

Comparison between direct and indirect estimates of sex-biased dispersal

Direct and indirect methods to estimate (sex-biased) dispersal have different strengths and weaknesses; also both methods should be used if possible to compare their results. Direct methods are often difficult to apply in natural populations but indirect methods based on neutral markers are sometimes not powerful enough to detect an effect. Our results from capture-mark-recapture based on long-term monitoring of our

barn owl populations highlighted an overall high dispersal rate in barn owls (71% of immigrants among breeding adults), which is in line with the low genetic differentiation at the scale of continental Europe as a consequence of intense gene flow (Antoniazza *et al.* 2010) and which seems to be common in other birds species as well (Schaub *et al.* 2013; Altwegg *et al.* 2014; Schaub *et al.* 2015). We also revealed that dispersal is female-biased in barn owls, with 78% and 62% of females and males being immigrants, respectively. However, the rate of immigration is so high that the genetic (indirect) methods failed in detecting sex-biased dispersal, with only the *vAIC* tests being significant). Indeed, using simulations, we find an extremely low power of the *mAIc* and *vAIc* tests in detecting the observed female-biased dispersal. Accordingly, simulating a lower dispersal rate resulted in a much higher power to detect sex-biased dispersal using neutral genetic markers. In addition, the statistical power of the tests was reduced if the tests were performed on a single rather than several populations. Because the assignment index tests are commonly performed on a single population or on small spatial scales (Chambers & Garant 2010; Liebgold *et al.* 2013; Harrison *et al.* 2014), we advise researchers to use several methods to estimate sex-biased dispersal in order to decrease the risk of type I and II errors. Although, our simulations involved one type of dispersal scheme (i.e. island model), these findings must be cautiously considered and more simulations should be performed with other dispersal models.

Evolution of male-biased philopatry

Several hypotheses have been proposed to explain the evolution of sex-biased dispersal. The most acknowledged one is in Greenwood's seminal paper (1980), which linked the directionality of sex-biased dispersal in birds and mammals to mating systems. However, a recent review by Trochet *et al.* (2016) using a database of 257 species and phylogenetic approaches, proposed that the evolution of sex-biased dispersal was linked to parental care and sexual dimorphism rather than the mating systems *per se*, a pattern congruent to previous finding at least in birds (Mabry *et al.* 2013). Among raptors for example, which include barn owls, parental roles are asymmetric in the sense that females incubate, brood and partition prey for the nestlings, whereas males hunt unassisted during most of the nestling period (Sonerud *et al.* 2014). Thus, the survival of the brood relies strongly on the hunting efficiency of males. Familiarity with the environment to acquire resources and potentially to

attract females should therefore restrict male's dispersal and favor their philopatry. Concordant with this hypothesis, the results showed that locally born individuals have a higher apparent survival than immigrants in our study site, particularly in males. Locally born individuals also tended to produce slightly more fledglings than immigrants. Although, our survival analyses could not distinguish between mortality and emigration of adults, it conclusively indicates that locally born males have a higher probability to reproduce inside our study area compared to females and immigrants. Importantly, a previous study on barn owl's survival showed that emigration outside our study area is almost inexistent (emigration rate: 0.010, SE = 0.014; Altwegg *et al.* 2003), suggesting that our estimate of apparent survival relates more to true survival of adults than emigration.

Non-random dispersal

Dispersal is often related to morphological, behavioral or ecological traits, also known as dispersal syndromes (Cote *et al.* 2010; Ronce & Clobert 2012; Chakarov *et al.* 2013). Those traits can be sex-specific when dispersal is sex-biased and may explain why sexual dimorphism is often associated with sex-biased dispersal. This co-variation arises from phenotype-related costs and benefits associated to dispersal (Tarka *et al.* 2014; Pakanen *et al.* 2016), as suggested for example with the evolutionary loss of female coloration with migration among wood-warblers (Simpson *et al.* 2015). In barn owls, previous studies found evidence that females move farther than males during natal and breeding dispersal, although individuals' pheomelanic coloration explained better natal dispersal than the effect of sex (van den Brink *et al.* 2012; Roulin 2013). The relationship between dispersal and color could be easily explained by either a greater net costs of being colorful while dispersing (i.e. being dark-rufous is expected to be a cryptic coloration) owing to risks of being detected by visual predators (Simpson *et al.* 2015) or to the fact that the melanocortin system pleiotropically regulates different phenotypic traits such as melanism, physiology and behavior (Ducrest *et al.* 2008; Roulin & Ducrest 2011; Reissmann & Ludwig 2013).

In any case, our results are concordant with previous findings and showed that females immigrate more than males and also carry an excess of heterozygosity for *MC1R*, a gene with a valine-to-isoleucine substitution explaining ~30% of the

pheomelanic coloration in this population (San-Jose *et al.* 2015). Because homozygotes for the $MC1R_{RUFOUS}$ allele are rare inside our Swiss population but also in other populations (Burri *et al.* 2016), the higher frequency of heterozygosity results mostly from a decreased frequency of homozygote for the $MC1R_{WHITE}$ allele in immigrant females. In this species, the pheomelanin-based coloration is sexually dimorphic with females displaying on average a darker-rufous coloration than males and *MC1R* contribute to this sexual dichromatism (San-Jose *et al.* 2015). Also the strong sexual dimorphism in coloration makes difficult to determine whether color (and *MC1R*), sex, or both factors, affect dispersal and how this co-variation affects dispersal at different life stages. Using the European Union for Bird Ringing (EURING) dataset on barn owls, we detected a clear female-biased dispersal over large scales (Ducret *et al.*, unpublished data) and it would possible to quantity how and if this sex-biased dispersal varies between populations having different level of melanism. It would be also possible to compare individual's dispersal distance in populations where individuals are mainly white or dark-rufous to understand the effect of pheomelanin-based coloration on dispersal. Obviously, dispersal propensity can vary with the environment experienced by individuals in the different populations independently of their phenotype or genotype and inclusion of environmental conditions to control for their effect would be necessary.

Effect of sex-biased dispersal and non-random gene flow on fitness

Dispersal is often sex-biased in vertebrates (Palo *et al.* 2004; Biek *et al.* 2006; Berg *et al.* 2009; Paquette *et al.* 2010; Trochet *et al.* 2016) and should conduct dispersal and gene flow to differentially impact male and female fitness (Tarka *et al.* 2014; Camacho *et al.* 2013). As a matter of fact, theoretical and empirical studies have demonstrated that immigration of maladaptive alleles conduct to a reduction in mean fitness of the recipient populations, also known as "migration load" (Garcia-Ramos & Kirkpatrick 1997; Bolnick & Nosil 2007). Also, if one sex has evolved to remain in their native site (philopatry), migration load will impact specifically this sex and the difference in mean fitness between the philopatric and the dispersing sex. Thus, describing gene flow based on particular genotypes or gender is important to understand how dispersal could favor or constrain the adaptive effects of natural selection within populations in a sex-specific manner (Edelaar & Bolnick 2012).

To test the effect of non-random gene flow regarding *MC1R* on survival and reproductive success of male and female barn owls breeding in our study area, we modeled apparent survival and the number of eggs, fledging success and number of fledglings produced between *MC1R* genotypes, sex and dispersal status (locally born individuals vs. immigrants). First, the analysis of apparent survival showed that immigrants survived less well than locally born individuals. Also, if immigration could decrease the mean fitness of the population by introducing maladapted alleles, this effect should be reduced by the lower survival, and consequently reproduction, of immigrants compared to locally born individuals. Regarding the impact of non-random gene flow at *MC1R*, we found that in the second best model of apparent survival, the $MC1R_{RUFOUS}$ allele had only a weak and non-significant effect of decreasing apparent survival. In addition, individuals carrying at least one copy of the $MC1R_{RUFOUS}$ allele did not produce significantly fewer eggs or fledglings. Both results suggest that, despite a higher immigration of heterozygote females at *MC1R*, the lack of effect of *MC1R* on survival and reproductive success should not conduct to a migration load regarding *MC1R*. However, the presence of *MC1R* in the second best survival model could suggest that selection is operating against the rufous coloration but the power to detect this effect is too low due to the fact that *MC1R* only explains a third of the variation in color in this population. Future studies combining survival and reproductive success of differentially colored individuals into a general framework, using for example demographic models and elasticity approach, are needed to validate this hypothesis.

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Supporting information

Table S1: F_{IS} values in adult male and female barn owls for 10 microsatellite loci and averaged over 1998 to 2012. *P* values correspond to the proportion of randomizations that gave a LARGER F_{IS} than the observed. Bold *P* values indicate significant deviation from HW proportion under a risk ∝ = 0.05 and after Bonferroni correction.

	Males		***Females***	
	F_{IS}	*P* values	F_{IS}	*P* values
Ta202	0.154	**<0.001**	0.037	0.108
Ta204	0.010	0.340	0.008	0.362
Ta212	-0.030	0.942	0.062	**<0.001**
Ta214	0.016	0.253	0.029	0.087
Ta215	-0.025	0.820	0.046	0.049
Ta305	-0.023	0.831	0.047	0.033
Ta310	-0.100	**1.000**	0.036	0.068
Ta402	-0.012	0.847	-0.028	0.991
Ta408	0.009	0.338	-0.051	0.997
Ta413	-0.061	**1.000**	-0.003	0.609

Table S2. Model selection results of the general structure of recapture (ρ) and survival probabilities (φ) as a function of time (t: year) and group (g: sex, status, *MC1R*), with a constant (.), additive (+) or interactive (*) effect.

Model	**AICc†**	**ΔAICc§**	***w*‡**	**K**	**Deviance**
φ(g + t) ρ(t)	2199.05	0.00	0.60	42	782.41
φ(g + t) ρ(sex + t)	2199.85	0.79	0.40	43	781.07
φ(g + t) ρ(sex * t)	2214.12	15.07	0.00	60	758.74
φ(t) ρ(sex + t)	2223.57	24.52	0.00	36	819.62
φ(t) ρ(t)	2224.30	25.24	0.00	35	822.45
φ(g + t) ρ(.)	2227.16	28.10	0.00	26	844.11
φ(g + t) ρ(sex)	2229.21	30.16	0.00	27	844.09
φ(t) ρ(sex * t)	2236.05	37.00	0.00	53	795.86
φ(t) ρ(sex)	2247.85	48.80	0.00	20	877.21
φ(t) ρ(.)	2248.51	49.45	0.00	19	879.92
φ(g) ρ(t)	2278.70	79.65	0.00	26	895.65
φ(g) ρ(sex + t)	2278.83	79.78	0.00	27	893.71
φ(.) ρ(sex + t)	2290.22	91.16	0.00	20	919.57
φ(.) ρ(t)	2293.76	94.70	0.00	19	925.17
φ(g) ρ(sex * t)	2293.92	94.86	0.00	44	873.02
φ(.) ρ(sex * t)	2307.30	108.25	0.00	37	901.24
φ(g * t) ρ(t)	2366.83	167.77	0.00	161	673.83
φ(g * t) ρ(sex + t)	2369.02	169.97	0.00	162	673.48
φ(g * t) ρ(.)	2387.22	188.16	0.00	145	734.34
φ(g * t) ρ(sex)	2389.40	190.34	0.00	146	734.05
φ(g * t) ρ(sex * t)	2394.89	195.83	0.00	178	658.14
φ(g) ρ(.)	2420.50	221.45	0.00	9	1072.33
φ(g) ρ(sex)	2422.43	223.38	0.00	10	1072.22
φ(.) ρ(sex)	2437.19	238.14	0.00	3	1101.12
φ(.) ρ(.)	2438.01	238.95	0.00	2	1103.95

†AIC value corrected for small simple sizes
§Difference in a model's AICc to the best-ranked model's AIC
‡Model weight: probability of the model given the data
K: Number of parameter estimated

Table S3. Model selection results of apparent survival (φ) and recapture probabilities (ρ) of adult barn owls breeding in western Switzerland in function of sex, dispersal status (immigrant vs. resident) and *MC1R* genotypes (II/VI vs. VV). Included are the models with either time (t: year) or sex effect (sex) on the recapture probability (i.e. the two best models from the first modeling step (Table S2)) and within ΔAICc < 4.

Model	AICc†	ΔAICc§	*w*‡	K	Deviance
φ(sex + status + t) ρ(t)	2191.23	0.00	0.21	37	785.17
φ(sex + status + t) ρ(sex + t)	2192.02	0.79	0.14	38	783.84
φ(sex + status + *MC1R* + t) ρ(t)	2192.63	1.40	0.11	38	784.46
φ(sex + status + sex * status + t) ρ(t)	2193.34	2.11	0.07	38	785.17
φ(sex + status + *MC1R* + t) ρ(sex + t)	2193.42	2.19	0.07	39	783.14
φ(sex + status + *MC1R* + sex * *MC1R* + t) ρ(t)	2193.80	2.57	0.06	39	783.51
φ(sex + status + sex * status + t) ρ(sex + t)	2194.13	2.90	0.05	39	783.84
φ(sex + status + *MC1R* + *MC1R* * status + t) ρ(t)	2194.27	3.04	0.05	39	783.98
φ(sex + status + *MC1R* + sex * status + t) ρ(t)	2194.74	3.51	0.04	39	784.46
φ(sex + status + *MC1R* + *MC1R* * status + t) ρ(t + sex)	2195.06	3.83	0.03	40	782.66
φ(sex + status + *MC1R* + sex * *MC1R* + *MC1R* * status + t) ρ(t)	2195.14	3.91	0.03	40	782.73

†AIC value corrected for small simple sizes
§Difference in a model's AICc to the best-ranked model's AIC
‡Model weight: probability of the model given the data
K: Number of parameter estimated

Supporting results:

Hardy-Weinberg proportion at *MC1R*

Adult females showed a significantly higher excess of heterozygotes (average F_{IS} = -0.062, F_{IS} year-range = -0.231 to 0.053) than males (average F_{IS} = 0.022, F_{IS} year-range = -0.268 to 0.47) (Wilcoxon rank sum test, W = 96.5, P = 0.015).

Dispersal and gene flow

The marking-recapture data indicated that 32.4% of the adults breeding in our study area from 1998 to 2016 were born inside the study area. Also, we observed a higher proportion of breeding males born inside the study area compared to females (42% vs. 25%; Chi-square test, χ^2 = 33.6, P < 0.001; Table 1). In females, immigrants tended to be more often heterozygous for the *MC1R* gene than residents (Fisher's exact test, P = 0.084), while in males we did not find such a difference (P = 0.87; Table 1).

Table S4. Proportion and percentages of *MC1R* genotypes in philopatric and immigrant male and female barn owls breeding in Western Switzerland between 1998 to 2016 (II, homozygotes for the $MC1R_{RUFOUS}$ allele; VI, heterozygotes; VV, homozygotes for the $MC1R_{WHITE}$ allele).

		II	VI	VV	Total
Males	Philopatric	6 (3.1%)	53 (27.3%)	135 (69.6%)	194
	Immigrant	8 (3.0%)	80 (29.7%)	181 (67.3%)	269
Females	Philopatric	5 (3.5%)	32 (22.4%)	106 (74.1%)	143
	Immigrant	6 (1.4%)	128 (29.5%)	300 (69.1%)	434

Chapter 4

Climate drives color and *MC1R* variation in European Barn owls (*Tyto alba*)

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[2]These authors share seniority.

Personal contribution: I designed the project, performed the lab work (microsatellites genotyping), analysed the data and wrote the manuscript.

Abstract

Color variation offers good illustrations of the strong effect of natural selection because coloration has many functions allowing individuals to communicate or to adapt to their environment. Its genetic basis is commonly well known and those genes often play a similar role in multiple species, such as the melanocortin-1 receptor gene, *MC1R*. However, it often remains challenging to identify the exact selective agents when the functionality of a trait is not perfectly understood. In this study, we aimed at unraveling the environmental drivers of the European barn owl clines of plumage coloration and *MC1R*. In particular, we studied the importance of spatial distances, climatic and landscapes features using two spatial models (GLS and GDM) and a dataset of 434 unrelated barn owls from Iberia to the Balkans, including Great Britain. Apart from the effect of geographic distance, we observed that isothermality and precipitation seasonality explained the observed variation in color and *MC1R* and could be indirectly linked to the barn owls' preys' distribution or to individuals' ability to handle harsh environmental conditions. However, we also observed a discrepancy when we included the Great Britain samples into the spatial analyses explaining coloration but not *MC1R*. As we observed that Great Britain populations are differentiated at neutral markers from the rest of the continent, we suggest that the effect of neutral processes rather than differential selection is responsible for this discrepancy. Overall, these findings indicate that climatic factors can be important drivers of pheomelanin-based coloration and *MC1R*, although future studies should confirm a direct effect of those factors.

Introduction

Unraveling the effect of selection on phenotypic variation in wild populations has long attracted evolutionary biologists because it is a necessary step to understand how populations would be able to adapt in the face of environmental changes, such as climate warming or habitat destruction (Karell *et al.* 2011; Zeuss *et al.* 2014; Zimova *et al.* 2016). A major challenge remains to demonstrate that adaptation has occurred, as it requires evidence of population genetic changes and causal effect of natural selection. With the availability of a broad range of population genetic and spatial analyses, it is possible to acknowledge that adaptation rather than neutral processes better explain variation in a trait or a gene of interest (Merilä & Crnokrak 2001; Beaumont *et al.* 2002; Conover *et al.* 2006; Thomassen *et al.* 2010). However, it remains often difficult to identify the exact selective agents when the functionality of a trait is not perfectly understood.

Melanin-based coloration remains a suitable model to highlight adaptive variation. First, the genetic basis is commonly well known because those genes often play a similar role in multiple species. A recent series of papers has shown that a single gene, melanocortin-1 receptor (*MC1R*), is responsible for color polymorphism in a variety of vertebrates (Mundy 2005). Second, coloration has various functions that can be adaptive such as crypsis (Hoekstra *et al.* 2004, 2005; Munoz *et al.* 2013), social signaling (Hanlon *et al.* 1999; Krüger *et al.* 2001), thermoregulation (Galeotti *et al.* 2009; Dreiss *et al.* 2016, Koskenpato *et al.* 2016) or immunity (Jacquin *et al.* 2011; Roulin *et al.* 2011; Männiste & Hõrak 2014). A good example is the case of the Pocket mouse (*Chaetodipus intermedius*) in southern Arizona, where natural selection explained variation in the frequency of different color morphs, and underlying *MC1R* locus, to match the substrate color (Hoekstra *et al.* 2004).

European barn owls (*Tyto alba*) show strong clinal variation in their pheomelanic-based coloration present on their ventral body parts. Such color cline has been suggested to result from local adaptation rather than neutral processes, which was further supported by ABC simulations (Antoniazza *et al.* 2010, 2014). However, what local factors are driving selection on plumage coloration across Europe remains unknown. Therefore, we aimed at identifying the potential selective agent or agents behind color variation at such a large scale. We used and compared two different spatial approaches, Generalized Least Squares (GLS) and Generalized Dissimilarity Modeling (GDM), to explain the variation in rufous

pheomelanic-based coloration by climatic and landscape factors using a large dataset of GIS-based information of European barn owls. Those factors include among others temperature stability (isothermality), precipitation seasonality, vegetation density, habitat heterogeneity and disturbance (i.e. agricultural lands as intensive habitats, pasture as extensive habitats).

Because the expression of the pheomelanin-based trait in barn owls is weakly sensitive to the environment (Roulin 2003; Roulin & Dijkstra 2003; Roulin *et al.* 2010), variation in coloration among European barn owls would indicate adaptive evolution rather than phenotypic plasticity, therefore selecting underlying color-related genes, such as *MC1R*. Indeed, a previous study found a non-synonymous mutation on a candidate gene, the melanocortin-1 receptor gene (*MC1R*), which explains 47.2% of the total variance of pheomelanic pigment content (San-Jose *et al.* 2015). As for coloration, a steep cline in the frequency of this mutation has been found in Europe (Burri *et al.* 2016). Also, we will use the same spatial approaches to test for an association between environmental factors and variation at the *MC1R* gene, expecting that the same factors account for geographic variation in the degree of color variation and in the frequency of *MC1R* alleles.

Materials and methods

Study species

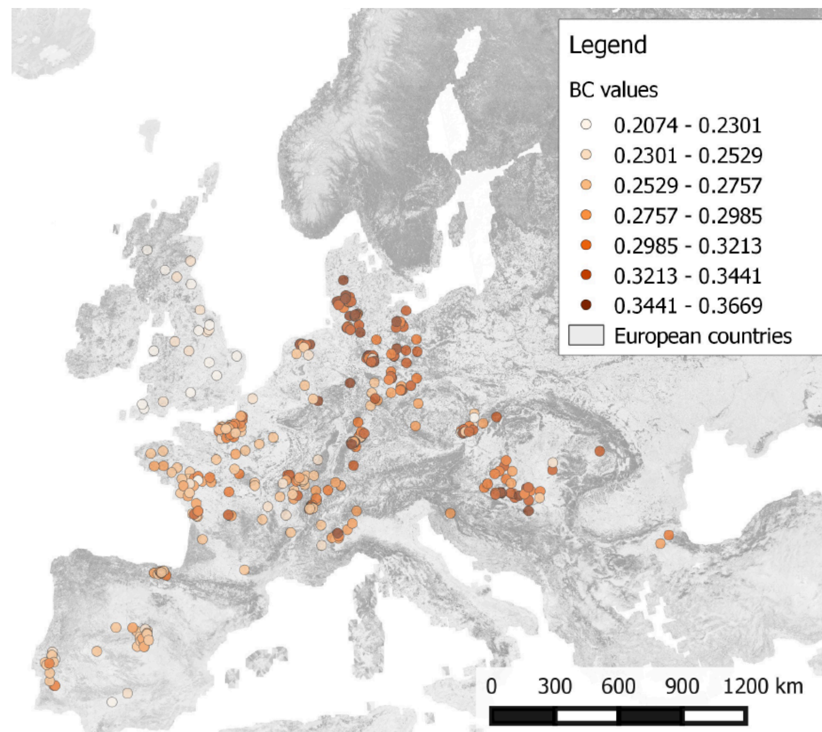
The barn owl is a nocturnal raptor that principally feeds upon small mammals captured in open landscapes. In Switzerland and Israel, and more generally in Europe, diet and pheomelanin-based coloration are correlated, with whiter individuals predating more often upon Muridae (i.e. woodmice species, *Apodemus spp.*), whereas rufous individuals predate more often upon voles (*Microtus arvalis*) (Roulin 2004a; Charter *et al.* 2012). This association could result from females' habitat nest choice that depends on their pheomelanin-based coloration, as observed in Switzerland. Indeed, whitish females were found to produce more fledglings when breeding in wooded areas, whereas rufous females when breeding in sites with more arable fields (Dreiss *et al.* 2012). Thus, landscape variables could be important factors explaining the distribution of color variants. In addition, there are numerous examples of similar clinal variation in pheomelanin-based coloration in the *Tytonidae* (South America, Africa and North America; Roulin *et al.* 2009). Particularly in North America, barn owls were found to be darker-rufous in colder regions (Roulin & Randin 2015). Thus,

climatic variables such as temperature and precipitation are good candidates for explaining color and *MC1R* variation.

Tissue sampling

The study included 434 unrelated barn owls captured alive or found dead between 2007 and 2010 across 15 European countries (Fig. 1). For all individuals, the GPS coordinates were recorded at their capture location with 1 km of resolution. Four to five feathers were kindly plugged out from the individuals' breasts and used for the genetic analysis as well as for color assessment (see below). The samples of 325 individuals were previously analyzed in Burri *et al.* (2016). Here, we added samples from 109 new individuals from continental Europe and Great Britain, which were processed following the same methodology as applied in Burri *et al.* (2016). DNA was extracted from the basal tips of several breast feather quills using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). DNA was used to sex the individuals using sex-specific molecular markers as described by Py *et al.* (2006) and for *MC1R* genotyping (see below).

Figure 1: Sampling location of 434 barn owls captured in Europe between 2007 and 2010. Color of the point represents the variation of the pheomelanin-based coloration among samples, which was measured by spectrophotometer on ventral feathers (Brown Chroma values).



Assessment of plumage coloration

We objectively measured plumage coloration as described in Burri *et al.* (2016). We used a spectrophotometer (OceanOptics usb4000, Dunedin, FL, USA) attached to a dual deuterium and halogen 2000 light source (Mikropackan, Mikropack, Ostfildern, Germany) to measure reflectance of the feathers within a range from 300 nm to 700 nm and with a standardized measure angle of 90° to the long axis of the feather (Montgomerie 2006). The measurements were done on at least 4 (max. 5) breast feathers per individual. For each feather, we measured reflectance at 4 different points of the upper part of the feathers' vanes). For each point, we derived a brown chroma index (hereafter referred to as BC), which was calculated as the ratio between reflectance at long visible wavelengths (600-700 nm) and total reflectance across the entire UV-visible range (300-700nm). Larger BC values indicate a larger relative contribution of long, yellow-to-red wavelengths and thus more rufous, pheomelanic colorations, whereas smaller BC values are indicative of a lower contribution of long wavelengths relative to all other visible wavelengths and thereby flatter reflectance spectra and whiter plumage colorations. A mean value of brown chroma was calculated per feather and, then, per individual considering the 4-5 feathers measured. A set of feathers from 12 different individuals used in Burri *et al.* (2016) was measured again to calibrate the BC values obtained from the new added 109 individuals and to obtain comparable values between these individuals and the individuals previously analyzed in Burri *et al.* (2016). Briefly, the BC values of the new 109 individuals were transformed using the estimates of the regression of the BC values taken on the 12 individuals in this study over the BC values of the 12 individuals in Burri *et al.* (2016).

Environmental variables

We collected a set of high-resolution and satellite remote-sensing variables that considered different climatic and landscape features. Because climatic variables are often highly correlated to each other, we first estimated their degree of correlation to later reduce the number of climatic variables in the analysis. Thus, 8 climatic variables (Fig. S1) were extracted for every individual's location from the WorldClim database at a 30 arc-second of resolution (Hijmans *et al.* 2005) using the R software v3.2.4 (R Core Team 2013). After checking their correlations (Figure S1), only three climatic variables (isothermality (BIO3), annual precipitation (BIO12), precipitation seasonality (BIO15)) were kept for the spatial

models Also, we added the elevation as predictor, extracted from the WorldClim database, and seven diverse landscape variables (see description Table 1) extracted from MODIS and from Corin Land Cover databases (European Environment Agency, 2006) using the QGIS software v2.2.0 (QGIS Development Team, 2009). The environmental data were extracted for all individuals within a buffer diameter of 10 km around the location where an individual was found, which corresponds to the species home range (Meek *et al.* 2003; Bond *et al.* 2005; Arlettaz *et al.* 2010). The landscape and climatic variables had a relatively low degree of correlation with each other ($-0.42 < r < 0.43$; Fig. 2) and were used directly as predictors in the models.

Figure 2: Correlation matrix of the variables performed on the full dataset (including Great Britain samples). The degree of correlation (r) between pairs of variables are indicated in each grid cell of the matrix and are presented by a gradient of color from red (negative) to violet (positive) values.

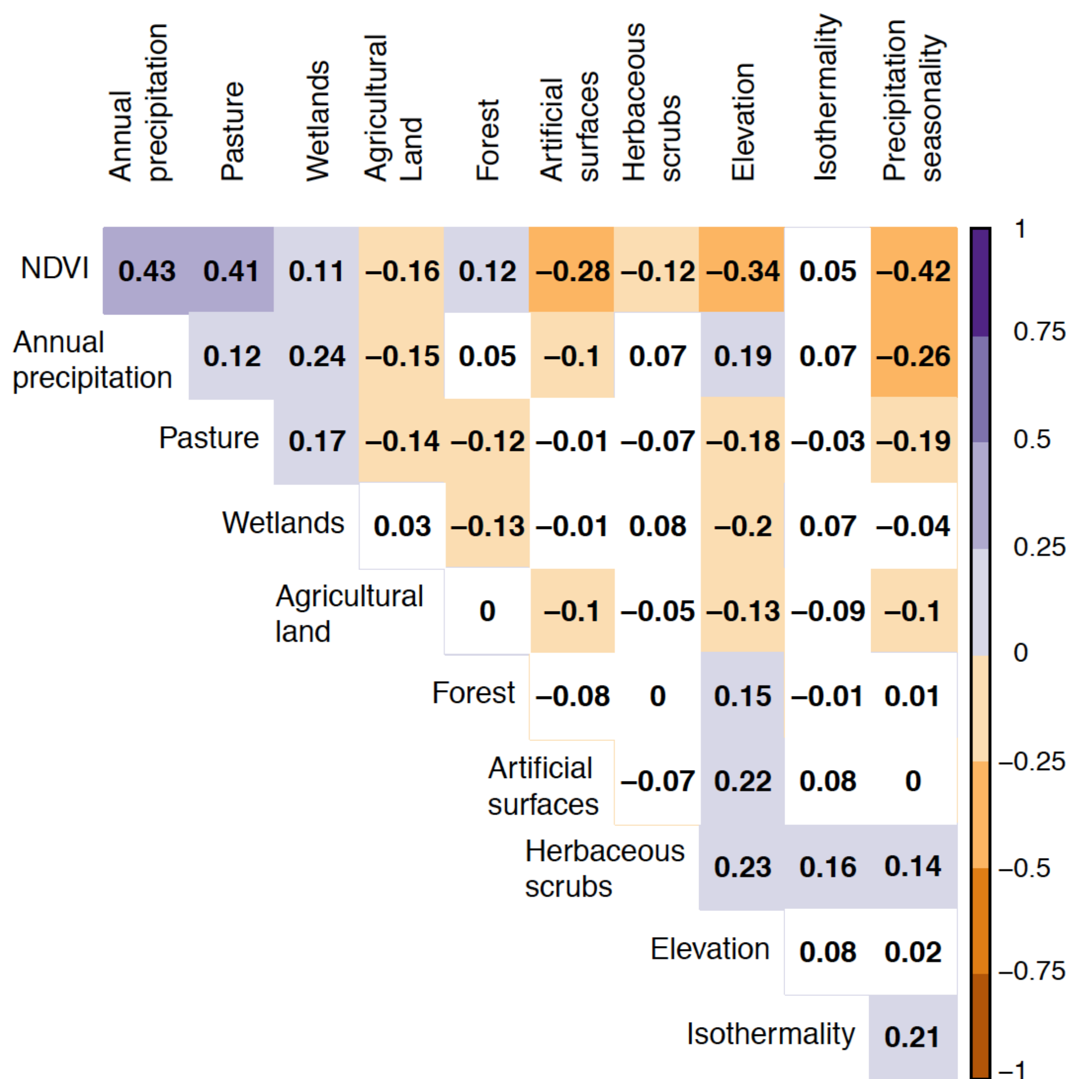


Table 1: Variables description used in the spatial analyses

Variables	Description
Isothermality (BIO3)	Mean diurnal temperature range (mean of monthly (max. – min. temp.) divided by the temperature annual range (annual max. –min. temp.)
Annual precipitation (BIO12)	Sum of monthly precipitation
Precipitation seasonality (BIO15)	Coefficient of precipitation variation
Artificial surfaces	Urban areas and fabric, industrial, commercial and transport units, mine, dump and construction sites
Arable land	Non-irrigated and permanently irrigated land, rice fields, permanent crops, vineyards, fruit trees, berry plantations and olives groves
Pastures	Land used for grazing
Forest	Broad-leaved forest, coniferous forest and mixed forest
Wetland	Inland marshes, peat bogs, salt marshes, salines, intertidal flats, water courses, water bodies and coastal waters
Scrubs-Herbaceous vegetation	Natural grasslands, moors and heathland, sclerohyllous vegetation and transitional woodland-shrub
NDVI (Normalized Difference Vegetation Index; MODIS)	Measure of “greenness” in the landscape as an indicator of red energy reflectance due to plants photosynthetic activity
Elevation	Altitude or height above sea level

***MC1R* and microsatellite genotyping**

The melanocortin-1 receptor (MC1R) contains a valine-to-isoleucine substitution at position 126 of the protein, where the valine allele ($MC1R_{WHITE}$ allele) is related to a white ventral plumage, whereas the isoleucine allele ($MC1R_{RUFOUS}$ allele) is strongly related to a rufous ventral plumage. The 109 new individuals were genotyped for *MC1R* (N = 70 homozygotes at the $MC1R_{WHITE}$ allele, N = 7 homozygotes at $MC1R_{RUFOUS}$ allele and N = 32 heterozygotes) using an allelic discrimination (AD) assay. The AD assay consisted of a first pre-amplication PCR to amplify the first part of *MC1R* where the mutation is located. The relative concentrations of the PCR products were adjusted to each other before the assay because initial DNA concentration is critical for AD assays. Then, the AD assays were run on an ABI 7500 qPCR machine (Applied Biosystems) where the fluorescent markers of mutant and the wild-type probes (respectively, ATOO550 and FAM, Microsynth) permitted to highlight the presence of each *MC1R* variants by quantitative amplification (see San-Jose *et al.* 2015 for further information on the method). In each assay, we always ran positive controls for each genotype and negative controls and each sample was run in duplicate using two different PCR products. We genotyped the new individuals for 22 microsatellite markers regrouped in five microsatellite multiplexes following the procedure of Antoniazza et al. (2014). Briefly, polymerase chain reactions (PCR) were performed using the Multiplex PCR Kit (Qiagen). Fragment analyses were run on an ABI 3100 sequencer with a ROX 500 size standard and allele lengths were assigned using Genemapper 4.0 (Applied Biosystems, Zug, Switzerland).

Statistics

All the statistical analyses were conducted with the R software v3.2.4 (R Core Team 2013).

Neutral genetic population structure

To infer the neutral population structure from continental Europe and Great Britain, we performed principal coordinate analyses (PCoA) based on pairwise F_{ST} between 20 localities using the function ‘genetic.dist’ of the package Hierfstat (Goudet & Jombart 2017). Eighteen localities regrouped the samples as in Burri *et al.* (2016) and two additional localities correspond to southern Great Britain (N = 13 individuals) and northern-center of Great Britain (N = 14 individuals).

The results from PCoA suggest that increased drift or reduced gene flow could have resulted in a lower genetic, and possibly phenotypic, variation in this population. This pattern related to demographic processes may blur the effect of the environment and we decided to perform each spatial analysis on either the full dataset of 427 individuals or excluding the 20 Great Britain samples.

Generalized Least Squares models

To predict the effect of the environmental variables and historical/demographic evolutionary processes on color variation taking into account spatial autocorrelation, we used Generalized Least Squares models (GLS) as implemented in the 'nlme' package (Pinheiro *et al.* 2016). All continuous covariates were standardized (i.e. centered and scaled) to allow model convergence. The full model contained the single effect of the 11 environmental variables, sex and the interaction between each environmental variable and sex to test for sex-specific divergent selection. Before performing stepwise selection models, we compared the full models with and without different spatial autocorrelation structure (i.e. none, linear, ratio, exponential, Gaussian) and compared their fit using Akaike Information Criterion values (AIC; Akaike, 1974). The four spatial structures gave similar AIC values and we kept the Gaussian correction as it gives the smallest AIC values on both datasets. Addition of this spatial structure on both dataset significantly improved the models fit (Full dataset: $\Delta AIC = 52.85$, $P < 0.001$; Continent: $\Delta AIC = 7.56$, $P = 0.003$). Full models were simplified by backward elimination of the terms using the function 'stepAIC' from package 'MASS' (Venables & Ripley 2002).

Generalized Dissimilarity Modeling

We tested the prediction that similar environmental factors explained variation at *MC1R* and at plumage coloration. We used the package 'GDM' in R (Manion *et al.* 2016) to perform Generalized Dissimilarity Modeling (GDM; Ferrier *et al.* 2007), a recently developed matrix regression technique that relates dissimilarities in predictor variables (e.g. environmental variables or geographic distance) to dissimilarities in response variables (e.g. genetic or phenotypic distances between sites). This method is appropriate for non-linearity in the response variables such as genetic distances and it can fit non-linear rate of change in the response variables along environmental gradients with I-spline basis function (Thomassen *et*

al. 2010). We computed *MC1R* and color dissimilarity matrix between the 20 localities in Europe based on F_{ST} values for *MC1R* using the package Hierfstat and on absolute differences for color. We computed a mean value per site for each environmental variable, which was used in the model to construct the matrix of environmental dissimilarity between sites.

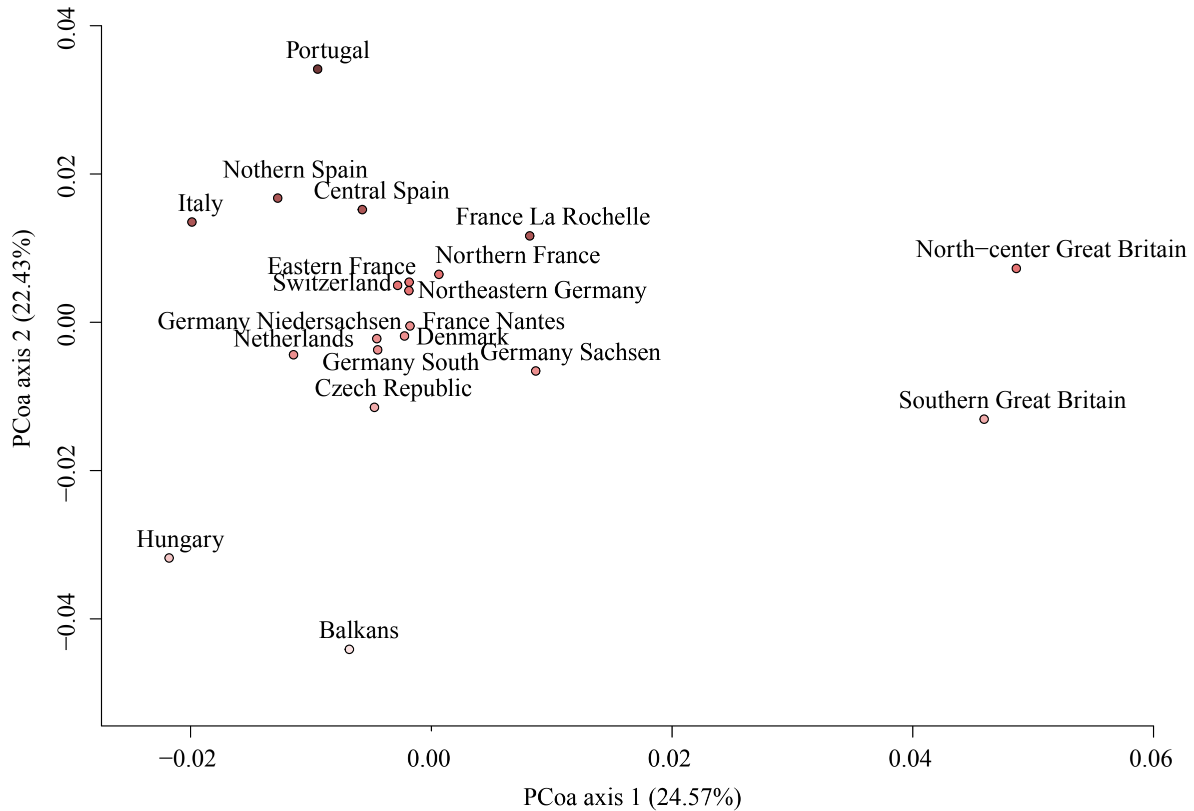
Because population structure resulting from drift-migration balance could also explain variation in phenotypes and underlying genotypes, it is important to control that environmental variables rather than variation at neutral markers explain variation at the gene of interest (here *MC1R*). We therefore added F_{ST} genetic distances between sites calculated on the 22 microsatellites as a predictor in the GDM analyses for *MC1R*. To test the variable's contribution in explaining the model deviance, we used a permutation test that randomized the values of the variable of interest between sites (N = 1000). If the observed model deviance explained by the environmental variable is higher than 95% of the values of model deviance after randomization, this variable is considered significant. Only variables with a significant contribution ($P < 0.05$) were retained in the final model.

Results

Neutral population structure

Genetic differentiation based on F_{ST} among barn owls localities in the PCoA resulted in a clear differentiation between Great Britain localities and continental Europe as observed in the first axis (24.57% variance explained; Fig. 3). The second axis of the PCoA indicated a southwest-to-east gradient of genetic differentiation (22.43% variance explained). However, the overall genetic differentiation in Europe was particularly low (overall F_{ST}: 0.047), as found in Burri *et al.* (2016; overall F_{ST}: 0.045).

Figure 3: Neutral population structure among European barn owls. PCoA was based on pairwise F_{ST} between 20 localities using 22 microsatellite markers. The 18 localities in continental Europe are similar to Burri *et al.* (2016) and 2 were added for Great Britain.

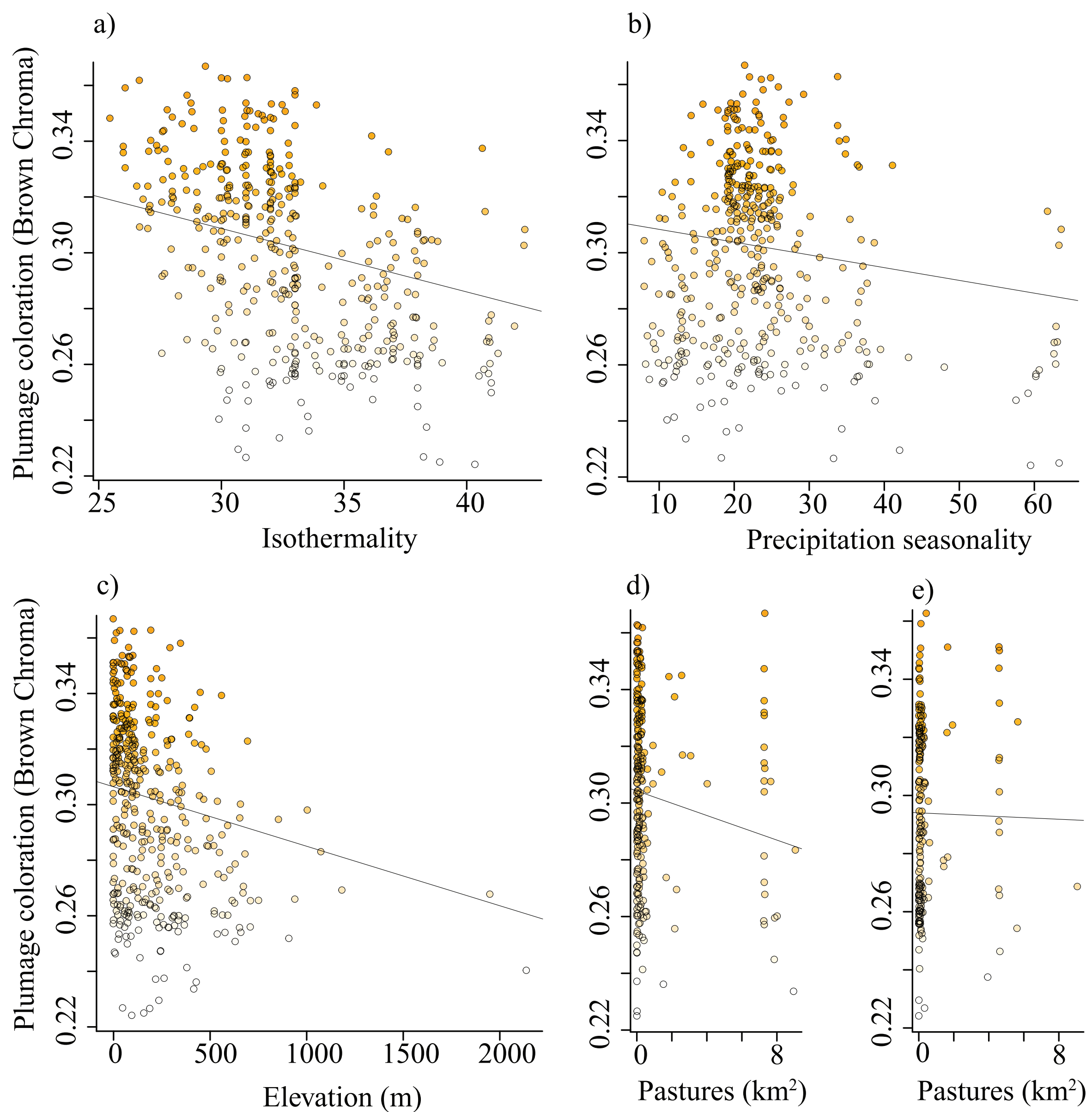


Generalized Least Squares models (GLS)

When the GLS analyses were performed including the samples from Great Britain, backward elimination of the terms highlighted only sex as a significant predictor of color variation (Sex: $\chi^2 = 16.99$, $P < 0.001$). Excluding the samples from Great Britain, sex was still significant in the model (Sex: $\chi^2 = 11.04$, $P < 0.001$). Additionally, we found a significant effect of isothermality ($\chi^2 = 10.30$, $P < 0.01$) and elevation ($\chi^2 = 8.64$, $P < 0.01$). Barn owls were whiter at high than low elevations ($t_{398} = -2.94$, $P = 0.004$; Fig. 4c) and in sites where values of isothermality were higher (i.e. when the day-to-night temperatures oscillate more closely to the annual temperature oscillation) ($t_{398} = -3.21$, $P = 0.0014$; Fig. 4a). Seasonal precipitation significantly improved the model fit ($\chi^2 = 3.85$, $P = 0.0498$), with a trend for whiter barn owls where seasonal precipitation increased ($t_{398} = -1.96$, $P = 0.051$; Fig. 4b). In addition, the final model contained a trend for an interaction between pasture and sex ($\chi^2 = 2.99$, $P = 0.084$). Indeed, females were darker in environments with low density of pastures ($t_{398} = -2.32$, $P =$

0.021; Fig. 4d), while no relationship with pastures was observed in males (t_{398} = -0.019, P = 0.85; Fig. 4e). Also, a non-significant interaction between the effect of wetlands and sex was included in the final model (χ^2 = 2.37, P = 0.12) but the effect of wetland was not significant in females (t_{398} = 0.75, P = 0.45) and males (t_{398} = -1.30, P = 0.20).

Figure 4: Plumage coloration (brown chroma values) of continental barn owls explained by isothermality (a), precipitation seasonality (b), elevation (c) and pastures in females (d) and males (e). Black lines represent the regression line from the GLS summary.



Generalized Dissimilarity Modeling (GDM)

The best statistical model of *MC1R* dissimilarities between sites in Europe contained geographic distance (*P* < 0.001), isothermality (*P* < 0.001) and seasonal precipitation (*P* = 0.004), which together explained 53.58% of *MC1R* variation (Table 3). Geographic distance was the most important variable explaining genetic dissimilarity (29.50% change in deviance explained by the full model and the deviance explained by a model fit with that variable permuted), followed by isothermality (8.03%) and seasonal precipitation (2.47%). The results were qualitatively similar if the samples from Great Britain were excluded from the analyses with geographic distance (30.55% change of deviance after permutation, *P* < 0.001), isothermality (5.97%, *P* = 0.002) and seasonal precipitation (4.69%, *P* = 0.004) as significant predictors. This model explained 55.51% of the observed *MC1R* variation at the continental scale (Table 3).

The best statistical model of brown chroma dissimilarities between sites in Europe contained only geographic distance (*P* < 0.001) and explained 21.60% of BC variation (Table 3). Note that the two previous best models included isothermality (11.59% change in deviance; *P* = 0.17) and wetlands (14.84% change in deviance; *P* = 0.06) and explained 28.22% of BC variation. Excluding the samples from Great Britain, geographic distance was the most important variable explaining BC dissimilarity (44.58%; *P* < 0.001), followed by isothermality (19.65%; *P* = 0.018). This model explained 37.10% of the observed BC variation at the continental scale (Table 3).

Table 3: Summaries of the full and the best-supported GDM models explaining *MC1R* dissimilarity (F_{ST}) as well as Brown Chroma (BC) dissimilarity by environmental and geographic distance between localities. We added neutral genetic distances (F_{ST}) as a predictor in the GDM for *MC1R* variation. The best-supported models contained only significant variables ($P < 0.05$) tested by permuting the data within each predictor (N = 1000 permutations). GDM were performed on the full dataset (i.e. continent and UK) and on the continent alone. The best-supported model for the GDM performed on Brown Chroma on the full dataset contained only the geographic distance as significant variable.

	MC1R		**Brown Chroma**	
	Cont. + UK	**Continent**	**Cont. + UK**	**Continent**
Full models				
Model deviance	24.64	17.89	30.26	12.85
Percentage deviance explained	53.77	55.90	32.53	41.03
Variable importance				
Geographic distance	24.56	21.55	26.61	27.31
Isothermality	5.42	5.04	12.13	17.1
Annual precipitation	0.00	0.32	0.00	2.12
Seasonal precipitation	2.47	3.02	9.45	4.35
Wetlands	0.00	0.00	2.84	0.00
Agricultural lands	0.00	0.00	0.00	0.00
Pastures	0.00	0.00	0.00	0.00
Forest	0.00	0.00	0.20	0.00
Scrubs-Herbaceous	0.33	0.07	6.00	0.45
Artificial surface	0.00	0.00	0.00	0.00
Annual vegetation density	0.00	0.00	0.00	0.00
Elevation	0.00	0.00	0.00	1.00
Neutral genetic distance	0.00	0.00	-	-
Best-supported models				
Model deviance	24.74	18.05	35.16	13.71
Percentage deviance explained	53.58	55.51	21.60	37.10
Variable importance				
Geographic distance	29.50	30.55	-	44.58
Isothermality	8.03	5.97	-	19.65
Seasonal precipitation	3.94	4.69	-	-

Discussion

The various functions of coloration in the animal kingdom have provided remarkable illustrations of the strong effect of natural selection and local adaptation to divergent environments (Nachman *et al.* 2003; Hoekstra *et al.* 2004; Rosenblum *et al.* 2010). Previous studies suggested that local adaptation, rather than neutral processes, maintains clinal color variation and underlying *MC1R* variants at the European scale in barn owls (Antoniazza *et al.* 2010, 2014; Burri *et al.*, 2016). However, the selective agent still needed to be discovered. The pronounced geographic structure shaping color and genetic variation in Europe highlighted the necessity to use spatial modeling to take into account geographic distance and spatial autocorrelation. In the present study, we aimed to explain variation in the pheomelanic-based coloration present on the ventral body part of European barn owls by climatic and landscape variables. Also, we tested for an association between environmental factors and variation at the *MC1R* gene, expecting that the same factors will underlie both color and genotypic variation. The two spatial analyses GLS and GDM often show an effect of isothermality and precipitation seasonality, as well as geographic distance and sex, in explaining both color and *MC1R* variation. However, those effects disappeared when the Great Britain samples were considered in the analyses and could suggest that neutral processes such as increased drift are important factors shaping phenotypic and genetic variation in this population.

Effect of demographic processes on neutral and phenotypic differentiation

Neutral genetic divergence between populations depends on a drift-migration balance where drift increases genetic divergence between populations and gene flow through migration will homogenize genetic variation (Wright 1943; Allendorf & Luikart 2007). Previous studies on European barn owls have described a pattern of isolation-by-distance from Iberia to northeast Europe that shape neutral genetic differentiation (Antoniazza *et al.*, 2010; Burri *et al.*, 2016). Based on our dataset that include new individuals on the continent, we observed a similar pattern of gradual genetic differentiation from southeast to northeast Europe in the second PCoA axis. However, the pattern observed in the first PCoA axis was quite different. It showed that Great Britain and populations on the continent are remarkably differentiated at neutral markers, implying that large water body such as sea could constrain gene flow between barn owls populations. Indeed, when gene flow is drastically reduced over short

distances due to geographical barriers preventing migration, genetic drift will cause isolated populations to become genetically and phenotypically distinct (Slatkin 1987; Mila *et al.* 2009).

Previous studies described that birds of the Great Britain and British Islands are particularly white compared to the rest of continental Europe (Roulin 2003; Roulin & Randin 2016) and as observed in figure 1. Thus, it is not clear if neutral and color differentiation between Great Britain and the continent results from a reduced gene flow and an increased effect of drift, as explained above, which balances the effect of selection, or from a different selective agent operating on the Great Britain population. Indeed, the results from the GLS and GDM using coloration or *MC1R* as response variable were somehow contrasted. When the British samples were included in the spatial analyses, only sex or geographic distances were significant to explain color variation in the GLS and GDM analyses. However, isothermality and precipitation seasonality were significant in explaining *MC1R* variation when the Great Britain samples were included. Two hypotheses could explain this discrepancy. First, different results between GLS and GDM may arise from the fact that those models are based, respectively, on individual and population, thus harboring different sample sizes and variance in the response variable. The second possibility could come from a different balance of selection-drift-migration acting on color and *MC1R* between the Great Britain and the continent. Therefore, future studies are necessary to disentangle the effect of selection and demographic processes in shaping the Great Britain population diversity. For example, a study based on the Berthelot's pipit (*Anthus berthelotti*) populations distributed on 13 islands revealed that isolation-by-colonization (i.e. founder effects), rather than isolation-by-distance or adaptation was responsible for both genetic and phenotypic divergence between populations (Spurgin *et al.* 2014).

Selective agent at the continental scale

Among continental European barn owls, previous studies highlighted a pronounced geographic structure of coloration with white individuals located in southeast Europe and coloration becoming gradually darker-rufous morph in northeast Europe (Roulin, 2003; Antoniazza et al., 2010, 2014). Variation between populations appeared to be strongly determined by spatial proximity, which may reflect adaptation to similar environment (Burri *et al.* 2016). In our spatial analyses, we also found a strong effect of geographic distance and

spatial structure in explaining the variation in coloration and *MC1R*. However, our results also highlighted few environmental variables that are significant predictors of phenotypic and genetic variation. Individuals were found to be darker-rufous and more frequently homozygotes for the $MC1R_{RUFOUS}$ allele in sites with lower isothermality values (i.e. a ratio value closer to 0) and lower elevation. Isothermality quantifies how large the day-to-night temperatures oscillate relative to the summer-to-winter (annual) oscillations. This variable is highly correlated to annual temperature and temperature seasonality (respectively, r = 0.65 and r = -0.61; Fig. S1). Noticeably, seasonality for precipitation was also, although moderately, a significant factor explaining color and *MC1R* variation. Also, darker-rufous individuals seemed to be present in sites with less oscillating temperature and precipitation.

However a positive association does not necessarily imply causation and a direct effect of the variables. For example in the Swainson's thrush (*Catharus ustulatus*), Ruegg *et al.* (2006) found that song evolution among populations was not directly linked to climate but rather to the forest type, itself affected by climate. In barn owls, variation in melanin-based coloration explained by climatic variables has been suggested to arise from indirect effects linked to individuals' abilities to handle stressful conditions or elevated parasitism (Roulin 2004b; Roulin *et al.* 2007; Gangoso *et al.* 2011; Saino *et al.* 2013). In addition, previous studies at different scales in Europe found a relationship between the pheomelanic-based coloration and diet, with whiter individuals predating more often upon Muridae (*Apodemus spp.*), whereas rufous individuals predate more often upon voles (*Microtus arvalis*) (Roulin 2004a; Charter et al. 2012). Also, color variation could be indirectly explained by stability in temperature and to a lesser extent in precipitation seasonality, because those variations might be affecting the distribution and abundance of rodent species in Europe. Further studies investigating the effect of climatic variables on the distribution of prey's species eaten by barn owls may offer a better view of the relationship between climate and pheomelanin-based coloration in this species and potentially in other raptor species showing variation in melanin-based coloration.

Sex-specific selection

In European barn owls, females are on average darker-rufous than males, which could suggest antagonistic selection operating on pheomelanic-based coloration with males and females respectively selected to harbor a white plumage or a dark-rufous plumage (Roulin & Jensen

2015; San-Jose *et al.* 2015; Ducret *et al.* 2016). Using the GLS analyses, we modeled an interaction between sex and environment in order to highlight potential sex-specific divergent selection. Our results showed a significant effect of pasture on female but not male coloration at the continental scale, with females being darker-rufous in habitats with low pasture density (i.e. pasture reflects dense grass cover not included in an agricultural rotation system and mainly used for grazing, including areas with hedges). This result is partly concordant to previous finding in Switzerland showing that rufous females have a higher fitness when breeding in sites surrounded by more arable field and meadow, which included pasture (Dreiss *et al.* 2012). Also, the weak effect observed in our study between pasture and coloration in females can be expected. Indeed, the recent work of Connallon (2015) modeling the geography of sex-specific selection, local adaptation and sexual dimorphism showed a variation in intensity of sexual antagonism across species' range, with subpopulations near the range center exhibiting hotspots for antagonistic selection. In other words, detecting sex-specific selection is potentially easier at a local scale rather than a large scale where sexual antagonism could vary in intensity.

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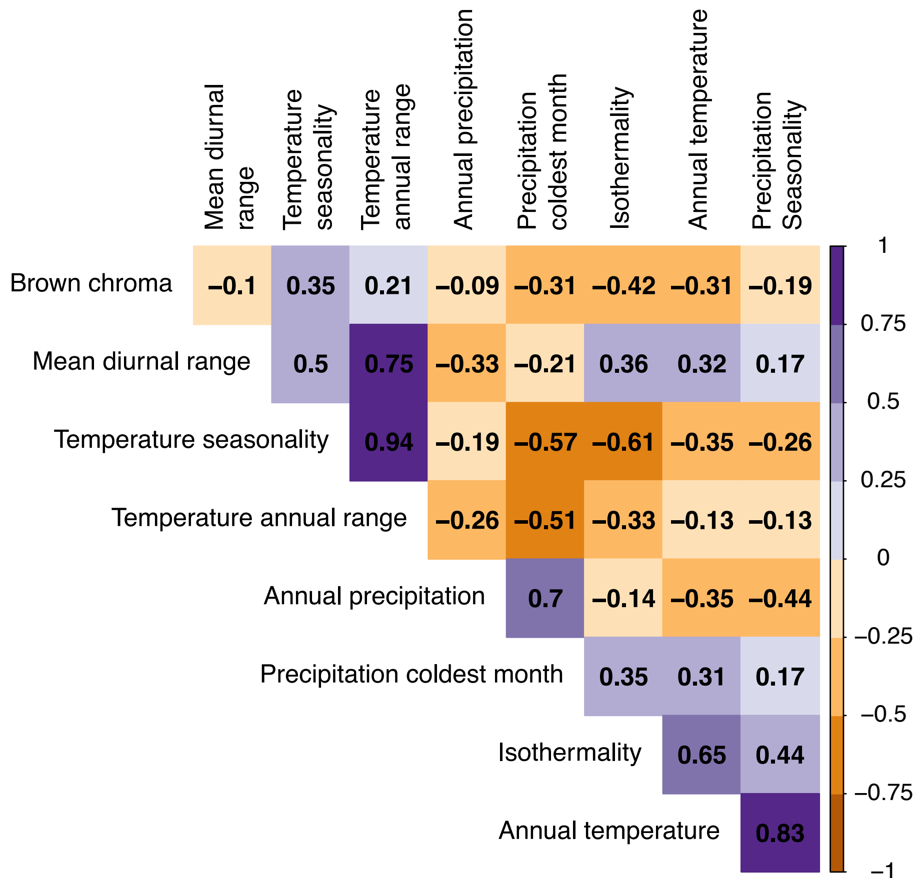
Figure S1: Correlation matrix of the 8 climatic variables performed on the full dataset (including Great Britain samples).. The degree of correlation (r) between pairs of variables are indicated in each grid cell of the matrix and are presented by a gradient of color from red (negative) to violet (positive) values.



General discussion and perspectives

Unraveling the distinct evolutionary mechanisms affecting phenotypic and genetic diversity has been a major focus in evolutionary and conservation biology. It is a necessary step to understand how populations can potentially adapt and evolve. In this thesis, I aimed to reveal the effect of selection (e.g. natural or sexual selection, sex-specific natural selection) and demographic processes (e.g. dispersal), on the evolution of pheomelanin-based coloration and its underlying genetic variants, *MC1R*, in barn owls. The diverse aspects covered during this work permitted to highlight several interesting features such as the relationship between *MC1R*-color variants and the degree of sexual dimorphism or the existence of non-random *MC1R* gene flow in relation to female-biased dispersal. However, there are still many questions unanswered due to the limitation of our dataset or a lack of comparative studies in natural populations. For example, it is still not clear how transmission ratio distortion of *MC1R* variants evolved in the context of sex-specific selection and sexual conflict in barn owls. Therefore, this work opens a large avenue for future studies on the evolution of color polymorphism and sexual dichromatism.

Effect of *MC1R* on melanin-based color variation and sexual dimorphism

In wild animals, missense mutations at the melanocortin-1 receptor gene, *MC1R*, result in abrupt colour changes that lead to the occurrence of alternative colour morphs within species (Mundy 2005). Among 121 Swiss barn owls, we identified four mutations at *MC1R* with two mutations being synonymous (T3T and T25T) and two mutations being non-synonymous (R8H and V126I) with the following frequencies of the derived alleles 4.3%, 3.0%, 0.5% and 15.4%, respectively. Also, we characterized the degree of color changes accounted for the frequent V126I non-synonymous mutation at *MC1R* (San-Jose *et al.* 2015; Chapter 1). The *MC1R* genotypes explained 47.2% and 34.1% of the total variance in pheomelanin and eumelanin feather contents, respectively. We also estimated that *MC1R* explained 33.7% of the total variance of the dark-rufous coloration, whereas it explained only 0.2% and 0.05% of the total variation in the number and size or black feather spots, respectively.

However, the impact of *MC1R* on all plumage traits differed between sexes in nestling and adult of different age classes. This suggests that the *MC1R* interacts in a non-additive

manner (i.e. epistasis) with factors responsible for color variation between sexes in the barn owl. Also, we observed that *MC1R* affects the degree of sexual dimorphism of black feather spots, a trait that has been shown to be under sexually antagonistic (SA) selection (Roulin *et al.* 2010). Homozygous females for the $MC1R_{RUFOUS}$ allele exhibited larger breast spots and homozygous males for the $MC1R_{WHITE}$ allele exhibited smaller breast spots than other genotypes. Thus, sex-specific effect of *MC1R* on plumage traits allows each sex to reach its fitness optimum for this trait. However, we also observed that the $MC1R_{WHITE}$ allele allows for larger differences between sexes in breast spot size, supporting that this allele could still have a slightly higher advantage as it allows producing more sexually dimorphic offspring. Under this scenario, we expected the $MC1R_{WHITE}$ allele to be more successful than the $MC1R_{RUFOUS}$ allele under sexual selection or sex-specific natural selection.

Hardy-Weinberg deviation at *MC1R* as a tool for detecting selection

We used diverse approaches combining population genetics, simulations and modeling that were relevant for testing several hypotheses relative to natural selection, sexual selection and sex-specific selection at *MC1R* in Swiss barn owls. As expected based on previous studies, we only find a slight positive assortative pairing (8.7%) regarding *MC1R*, suggesting a small effect of sexual selection on *MC1R* (Ducret *et al.* 2016; Chapter 2). Among fledglings, we detected a significant deviation from Hardy-Weinberg proportion at *MC1R* with 7.5% and 12.9% of heterozygotes deficit in males and females, respectively. However, this pattern was reversed for females at the adult stage (Chapter 3). Our results highlighted that, despite a stable deficit of heterozygotes at *MC1R* in adult males, adult females showed an excess of heterozygotes. Indeed, high immigration of female heterozygotes at *MC1R* from outside the study area may compensate the deficit observed at the fledgling stage. Non-random gene flow at *MC1R* combined with female-biased dispersal in barn owls suggests that females obtain a benefice to carry particular *MC1R* genotypes during dispersal and need further inspection. Contrary to our prediction in San-Jose *et al.* (2015), we did not highlight sex-specific natural selection on *MC1R* and particularly, we did not detect a fitness advantage in terms of survival or reproductive success in adult males when carrying $MC1R_{WHITE}$ allele. However, the observed higher transmission of $MC1R_{WHITE}$ allele observed in the male lines (Chapter 2)

could still suggest a sexual conflict at *MC1R* and a need for further investigation of fitness differences between males and females depending on *MC1R* or color.

Transmission ratio distortion

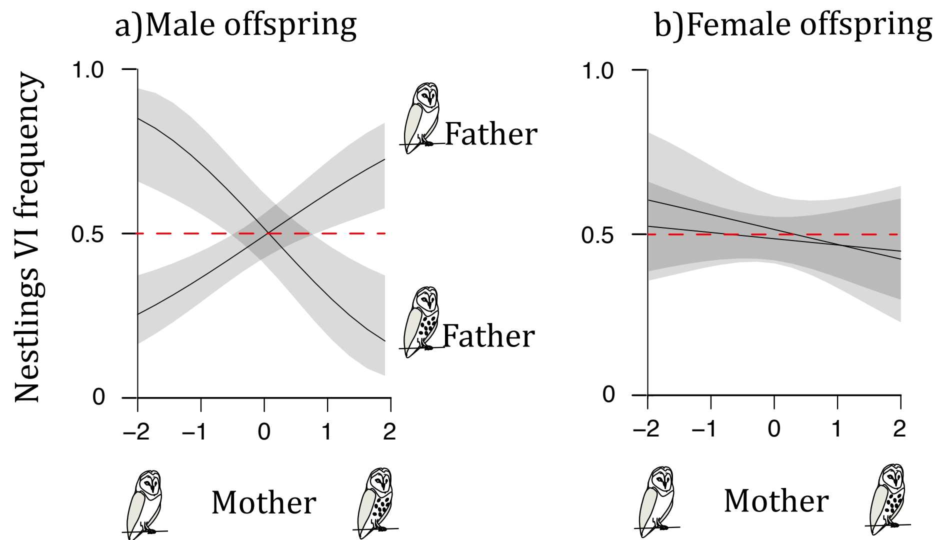
Departure from Mendelian inheritance, also called transmission ratio distortion (TRD), is detected when a given parental allele is transmitted to the next generation at lower or higher frequency than expected under random segregation of alleles (for a review, see Huang *et al.* 2013). Transmission ratio distortion (TRD) may arise from (i) selection of a particular allele, (ii) origin of the parental chromosomes (i.e. genomic imprinting), (iii) chromosomal rearrangements (e.g. inversions) or (iiii) complex interactions between chromosomal regions (Haig 2000). Although TRD has been widely observed in laboratory animal and plant species (de la Casa-Esperón *et al.* 2000; Dyer *et al.* 2007; Eversley *et al.* 2010; Seidel *et al.* 2011; Leppala *et al.* 2013) and in few wild species (Aparicio *et al.* 2010; Alcaide *et al.* 2012; Gagnaire *et al.* 2013), the underlying mechanism often remains obscure. After controlling for a low extra-paternity rate, we observed in barn owls a recurrent lower transmission of the $MC1R_{RUFOUS}$ allele from father to sons (40% instead of expected 50%). We proposed several mechanisms to explain this TRD and first, we could reject the meiotic drive hypothesis. Meiotic drive characterizes a selfish element that biases its Mendelian segregation by moving away from dead-end polar bodies into the functional egg during oogenesis (Johns *et al.* 2005; Wilkinson *et al.* 2006; Gell & Reenan 2013; Friberg & Rice 2014; Zanders & Malik 2015). In our case, this scenario is unlikely because the recurrent pattern of segregation distortion occurs in the parental male line and thus, it cannot explain the pattern of sex-specific *MC1R*-allele transmission. Indeed, even if a form of meiotic drive had occurred during spermatogenesis (e.g. sperm selection through motility; Holt & Van Look 2004), this mechanism would have implied an overall deficit of spermatozoids possessing the $MC1R_{RUFOUS}$ allele that would have affected both sons and daughters. Further, we did not find higher egg or nestling mortality in breeding pairs showing TRD but we do not exclude the possibility that embryos carrying particular *MC1R* variants were lost during their development. Although it remains unknown how this TRD at *MC1R* evolved in barn owls,

future studies should focus on the possibility that the transmission of *MC1R* variants could rely on the presence of other color-related gene variants.

Investigation1: Genetic correlation and multivariate approaches

In the barn owls, the three melanin-based traits (i.e. rufous coloration, number and size of black feathers spots) are highly heritable and strongly correlated (Roulin & Dijkstra 2003; Roulin *et al.* 2010; Roulin & Jensen 2015), which imply that the evolution of the pheomelanin-based coloration is constrained by the evolution of the two other eumelanin-based trait (number and size of black feather spots). Particularly, the genetic correlations in males were on average 1.6 times stronger than in females and therefore, a white male will have more chance to express also less and smaller black feathers spots (Roulin & Jensen 2015). Therefore, the redundant function of the three melanin-based traits in males could have evolved because those traits are co-adapted in this sex, which is concordant with results from Chapter 1 and 2. Furthermore, preliminary results from Generalized Linear Mixed Models (GLMMs) seemed to point out that the transmission of the $MC1R_{RUFOUS}$ allele in males depends on the presence of genes coding for the number of breast spots in fathers and mothers (Fig. 3). Sons would inherit more often than expected the $MC1R_{WHITE}$ allele when both parents carry genes that make them immaculate (i.e. without spots) or highly spotted. Also, there is a need for further investigation of how genetic correlations between the three melanin-based traits constrain or promote fitness in males and females, which would require to use, for example, multivariate approaches and quadratic regressions (Blows *et al.* 2003; Martin & Wainwright 2013; Devigili *et al.* 2015). It will permit to understand how gene interactions may constrain the evolution of sexual dimorphism in individual traits (Berger *et al.* 2014).

Figure 3: Heterozygote frequency at *MC1R* in fledgling male (a) and female (b) barn owls and depending on the interaction of father and mother spottiness. The red line show expected proportion of heterozygote at *MC1R* based on Mendelian segregation.



Investigation 2: Linkage disequilibrium or pleiotropic effect

Genetic correlation between characters can arise from two deterministic mechanisms: pleiotropic effects of genes and linkage disequilibrium (i.e. non random associations of alleles) between loci affecting different characters (Lande 1984). Determining the cause of genetic correlation could help understand how TRD evolved in barn owls. A good example is the case observed in the 1950's of a segregation distorter (SD) of Mendelian transmission in *Drosophila melanogaster*. Males heterozygous for the distorter (SD/SD^+) sire almost exclusively SD-bearing progeny because sperm carrying the locus SD^+ develop distorted (Sandler *et al.* 1959; Hartl *et al.* 1967). Fifty years later, and following a rich work on genetics and theories, the molecular basis of this segregation distortion became clearer. The distorter evolved through a complex of co-adapted genes on chromosome 2 tightly linked by chromosomal inversions (Presgraves *et al.* 2009).

Chromosomal inversions are often studied in the context of speciation and local adaptation. Indeed, inversions minimize recombination between sets of locally adapted genes and can lead to reproductive isolation within or between species through their effect on fertility (i.e. hybrid sterility) or congenital anomalies (Noor *et al.* 2001; Kirkpatrick & Barton

2006; Honeywell *et al.* 2012). A fascinating study case is the hooded and carrion crows in Europe where their status of species or subspecies were long debated. Studies observed the presence of slight non-random mating between the two types of crows (Randler 2007). However, despite a striking differentiation for color, they present a low neutral genetic differentiation that does not exceed differentiation within taxa (Poelstra *et al.* 2014). Further inspection using genomics, RNA-sequencing and gene expression, detected a region on chromosome 18 that contain a complex pattern of inversions and revealed differential expression of several genes involved in the regulation of pigmentation, visual perception and hormonal balance (de Knijff 2014; Poelstra *et al.* 2014, 2015). Whether the barn owl is at an early stage of speciation and presents a similar pattern of inversion at co-adapted color-related genes, which may have conduct to TRD, still need to be answered.

Investigation 3: Clinal variation in different geographic regions

Clinal color variation offers remarkable illustrations of spatially varying selection, particularly for substrate color matching and camouflage (Hoekstra *et al.* 2004; Mullen & Hoekstra 2008; Willink *et al.* 2013). However, in the barn owl climatic variables rather than landscape were important predictors of the pheomelanin-based color and *MC1R* variation in Europe. Because climatic variables were highly correlated between each other and could not be all used directly in the same statistical model, it is still unclear which one predicts better this color cline. Previous studies on the three melanin-based traits in barn owls (i.e. rufous coloration, number and size of black feather spots) only highlighted a relationship between eumelanin-based traits and thermoregulation or spatial variation in temperature and precipitation (Roulin *et al.* 2009; Roulin & Salamin 2010; Dreiss *et al.* 2016). However, the pheomelanin-based color cline observed in North American *Tyto alba* was significantly explained by ambient temperature. Owls were darker-rufous in colder regions, particularly when cold during summer. Future studies should confront the effect of several climatic variables concurrently on different color cline observed in *Tyto alba* worldwide. Identification of the precise climatic variables explaining color variation in this species would have a significant impact in conservation for understanding how the populations will adapt and evolve in future climate.

Conclusion

The work performed during this thesis permitted to advance in our understanding of the different mechanisms affecting phenotype and genotype variation, particularly in the context of melanin-based coloration. The vast amount of *MC1R* genotypes data generated was used in several studies apart from this thesis and will continue to be used in future, such in the whole-genome sequencing study aiming at unravelling other genes affecting the three melanin-based coloration in barn owls, controlling for the effect of *MC1R*. Moreover, future studies on intralocus sexual conflict and genomic architecture may hopefully provide new insights into the resolution of sexual dimorphism in complex traits system such as melanin-based coloration and the melanocortin system. We also hope that more studies will be achieved on wild populations because most studies of genetic conflict used laboratory species, which does not reflect the wide complexity observed in natural populations. Finally, coloration is widely studied in evolutionary biology, we believe that this work will be helpful for future studies and that those methods can be applied for a various range of species expressing color polymorphism.

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SPECIAL ISSUE: THE MOLECULAR MECHANISMS OF ADAPTATION AND SPECIATION: INTEGRATING GENOMIC AND MOLECULAR APPROACHES

MC1R variants affect the expression of melanocortin and melanogenic genes and the association between melanocortin genes and coloration

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Abstract

The melanocortin-1 receptor (*MC1R*) gene influences coloration by altering the expression of genes acting downstream in the melanin synthesis. *MC1R* belongs to the melanocortin system, a genetic network coding for the ligands that regulate MC1R and other melanocortin receptors controlling different physiological and behavioural traits. The impact of *MC1R* variants on these regulatory melanocortin genes was never considered, even though *MC1R* mutations could alter the influence of these genes on coloration (e.g. by decreasing *MC1R* response to melanocortin ligands). Using barn owl growing feathers, we investigated the differences between *MC1R* genotypes in the (co)-expression of six melanocortin and nine melanogenic-related genes and in the association between melanocortin gene expression and phenotype (feather pheomelanin content). Compared to the *MC1R rufous* allele, responsible for reddish coloration, the *white* allele was not only associated with an expected lower expression of melanogenic-related genes (*TYR, TYRP1, OCA2, SLC45A2, KIT, DCT*) but also with a lower *MC1R* expression and a higher expression of *ASIP*, the *MC1R* antagonist. More importantly, the expression of *PCSK2*, responsible for the maturation of the MC1R agonist, α-melanocyte-stimulating hormone, was positively related to pheomelanin content in *MC1R white* homozygotes but not in individuals carrying the *MC1R rufous* allele. These findings indicate that *MC1R* mutations not only alter the expression of melanogenic-related genes but also the association between coloration and the expression of melanocortin genes upstream of MC1R. This suggests that *MC1R* mutations can modulate the regulation of coloration by the pleiotropic melanocortin genes, potentially decoupling the often-observed associations between coloration and other phenotypes.

Keywords: barn owl, colour genetics, colour polymorphism, gene expression, melanin, pleiotropy

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Introduction

Understanding the genetic basis of phenotypic variation is one of the main goals in evolutionary biology. In this field, the melanocortin-1 receptor (*MC1R*) gene has become a textbook example to understand phenotypic convergence. Mutations at this gene explain variation in melanin-based colour traits in distantly related taxa such as mammals (Eizirik *et al.* 2003; Makova & Norton 2005; Hoekstra *et al.* 2006; Nowacka-Woszuk *et al.* 2013), birds (Doucet *et al.* 2004; Mundy *et al.* 2004; Gangoso *et al.* 2011; Cibois *et al.* 2012; Janssen & Mundy 2013; San-Jose *et al.* 2015), nonavian reptiles (Rosenblum *et al.* 2004; Nunes *et al.* 2011) and fish (Gross *et al.* 2009).

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However, *MC1R* is embedded in the melanocortin system, a highly pleiotropic gene network responsible for the orchestrated expression of melanin-based colorations with multiple behavioural and physiological traits (sexual behaviour, aggressiveness, stress response, energy homeostasis, among others: Ducrest *et al.* 2008; Roulin & Ducrest 2011; Reissmann & Ludwig 2013). Although the molecular effect of *MC1R* on the melanin pathway is relatively well understood (García-Borrón *et al.* 2005), whether *MC1R* mutations also alter the function of the other melanocortin genes remain unknown. Unravelling how *MC1R* interacts with the other melanocortin genes and not only with melanogenic genes could help to understand how trait associations recurrently found in animals arise (e.g. melanin-based ornaments and aggressiveness: West & Packer 2002; Mafli *et al.* 2011; Santos *et al.* 2011; Reiter *et al.* 2014; Johnson & Fuller 2015).

MC1R governs melanin synthesis by regulating the expression of different genes belonging to the melanin pathway (Vachtenheim & Borovanský 2010). Mutations at this gene often induce a change in MC1R affinity to bind its ligands (the α-melanocyte-stimulating hormone, α-MSH, and the agouti signalling protein, ASIP), in the MC1R G protein coupling activity, or in MC1R location (Dessinioti *et al.* 2011). Variation in the activity of the MC1R protein finally alters the expression and activity of genes downstream of MC1R in the melanin pathway. However, mutations at the *MC1R* gene might also modify the influence of the other genes in the melanocortin system that also regulate melanin synthesis and coloration (i.e. those coding for hormonal agonists and antagonists of the MC1R: *POMC*, *PCSK1/3*, *PCSK2* and *ASIP*). The epistatic (Wolf *et al.* 1978; Steiner *et al.* 2007) and pleiotropic (Gangoso *et al.* 2011; Maresca *et al.* 2015) effects of different *MC1R* alleles as well as the coevolution among the genes of the melanocortin system are plausible mechanisms to account for such effects. If a *MC1R* mutation results in a constitutively active receptor, like the epistatic *sombre* allele in the murine *MC1R* (Robbins *et al.* 1993), variation in the expression of genes leading to the production of α-MSH or ASIP is expected to have no incidence in colour expression, given that this type of mutations will stop *MC1R* from responding to its ligands. Consequently, the association between colour traits and the melanocortin system, as shown in several species (Emaresi *et al.* 2013; Monti *et al.* 2013; Ducrest *et al.* 2014; Poelstra *et al.* 2015), will only exist in the wild type but not in the derived type. Less dramatic changes in the functioning of the MC1R protein (e.g. a mutation inducing a partial loss of function of *MC1R* by decreasing the efficiency with which the receptor responds to α-MSH, Rosenblum *et al.* 2010) might also affect the association between the melanocortin system and coloration. Although to a lower degree, the effects of these mutations could also have important consequences because they could alter the associations between coloration and the other traits regulated by the melanocortin system. For instance, if an association between coloration and aggressiveness exists in the *MC1R* wild type due to the action of α-MSH on MC1R and MC5R (Ducrest *et al.* 2008), the derived *MC1R* type will exhibit, for the same level of α-MSH, the same level of aggressiveness than the wild type but with a different coloration.

From an ultimate point of view, selection acting on the colour differences generated by the *MC1R* can also select for changes in the expression of other melanocortin genes. On the one hand, selection might favour changes in the expression of melanocortin genes resulting in colour changes in the same direction to those induced by a selected *MC1R* mutation (Steiner *et al.* 2007). For instance, if selection on coloration favours a *MC1R* mutation inducing more melanic traits, it could also favour mutations at other genes inducing further melanization and consequently the association of *MC1R* mutations with further genetic differences. Similarly, given that the adaptive value of coloration may depend on other physiological and behavioural traits (Kingsolver 1987; Forsman *et al.* 2002; Kim *et al.* 2013), selection on coloration is likely to be correlated to selection exerted on other phenotypes (Sinervo & Svensson 2002). Therefore, selection on a given melanin-based trait can indirectly promote the evolution of further phenotypic/genotypic differences, for instance in those traits that are often observed to be related with melanin-based traits and that are potentially under the control of the highly pleiotropic genes of the melanocortin system (Ducrest *et al.* 2008; Roulin & Ducrest 2011). In conclusion, although the effects of the *MC1R* on coloration are relatively simple, usually having major effects on continuous colour traits (San-Jose *et al.* 2015) or underlying the inheritance of discrete Mendelian colour traits (Hoekstra *et al.* 2006), *MC1R* could have more complex effects on the other genes of the melanocortin system and the complete consequences of the observed variation in the nucleotide sequence of the *MC1R* have not been fully explored.

In this study, we investigated to what extent the expression of melanocortin and melanogenic genes in growing feathers of barn owls, *Tyto alba*, is altered by different *MC1R* alleles. In the European population of barn owls, *MC1R* is polymorphic for the amino acid 126, where a valine-to-isoleucine substitution explains ~30% of variation in ventral coloration (San-Jose *et al.* 2015; Burri *et al.* 2016). The valine allele (hereafter referred to as *white*, W, allele) is strongly related with whiter plumage colorations, whereas the isoleucine

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allele (hereafter referred to as *rufous*, R, allele) relates to redder plumage colorations (Fig. 1). Variation in plumage coloration may have several adaptive functions (e.g. Roulin *et al.* 2012) in relation to habitat and/or predator–prey interactions (Roulin 2004; Dreiss *et al.* 2012). To a minor extent and depending on the ventral body part considered, the polymorphic site V126I also explains variation in the number and size of eumelanic black spots that spattered the body of the barn owl. On the breast, the body part studied here, the *white* and *rufous* alleles confer smaller and larger black spots, respectively, and fewer spots although only in males homozygous for the *white* allele (San-Jose *et al.* 2015). The size and number of these spots are used as a criterion in mate choice (Roulin 1999), signalling aspects of genetic quality (Roulin & Ducrest 2011).

We monitored a set of six melanocortin genes (*POMC*, *PCSK2*, *PCSK5*, *AGRP*, *ASIP* and *MC1R*) and nine melanogenic-related genes (*CREB1*, *MITF*, *KIT*, *SLC45A2*, *SLC7A11*, *DCT*, *OCA2*, *TYR* and *TYRP1*) that covers the main paths in the regulation and synthesis of melanin (see fig. 3 in Poelstra *et al.* 2014; and figs 2 and 3 in Ducrest *et al.* 2008). We measured the expression of these genes using qPCR, because the aim of the study focuses on the specific effect of *MC1R* alleles on the expression of melanocortin genes and melanogenic-related genes rather than on general effects on gene expression in feathers. qPCR is more adequate to test the above stated hypothesis than alternative methods such as whole transcriptome shotgun sequencing (RNA-seq) because it ensures sensitive detection of the genes of interest and obtaining more precise estimates of the expression of different isoforms (Robles *et al.* 2012). We first investigated whether the *MC1R* genotypes found in the barn owl differentially express melanocortin genes and melanogenic-related genes in developing feathers and whether *MC1R* genotypes differ in the patterns of gene coexpression. Finally, we investigated whether the regulatory role of the melanocortin genes on melanin synthesis differs between *MC1R* genotypes, by testing for differences between genotypes in how melanocortin genes and feather pigment content are associated.

Materials and methods

Tissue sampling and assessment of melanin pigments

The study was conducted in a population of wild barn owls breeding in nest boxes in western Switzerland. During the breeding season of 2011, we collected blood and developing feathers (i.e. feathers surrounded by a peripheral epidermal sheath enclosing the feather follicle where melanogenesis and feather growth occur; Lin *et al.* 2013a,b) from the breast of 117 nestlings (mean age ± SD: 47 days ± 4.6) from 43 different nests. Tissue samples were immediately frozen in dry ice and stored at −80 °C for the genetic (blood), gene expression, and melanin content (feathers) analyses. For each individual, sex was identified using sex-specific molecular markers

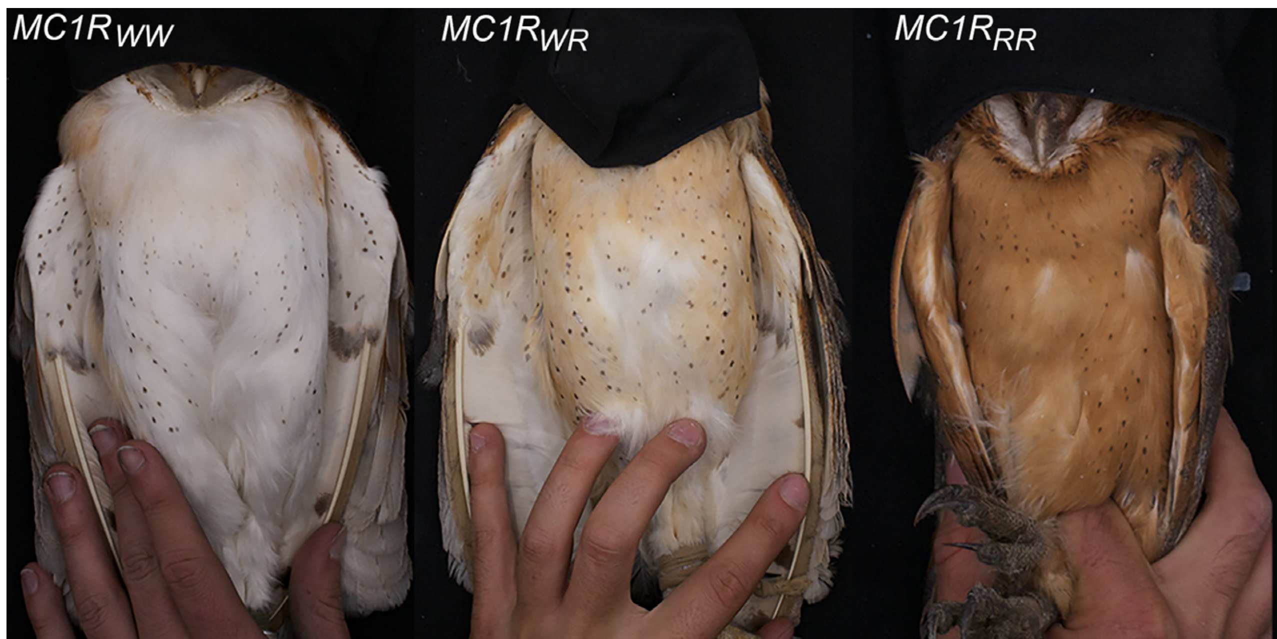

Fig. 1 Plumage coloration of *MC1R* genotypes in the barn owl. Shown are three examples of the colour differences between individuals of different genotypes for the *rufous* (R) and *white* (W) alleles in the barn owl.

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(Py *et al.* 2006) and *MC1R* genotype at the position V126I was ascertained by allelic discrimination as described in San-Jose *et al.* (2015). Final sample sizes for the three *MC1R* genotypes and sexes were as follows: 73 $MC1R_{WW}$ individuals ($N_{males} = 31$, $N_{females} = 42$), 38 $MC1R_{WR}$ ($N_{males} = 18$, $N_{females} = 20$) and six $MC1R_{RR}$ ($N_{males} = 1$, $N_{females} = 5$). Given the low frequency of the $MC1R_{RR}$ genotype, only $MC1R_{RR}$ and $MC1R_{WR}$ individuals were considered for the statistical analysis. There were no significant differences in nestling age (all $P > 0.14$), sampling hour (all $P > 0.49$) and sampling date (all $P > 0.091$) between *MC1R* genotypes, sexes or their interaction.

We analysed the amount of pheomelanin and eumelanin in the same feathers as for RNA extraction as described in Roulin *et al.* (2013). After removing the basal part of the feathers for RNA extraction, the remaining upper part (i.e. the part of the feather vane already developed) was used to measure pigment concentration. Therefore, pigment content corresponds to the melanin already synthesized at the moment of gene expression analysis. In most cases, feather spots were already developed given that they are mainly present in the apical part of the feather. Thus, the measured levels of gene expression will unlikely reflect the process of spot formation but mainly the process of pheomelanin deposition and development of plumage redness. Feathers were not homogeneously pigmented in all the individuals (except in purely white and purely red individuals), showing a slight depigmentation from the apical part to the basal part of the feather. In this sense, our analyses are conservative because they increase the likelihood of missing an association between pigment content and gene expression. Pheomelanin and eumelanin contents were highly correlated ($r = 0.91$, $N = 117$, $P < 0.001$). We therefore run the analysis with pheomelanin, which shows a higher relative abundance than eumelanin (pheomelanin: eumelanin ratio = 18.54 ± 14.6 SD). The statistical analysis of eumelanin content was nevertheless similar to those of pheomelanin (see Results).

Gene expression and sequencing analyses

We measured the expression of *POMC*, *PCSK2*, *PCSK5*, *AGRP*, *ASIP* (three alternative splice variants and total levels), *MC1R*, *CREB1*, *MITF* (two alternative splice variants), *KIT*, *SLC45A2*, *SLC7A11*, *DCT*, *OCA2*, *TYR* and *TYRP1*. *POMC* codes for the melanocortin peptides that binds to and activate MC1R (e.g. α-MSH; Chakraborty *et al.* 1996; Yoshihara *et al.* 2011). POMC prohormone is cleaved into different melanocortin peptides by the convertases encoded by the genes *PCSK1/3* and *PCSK2*. We did not detect *PCSK1* mRNA in the feathers and decided to focus on *PCSK5*, which is also located in the chromosome Z in the chicken (GenBank gene ID: 395456). Two transcripts, one short that corresponds to the soluble form of the protein and one long which is a membrane-anchored form (respectively, called PC5/6A and PC5/6B in human) (Seidah 2011), were identified. We quantified by qPCR the total amount of both transcripts and refer to them as *PCSK5*. Antagonist binding to MC1R is mediated by ASIP (Sakai *et al.* 1997) and results in a switch of eu- to pheomelanin pigments in mammals. The agouti-related protein (AgRP), involved in the regulation of energy homeostasis in the brain (Warne & Xu 2013), was also monitored given that it is also expressed in the skin in chicken and is thought to play a role in melanogenesis in birds (Takeuchi *et al.* 2000). Melanogenesis proceeds mainly through the activation of MC1R by its ligand agonist, α-MSH. This activates the adenylate cyclase, increasing cAMP signalling, which activates the expression of the microphthalmia transcription factor (MITF) gene via the cAMP-responsive element binding protein (CREB). MITF protein activity is also regulated by the c-Kit receptor (KIT); the latter can also be transactivated by the MC1R (Herraiz *et al.* 2011). MITF finally acts as a central transcription factor that upregulates the expression of different genes essential for the production of melanin and melanosome maturation (tyrosinase, *TYR*, tyrosinase-related proteins 1 and 2, *TYRP1*, and *DCT*, respectively; Vachtenheim & Borovanský 2010). We also monitored a gene coding for a transmembrane melanosomal protein controlling melanosome pH, *SLC45A2* (*MATP/OCA4*), the *OCA2* gene, another membrane transporter of the melanosomes also known as the pink-eyed dilution gene or p-gene, and *SLC7A11*, which encodes the plasma membrane cysteine/glutamate exchanger, xCT (Chintala *et al.* 2005) and that may play a role in pheomelanin synthesis.

Total RNA was obtained from the basal parts of one or two developing feathers. All tissues contained in the basal part of the feathers were sampled (dermal papillar, central and peripheral pulps, ramogenic zone, dermal sheath and barbules, Lin *et al.* 2013b). Samples were grounded in liquid nitrogen with pestle in 1.5-mL tubes and resuspended in RLT buffer of the RNAeasy mini kit (Qiagen, Hombrechtikon, Switzerland). Keratin-containing sheaths and barbs and barbules were removed by filtration in Qiashredder columns (Qiagen) prior extraction. During RNA extraction, a Qiagen RNase-Free DNase treatment was included. One aliquot of total RNA was used to assess its quantity with Qubit fluorometer (Life Technologies, Zug, Switzerland) and its quality using the Fragment analyser (Advanced analytical, Labgene, Switzerland). Only total RNA with RQN > 9.0 was used for gene expression.

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To get the sequences of the different genes, cDNA was prepared by reverse-transcribing 1 μg of total RNA in 20 μL with 2.5 μM oligo(dT)$_{20}$ primers or specific primers for rare transcripts that are *POMC*, *PCSK2*, *PCSK5*, and 200 U of Superscript III according to the manufacturer's protocol (Life Technologies). The total amount of ASIP mRNA (*ASIP*) and three different tissue-specific 5′ splice variants of ASIP (*ASIP-AC*, *ASIP-AD* and *ASIP-BC*) were monitored (see Appendix S1, Supporting information, Yoshihara *et al.* 2012). PCR primers were designed using PRIMER 3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) on conserved regions between *Gallus gallus* and *Taeniopygia guttata* sequences. PCR fragments of the expected length were purified (Minelute Kit; Qiagen) and sequenced directly or TA-cloned (Promega, Dübendorf, Switzerland) and sequenced using Big Dye® V 3.1 terminator chemistry (Life Technologies). Sequencing reactions were run on ABI3130XL Genetic Analyzer (Life Technologies), and PCR fragments and bacterial clones were sequenced directly by Microsynth (Microsynth, Balgach, Switzerland). Gene sequences were edited and aligned in CodonCode Aligner 3.7.1.2 (CodonCode Corporations). After sequencing of the different genes (see Table S1, Supporting information for Accession Nos), TaqMan probes and primers were designed to span exon–intron boundary except for *MC1R* (one exon gene), *AGRP* (pre-mRNA) and *POMC* (exon 3 primers) and synthesized by Microsynth (Microsynth, Balgach, Switzerland) and Eurofins MWG Operon (Ebersberg, Germany) (Table S2, Supporting information). The PCR fragment amplified for *AGRP* spans the first intron and the second exon, and it therefore corresponds to the pre-mRNA of *AGRP*. We quantified this pre-mRNA because it was not possible to optimize the conditions to detect the mature mRNA of *ARGP* using qPCRs. A similar detection problem was encountered with *POMC*. We used 5′ RACE kit to identify its transcription start site (see Appendix S1, Supporting information). However, in feathers and with the design qPCR primers, the expression of the full-length *POMC* transcript was almost undetectable in feathers (A.L. Ducrest & A. Roulin, unpublished data) and we mainly detected a truncated transcript starting in exon 3 (15-bp downstream of the exon 2-3 boundary). The translated protein would lack the signalling peptide responsible for correct trafficking of the protein into the secretory vesicles where posttranslational modification takes place (Cawley *et al.* 2016). We measured *POMC* transcripts with exon 3 primers as total amount of *POMC* transcripts, although it mainly contained the truncated form. In addition, we used 5′ RACE for *ASIP*, *PCSK2*, *PCSK5* and *MITF* to detect their transcription start site (see Appendix S1, Supporting information).

For gene expression analysis, a second DNase I treatment was applied to 1 μg of total RNA with DNase I; then 100 ng of the DNAse I treated RNA was reverse-transcribed as described in Emaresi *et al.* (2013). We then precipitated the cDNA with one volume 5 M NH_4OAc (pH 8.0) and 2.5 volumes of cold ethanol 95%, and the pellet was resuspended in one volume 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (TE) buffer. Because of the low expression of some genes (*POMC*, *PCSK2*, *PCSK5*), we preamplified the cDNA with the TaqMan PreAmp Master Mix kit (Life technologies) with 14 cycles. Preamplification uniformity was assessed with the delta–delta C_t values that are ΔC_t of the preamplified gene—ΔC_t of the cDNA (C_t values of the target genes − C_t values of one of the reference genes). As expected, values were between 0 ± 1.5. We set up the qPCR conditions with various primers and probe concentrations with different concentrations of templates (plasmids or PCR-purified products) to get PCR efficiency between 95% and 105% (Table S2, Supporting information). A total of 117 individuals were tested in duplicates (ABI 7500) or triplicates (ABI 7900HT) in qPCR using 1× qPCR Mastermix plus low Rox (Eurogentec SA, Liège, Belgium), in 20/10 μL with 2/1 μL of preamplified cDNA diluted 10× depending on the qPCR machine, ABI 7500 and ABI 7900HT, respectively. When C_t values for duplicates or triplicates differed in more than 0.3 C_t, the qPCR was repeated. To control for interplate variation, three pools of different preamplified cDNAs were introduced into each plate. QBASEPLUS 1.3 software (Biogazelle, Zwijnaarde, Belgium) was used to calculate relative expression of the genes to the reference genes: elongation factor 1A (*EEF1A*), hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and ribosomal protein L13 (*RPL13*). GeNorm *M*-values for *HPRT1*, *EEF1A* and *RPL13* were 0.272, 0.262 and 0.238, respectively, and GeNorm *V* value was 0.0905. Mean relative quantities values were used in the subsequent statistical analyses.

Statistics

All the statistical analyses were conducted with R 3.0.2 (R Core Team, Vienna, Austria). We used a MANOVA test to test for the differences in gene expression between *MC1R* genotypes and sexes. To assess the importance of each gene in determining the differences between *MC1R* genotypes, we conducted a permutational discriminant function analysis (pDFA) and univariate tests on each gene (Quinn & Keough 2002). A pDFA was chosen because it allowed us to account for the lack of statistical independence among siblings (Mundry & Sommer 2007). The predetermined categories considered in the pDFA were set according to a four-level factor resulting from combining sex and *MC1R* genotypes: $MC1R_{WW}$ and $MC1R_{RW}$. Significance of the classification

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success and genes discriminability power was calculated from 999 permutations where the nest of origin of the nestlings, that is, the cause of nonindependence among our observations, was randomized in each permutation. In parallel, we ran univariate linear mixed models, LMM (*lme* function, 'NLME' package), to test for differences between *MC1R* genotypes and sexes on each of the candidate genes. Models included *MC1R* genotype, sex and nestling age as predictors and 'nest of origin' as a random factor. The *P* values from these tests were adjusted following the method described by Benjamini & Hochberg (1995).

Because differences between genotypes can result from population stratification (i.e. owing to admixture of two different populations, each one carrying different *MC1R* alleles as well as different alleles at other QTLs), we ran a robust transmission disequilibrium test (TDT; Hernández-Sánchez *et al.* 2003; Gratten *et al.* 2008) on the genes showing significantly differentiated expression between genotypes. This test is based on decomposing allelic effects in two independent coefficients that measure within- and between-family information (for further information, see table 2 in Hernández-Sánchez *et al.* 2003). The within-family coefficient (transmission disequilibrium coefficient, b_{TD}) is unbiased and significant when the transmitted allele is associated (or physically linked to a QTL associated with) the trait of interest (here, gene expression) (Gratten *et al.* 2008). Contrarily, the between-family coefficient (population disequilibrium coefficient, b_{PD}) is biased and prone to be significant in scenarios of population stratification.

To test whether *MC1R* genotypes and sexes differed in gene coexpression patterns, we conducted pairwise Pearson's correlations among all genes and for each combination of genotype and sex. We then used the Mantel's tests to examine whether the matrices of gene pairwise correlations generated for each group correlate between each other, which allowed testing to what extent gene coexpression patterns differed between groups. To investigate which genes have a major contribution to similarities and dissimilarities between groups, we reran the Mantel's tests by excluding one gene at a time. The difference in the correlation coefficient after excluding each gene was calculated for each pairwise comparison between groups and the mean value, and its 99% interval of confidence was calculated for each gene. Genes with positive means indicate that their exclusion resulted in lower correlations on average and therefore that they had similar coexpression patterns between groups. Contrarily, genes with negative mean values indicate that their exclusion resulted in higher correlations and that they had divergent coexpression patterns between groups.

To investigate whether *MC1R* genotypes differ in the association between the expression of melanocortin genes and feather pheomelanin content, we first investigate the expression of which genes is associated with variation in pheomelanin content within each genotype. We ran a set of competing models for each genotype and ranked them based on the Akaike's information criterion corrected for small sample sizes (AICc: Burnham *et al.* 2011). The best model or models were selected according to a ΔAICc < 2. A ΔAICc < 2 is considered to highlight significant differences between models although it can leave out models supported by the data (Murtaugh 2009 and references there in). However, we opted for this threshold because it is conservative for the aim of this study. A larger ΔAICc will likely result in a larger set of top models, a larger number of genes to test for a different role on each genotype and a higher probability of making type I errors. In these models, we used pheomelanin content as dependent variable and age, sex and the expression of melanocortin genes as predictor variables in the models. We built all the potential models including a maximum of six predictor terms (considering also the interactions between sex and gene expression) to avoid overparameterization of the models. To confirm that genes really have a different association between genotypes, we reran all the best models using the data set for both genotypes and modelled the interactions between all the terms in the model and *MC1R* genotype. Models were simplified by backward elimination of nonsignificant interactions ($P > 0.1$). The presence of a significant interaction between the expression level of a given gene and the *MC1R* genotypes confirmed that the role of this gene in explaining variation in melanin content differed between genotypes. We considered a significance level of 0.05 (two-tailed).

Results

Differences between MC1R genotypes in the expression of melanocortin and melanogenic-related genes

The MANOVA showed that genotypes and sexes differ in their gene expression levels (Pillai trace = 0.46, $F_{19,89} = 4.05$, $P < 0.001$, Pillai trace = 0.60, $F_{19,89} = 7.03$, $P < 0.001$, respectively) and that the interaction between genotype and sex was not significant (Pillai trace = 0.23, $F_{19,89} = 1.41$, $P = 0.145$). Similarly, the discriminant function successfully discriminated between *MC1R* genotypes and sexes (mean percentage of correctly classified individuals = 77.50%; expected percentage based on 999 permutations = 54.64%, $P = 0.001$; Fig. 2A). Classification success was high and significant for all groups ($MC1R_{WW}$ males: 87.09%, $MC1R_{RW}$ males: 77.77% and

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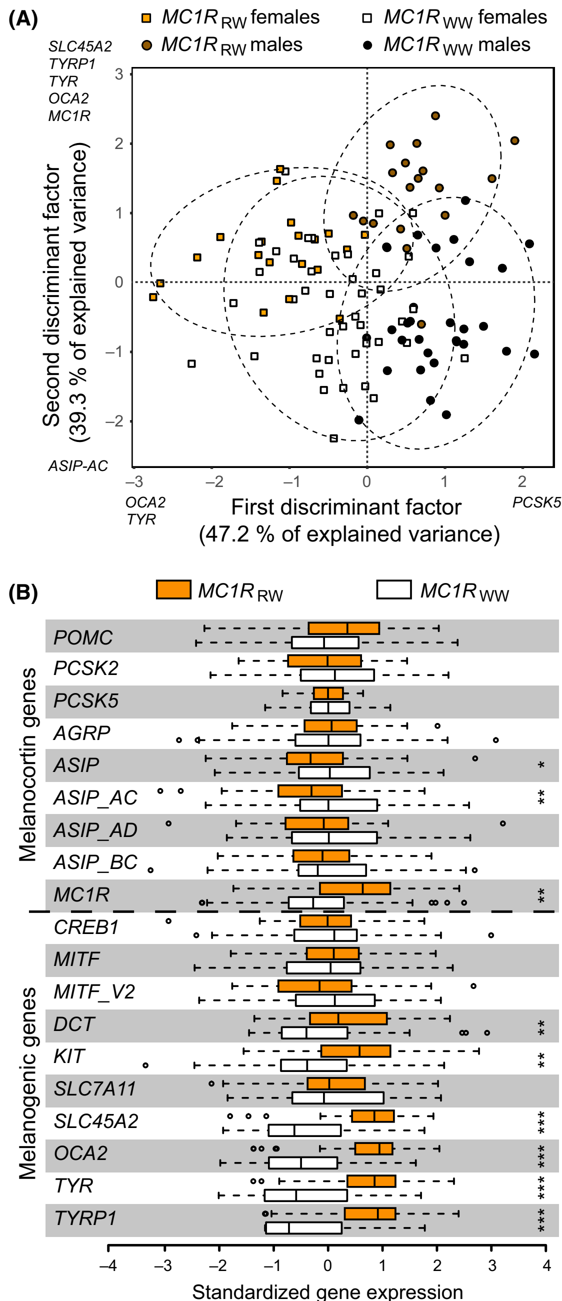

Fig. 2 Differences between *MC1R* genotypes on the expression of melanocortin and melanogenic-related genes in nestling barn owls. (A) Plot with the first two discriminant functions yielded by the DFA. Shown are the scores of each individual on the two axes and the 95% CI ellipses for each group. The genes with the highest positive and negative loadings for each axis are indicated on each axis. The third function (not shown) explained 13.48% of the variance, and it was mainly determined by DCT expression. (B) Boxplots on the standardized values of gene expression (RQ). Negative and positive values indicate expression values below or above mean gene expression, respectively. Significant differences between *MC1R* genotypes are denoted by the asterisks on the right sides of the figure, respectively. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

$MC1R_{RW}$ females: 75.00%, all $P = 0.001$) except for $MC1R_{WW}$ females (71.43%, $P = 0.494$). $MC1R_{WW}$ females were relatively often classified as $MC1R_{RW}$ females (25.00%) or $MC1R_{WW}$ males (16.66%) owing to a substantial overlap in the discriminant functions with these two groups (Fig. 2A). Genes whose expression had a major (and significant) contribution to discriminate among the four groups were mainly melanogenic-related genes (*TYR*, *OCA2*, *SLC45A2*, *TYRP1*, *KIT* and *DCT*; Table 1A). Among the melanocortin genes, the expression levels of *MC1R* had the largest significant contribution to discriminate among groups (Table 1A). *PCSK5* and *ASIP*-AC had also an important contribution although they were no longer significant after correcting for multiple testing (Table 1A). Genes with a low contribution to discriminate between groups were *AGRP*, *SLC7A11*, *PCSK2*, *MITF*, *MITF* variant 2, *CREB1* and *ASIP*-BC variant (Table 1A).

Univariate LMM showed that *MC1R* genotypes differ in the expression levels of several melanocortin genes (Table 1B, Fig. 2B). The expression of the *ASIP*-AC splice variant was significantly larger in $MC1R_{WW}$ individuals, whereas the expression of *MC1R* was lower in $MC1R_{WW}$ than in $MC1R_{RW}$ individuals. The TDTs (Table S3, Supporting information) confirmed that between-genotype differences in *MC1R* expression were associated with the transmission of *MC1R* alleles (transmission disequilibrium coefficient, b_{TD}: $t_{65} = 2.99$, $P = 0.004$) and indicated that the effect observed on the expression of the *ASIP*-AC splice variant might be associated with population stratification in relation to *MC1R* genotypes (b_{PD}: $t_{65} = 2.60$, $P = 0.011$) rather than to allelic transmission (b_{TD}: $t_{65} = 1.85$, $P = 0.07$). Among the melanogenic-related genes, *TYR*, *TYRP1*, *SCL45A2*, *OCA2*, *KIT* and *DCT* were all more expressed in $MC1R_{RW}$ individuals than in $MC1R_{WW}$ individuals (Fig. 2B, Table 1B). Except for *DCT*, the TD tests confirmed that the observed differences between genotypes in the expression levels of *TYR*, *TYRP1*, *SCL45A2*, *OCA2*, and *KIT* were associated with the transmission of *MC1R* alleles (all b_{TD}, $t_{65} > 2.49$, $P < 0.015$, Table S3, Supporting information). The differences between genotypes in the expression of *DCT* might be associated with population stratification (b_{PD}: $t_{65} = 2.91$, $P = 0.005$, b_{TD}: $t_{65} = 1.73$, $P = 0.09$).

The expression levels of only two genes were different between males and females, although the differences were found to be marginally significant after correcting for multiple testing. *PCSK5* had a higher expression in males than in females, and *TYR* had a lower expression in males than in females (Table 1B). The interactions between genotype and sex were significant for none of the genes (all $t_{65} < 1.21$, $P > 0.15$) and were dropped from the final models. Nestling age was positively associated with the expression of *PCSK2* and

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Table 1 Differences in gene expression between *MC1R* genotypes and sexes in nestling barn owls. (A) Results from a permutational discriminant function analysis (pDFA) using *MC1R* genotype and sex combined into a four-level grouping factor. Shown are the discriminability power of each gene (importance of each gene to classify individuals according to genotype and sex) and their significance based on 999 permutations. (B) Results from univariate linear mixed models (LMM). Shown are the means (±SE) standardized values for each *MC1R* genotype and sex and the statistics for the differences between *MC1R* genotypes and sexes and for the linear association with nestling age. None of the interactions between *MC1R* and sex were significant (not shown)

		(A) pDFA[†]			(B) Univariate LMM[‡]						
					$MC1R_{WW}$		$MC1R_{RW}$		*MC1R* differences	Sex differences	Nestling age
Gene transcript		Discriminability	Wilk's λ	F	Male Mean ± SE	Female Mean ± SE	Male Mean ± SE	Female Mean ± SE	t_{66}	t_{66}	t_{66}
Melanocortin genes	*POMC*	0.054	0.95	2.04	0.20 ± 0.21	−0.04 ± 0.20	0.11 ± 0.17	−0.13 ± 0.16	−0.47	−1.47	2.19
	PCSK2	0.008	0.99	0.28	−0.17 ± 0.20	−0.13 ± 0.19	0.12 ± 0.17	0.16 ± 0.15	1.41	0.22	2.56*
	PCSK5	0.075	0.93	2.89$^{M.S.}$	0.04 ± 0.09	−0.20 ± 0.08	0.15 ± 0.07	−0.08 ± 0.06	1.35	−3.05$^{M.S.}$	3.44*
	AGRP	0.006	0.99	0.22	0.07 ± 0.21	−0.02 ± 0.21	0.02 ± 0.18	−0.07 ± 0.16	−0.25	−0.47	−0.42
	ASIP	0.032	0.97	1.18	−0.23 ± 0.21	−0.39 ± 0.20	0.21 ± 0.17	0.05 ± 0.16	2.11$^{M.S.}$	−0.84	−0.73
	ASIP-AC	0.078	0.92	3.01$^{M.S.}$	−0.19 ± 0.19	−0.40 ± 0.18	0.32 ± 0.16	0.11 ± 0.14	2.63*	−1.21	−3.24*
	ASIP-AD	0.052	0.95	1.95	−0.09 ± 0.20	−0.37 ± 0.19	0.25 ± 0.16	−0.03 ± 0.15	1.69	−1.53	−3.37*
	ASIP-BC	0.019	0.98	0.68	−0.04 ± 0.21	−0.14 ± 0.20	0.11 ± 0.17	0.00 ± 0.16	0.69	−0.56	1.28
	MC1R	0.112	0.89	4.52*	0.38 ± 0.20	0.41 ± 0.19	−0.29 ± 0.16	−0.26 ± 0.15	−3.57**	0.17	−4.05*
Melanogenic-related genes	*CREB1*	0.021	0.98	0.75	0.09 ± 0.20	−0.21 ± 0.19	0.18 ± 0.17	−0.12 ± 0.15	0.42	−1.58	1.16
	MITF	0.010	0.99	0.34	−0.11 ± 0.21	−0.05 ± 0.20	0.03 ± 0.17	0.09 ± 0.16	0.69	0.31	1.56
	MITF-V2	0.010	0.99	0.35	−0.11 ± 0.20	−0.14 ± 0.19	0.12 ± 0.16	0.09 ± 0.15	1.12	−0.14	1.42
	DCT	0.091	0.91	3.59*	0.44 ± 0.20	0.32 ± 0.20	−0.12 ± 0.17	−0.24 ± 0.16	−3.07**	−0.79	−2.16
	KIT	0.128	0.87	5.24**	0.23 ± 0.19	0.49 ± 0.19	−0.43 ± 0.16	−0.17 ± 0.15	−3.37**	1.47	−2.63*
	SLC7A11	0.008	0.99	0.27	−0.11 ± 0.21	0.04 ± 0.20	0.01 ± 0.17	0.16 ± 0.16	0.55	0.77	0.63
	SLC45A2	0.297	0.70	15.05***	0.64 ± 0.18	0.65 ± 0.18	−0.50 ± 0.15	−0.49 ± 0.14	−6.60***	0.08	−0.6
	OCA2	0.283	0.72	14.08***	0.37 ± 0.18	0.66 ± 0.17	−0.61 ± 0.14	−0.31 ± 0.13	−5.69***	1.95	1.96
	TYR	0.343	0.66	18.66***	0.40 ± 0.18	0.76 ± 0.17	−0.68 ± 0.14	−0.33 ± 0.14	−6.47***	2.43$^{M.S.}$	−1.39
	TYRP1	0.277	0.72	13.67***	0.55 ± 0.18	0.61 ± 0.17	−0.50 ± 0.15	−0.44 ± 0.14	−5.97***	0.36	1.14

[†]Given are the discriminability; degree of correlation of each gene to the discriminant functions (larger values indicate a higher importance in discriminating among groups) and the Wilk's statistics and the associated p-value, which indicates whether the contribution of each gene to discriminate among group is significantly larger than by chance.
[‡]Univariate test were run for each gene separately considering the effect of both *MC1R* genotype and sex (the t statistics and associated p-values are given). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $^{M.S.}P < 0.05$ before applying Benjamini-Hochberg corrections for multiple testing.

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PCSK5 and, negatively, with the expression of *ASIP*-AC, *ASIP*-AD, *MC1R* and *KIT* (Table 1B).

Differences between genotypes in the coexpression of melanocortin and melanogenic-related genes

Gene coexpression patterns were relatively similar between all genotypes and sexes (matrices of pairwise correlations between genes were positively correlated among all groups, Table 2). Melanocortin genes with a larger contribution to dissimilarities among groups (i.e. their exclusion from the Mantel's tests resulted in higher correlations, Fig. 3) were *AGRP* and *ASIP* (mainly *ASIP*-AC and *ASIP*-AD variants). *AGRP* was coexpressed with *MC1R*, *ASIP*, *PCSK2*, *PCSK5* and most melanogenic-related genes (except for *TYR* and *TYRP1*, Fig. 4) in $MC1R_{WW}$ males, whereas it was coexpressed only with *ASIP*, *PCSK2*, *CREB1*, *MITF* and *SLC7A11* in $MC1R_{WW}$ females and with none of the studied genes in $MC1R_{RW}$ individuals. *ASIP*-AC variant was coexpressed with *DCT* and *MITF* variant 2 only in $MC1R_{WW}$ males and with *ASIP*-BC variant only in $MC1R_{WW}$ individuals (Fig. 4). This gene was coexpressed with *TYRP1* only in $MC1R_{RW}$ females. *ASIP*-AD variant was coexpressed with other *ASIP* variants, *MITF* (variant 2) and *DCT* in $MC1R_{WW}$ males and only to *ASIP* variants in $MC1R_{WW}$ females and $MC1R_{RW}$ individuals. Melanocortin genes contributing to similarities among groups (i.e. their exclusion from the Mantel's tests resulted in lower correlations; Fig. 3) were *MC1R*, and *PCSK2* and *PCSK5*. Among melanogenic-related genes, *MITF*-V2 and CREB1 significantly contributed to dissimilarities among groups due to being coexpressed with *AGRP* and *ASIP* variants only in $MC1R_{WW}$ males but not in the other groups (Figs 3 and 4). *SLC7A11*, *SLC45A2* and *TYR* significantly contributed to group similarities (Fig. 3).

Relationship between melanocortin gene expression and within-genotype variation in feather melanin content

In $MC1R_{WW}$ individuals, eight models on the association between pheomelanin content and gene expression of melanocortin genes were selected owing to their ΔAICc value below 2 (Table 3). The model with the lowest AICc value included sex, *MC1R* and *PCSK2* as well as the interactions between these two genes and sex. The next six models also included these terms (accumulated

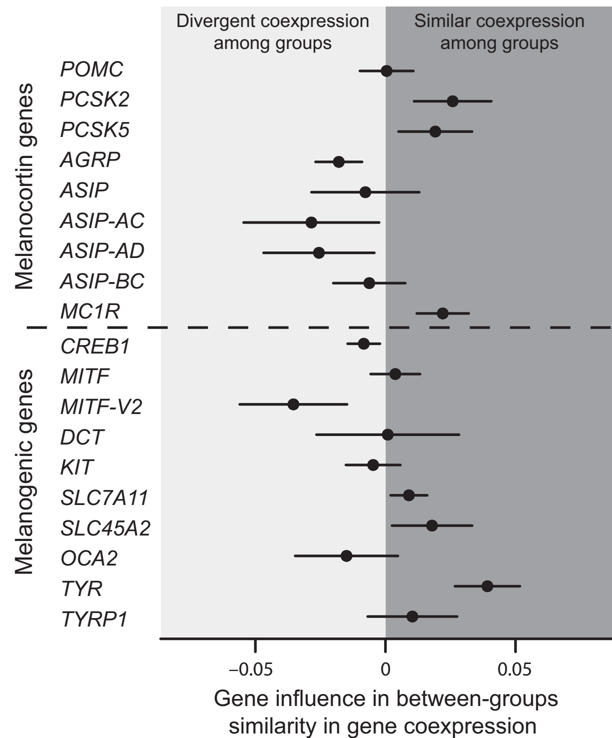

Fig. 3 Gene influence in between-groups similarities and divergence in gene coexpression in nestling barn owls. For each gene, we show the mean and the 99% IC of the difference in the correlation coefficients after excluding the gene from the Mantel's tests. Positive values indicate that gene exclusion resulted in lower correlations and that the excluded gene has a similar coexpression pattern between groups. Negative values indicate that gene exclusion resulted in higher correlations and that the excluded gene has a divergent coexpression pattern between groups. For instance, *PCSK2* shows a positive mean value given that it is expressed in a coordinated fashion with other genes of the melanocortin system and the melanogenic-related genes (Fig. 4).

Table 2 Results from the Mantel's tests on the between-groups correlations in gene coexpression in nestling barn owls

		$MC1R_{WW}$		$MC1R_{RW}$	
		Males	Females	Males	Females
$MC1R_{WW}$	Males	—	0.767*** (0.700–0.842)	0.660*** (0.551–0.756)	0.602*** (0.481–0.725)
	Females	—	—	0.798*** (0.738–0.865)	0.587*** (0.477–0.734)
$MC1R_{RW}$	Males	—	—	—	0.609*** (0.467–0.736)
	Females	—	—	—	—

Shown are the correlation values, which measure the degree of resemblance between the pairwise gene correlation matrices of two groups, and the associated 99% interval of confidence.
Asterisks denote the significance of the correlation (***$P < 0.001$).

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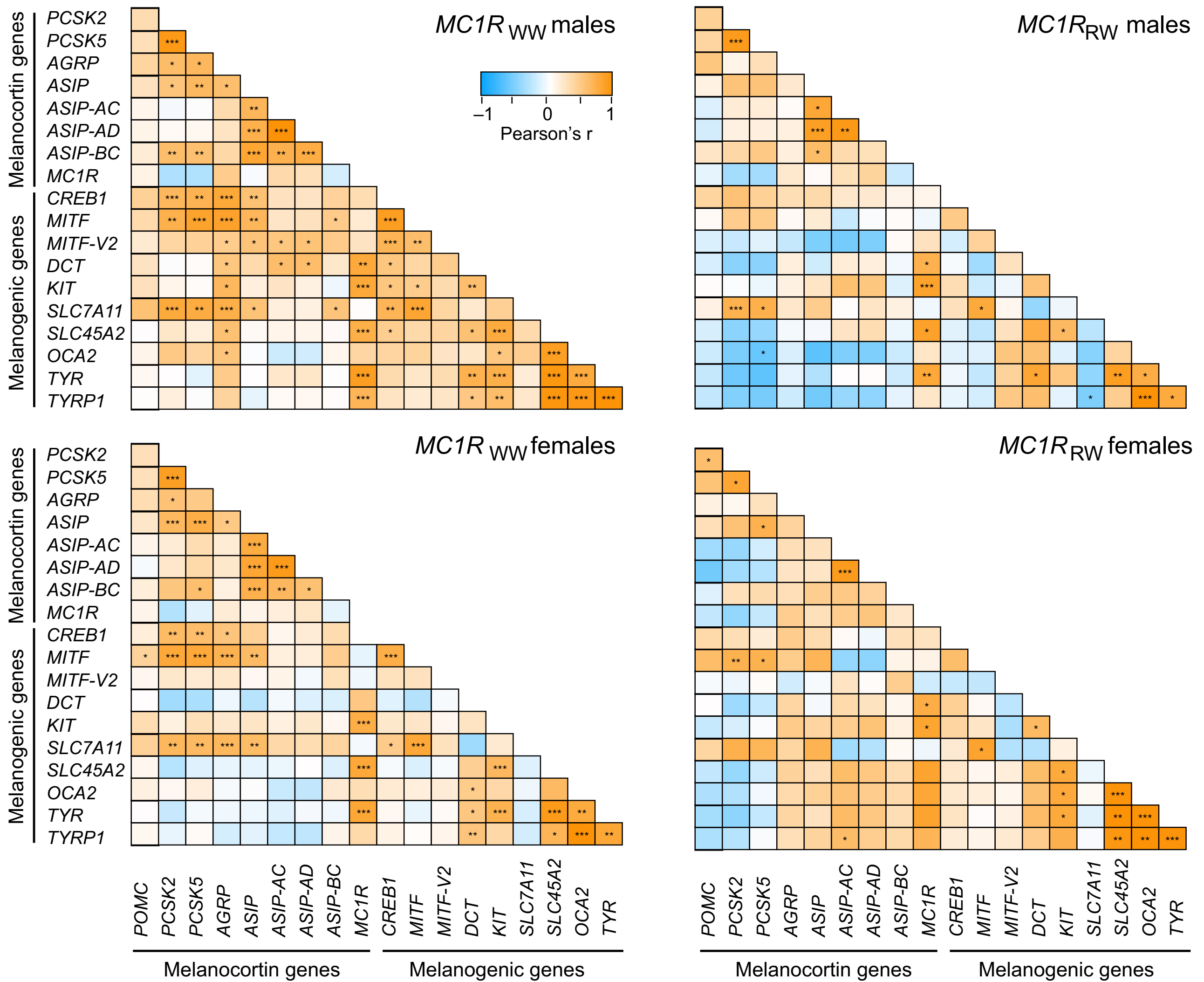

Fig. 4 Coexpression of melanocortin and melanogenic-related-related genes in nestling barn owls. Shown are the Pearson's correlation matrices for each *MC1R* genotype and sex. Significant pairwise correlations after correcting for multiple testing are denoted with an asterisk. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

model weight = 0.80) but considered the effect of one more gene at a time: *POMC*, *ASIP*-AC, *AGRP*, *ASIP*-BC, *ASIP* and *ASIP*-AD (listed according to the model ranks, Table 3). The eighth model considered sex, *AGRP* and its interaction with sex, and *PCSK2*. In $MC1R_{RW}$ individuals, four models were selected owing to their ΔAICc value below 2 (Table 3). All the models included sex (accumulated weight = 0.65) and three of the four best models included the effect of *POMC* (accumulated weight = 0.54). Additionally, the best model included *MC1R* and its interaction with sex as well as the effects of *ASIP*-AD and *ASIP*-BC. The second best model also included these terms except for the interaction between *MC1R* and sex (Table 3). Similar results were observed for eumelanin content except that for $MC1R_{RW}$ where *PCSK2*, total *ASIP* and age which were included in the top models (Table S5, Supporting information).

When individuals of both genotypes were considered together in the same analyses (Table S4, Supporting information), we could confirm that the expression of *PCSK2* (genotype × *PCSK2*: $F_{1,61} = 6.68$, $P = 0.012$) and *POMC* (genotype × *POMC*: $F_{1,64} = 6.24$, $P = 0.015$) was differently associated with pheomelanin content in each genotype. However, when the effect of *PCSK2* and *POMC* in interaction with genotype was considered together in the same model (second best model for $MC1R_{WW}$ individuals in Table 3, see also Table S4b, Supporting information), only the interaction between *PCSK2* and genotype was significant. The expression of *AGRP* was also associated with pheomelanin depending on the genotype and sex (genotype × sex × *AGRP*: $F_{1,60} = 4.84$, $P = 0.032$, Table S4, Supporting information), and its effect was not dependent on the effect of *PCSK2* (Table S4h, Supporting information). The

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Table 3 Relationship between the expression of melanocortin genes and feather pheomelanin content in nestling barn owls of different *MC1R* genotype

MC1R genotype	Predictors	AICc*	ΔAICc†	w‡
$MC1R_{WW}$	Sex + *MC1R* + *MC1R* × Sex + *PCSK2* + *PCSK2* × Sex	1023.3	—	0.18
	Sex + *MC1R* + *MC1R* × Sex + *PCSK2* + *PCSK2* × Sex + *POMC*	1023.8	0.53	0.13
	Sex + *MC1R* + *MC1R* × Sex + *PCSK2* + *PCSK2* × Sex + *ASIP-AC*	1023.8	0.56	0.13
	Sex + *MC1R* + *MC1R* × Sex + *PCSK2* + *PCSK2* × Sex + *AGRP*	1024.5	1.25	0.09
	Sex + *MC1R* + *MC1R* × Sex + *PCSK2* + *PCSK2* × Sex + *ASIP-BC*	1024.6	1.37	0.09
	Sex + *MC1R* + *MC1R* × Sex + *PCSK2* + *PCSK2* × Sex + *ASIP*	1024.6	1.39	0.09
	Sex + *MC1R* + *MC1R* × Sex + *PCSK2* + *PCSK2* × Sex + *ASIP-AD*	1024.7	1.45	0.09
	Sex + AGRP + AGRP × Sex + *PCSK2*	1025.0	1.76	0.07
$MC1R_{RW}$	Sex + *MC1R* + *MC1R* × Sex + *POMC* + ASIP-AD + ASIP-BC	329.2	—	0.24
	Sex + *MC1R* + *POMC* + ASIP-AD + ASIP-BC	329.5	0.29	0.21
	Sex	330.7	1.51	0.11
	Sex + *POMC*	330.9	1.74	0.10

Shown are the best models based on AICc (ΔAICc < 2) for each genotype pigment.
*AIC value corrected for small simple sizes.
†Difference in a model's AICc to the best-ranked model's AIC.
‡Model weight: probability of the model given the data.

expression of *PCSK2* was positively related with pheomelanin content in $MC1R_{WW}$ individuals (t_{61} = 3.58, P < 0.001) but not in $MC1R_{RW}$ individuals (t_{61} = 0.82, P = 0.41; Fig. 5A). *POMC* expression was negatively associated with pheomelanin content in $MC1R_{RW}$ individuals (t_{64} = 2.38, P = 0.020) but not in $MC1R_{WW}$ individuals (t_{64} = 0.82, P = 0.42; Fig. 5B). *AGRP* expression was significantly and positively associated with pheomelanin content in $MC1R_{WW}$ males (t_{64} = 2.71, P = 0.009), and no significant association was found in $MC1R_{WW}$ females or in $MC1R_{RW}$ individuals (all P > 0.39; Fig. 5C). No interactions with *MC1R* genotype were found for the expression of any other of the genes highlighted in Table 3: *MC1R*, *ASIP*, *ASIP*-AC, *ASIP*-BC and *ASIP*-AD (Table S4, Supporting information).

Discussion

MC1R plays a predominant role in explaining colour variation in wild species (Mundy 2005) because it is one of the major regulators of the melanin pathway (García-Borrón *et al.* 2005; Ito & Wakamatsu 2011). However, we barely understand the consequences of *MC1R* mutations on the melanocortin system, the genetic network that comprises the *MC1R* as well as the different genes coding for or processing the agonists and antagonists that regulate the MC1R (Gantz & Fong 2003; Ducrest *et al.* 2008). Here, we investigated using developing feathers of nestling barn owls how the expression levels of melanocortin and melanogenic genes and the association between pigmentation and the expression of melanocortin genes vary between *MC1R* genotypes. Our findings are therefore restricted to the studied age class and tissue, as well as to the developmental phase in which the feathers were sampled. We showed that the *MC1R* variant V126I present in European barn owls (San-Jose *et al.* 2015; Burri *et al.* 2016), in gyrfalcons, *Falco rusticolus* (Johnson *et al.* 2012; Zhan *et al.* 2012), and domestic ducks, *Anas platyrhynchos* (Yu *et al.* 2013), is associated with differences not only in the expression of melanogenic-related genes but also in the expression of important melanocortin genes, such as the *MC1R* itself and its antagonist, *ASIP* (Fig. 2B). Similarly, the patterns of coexpression of melanocortin genes slightly differed between *MC1R* genotypes, with important regulatory genes, such as *AGRP* or *ASIP*, being coexpressed with different melanocortin and melanogenic-related genes in each genotype (Figs 3 and 4). As observed in the tawny owl, *Strix aluco* (Emaresi *et al.* 2013), our results showed that colour variation within genotypes (i.e. the variation that is not explained by the *MC1R*) is associated with the expression of melanocortin genes, such as *PCSK2*, *POMC* and *AGRP*, but, more importantly, our results indicated that these associations are dependent on the *MC1R* genotype (Fig. 5). Together, these findings suggest that *MC1R* variants can associate with further changes in other components of the melanocortin system. This is of high importance because *MC1R* might alter how coloration relates to other traits regulated by the melanocortin system.

MC1R genotypes differ in the expression of melanocortin genes and melanogenic-related genes

Differences in gene expression allowed us to successfully distinguish between individuals of different *MC1R* genotype and sex, except for $MC1R_{WW}$ females whose

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intermediate coloration and gene expression levels between $MC1R_{WW}$ males and $MC1R_{RW}$ females could have rendered their classification more difficult. As expected, the pDF analysis highlighted that melanogenic-related genes involved in the melanin synthesis (*TYR*, *TYRP1*, and *DCT*), melanosome trafficking (*SLC45A2* and *OCA2*), and the *KIT* receptor, involved in the mitogen-activated protein kinase pathway, are more important to discriminate between genotypes. We observed that *MC1R* genotypes were associated with large differences in the expression of these melanogenic-related genes. All these genes showed a higher expression in the *rufous* genotype ($MC1R_{RW}$) than in the *white* genotype ($MC1R_{WW}$) and these differences in expression were significantly associated with the transmission of *MC1R* alleles except for *DCT*. A higher MC1R activity upregulates the transcription of *TYR*, *TYRP1*, *SLC45A2*, *OCA2* and *KIT* probably through *MITF* (reviewed in Levy *et al.* 2006; Cheli *et al.* 2010), suggesting that the *rufous* and *white MC1R* alleles code for a more and a less active MC1R protein, respectively (Hoekstra *et al.* 2006; Rosenblum *et al.* 2010), or affect MC1R protein trafficking (Sánchez-Laorden *et al.* 2009) or dimerization (Zanna *et al.* 2008). The fact that the polymorphic site is located in the third transmembrane domain of the MC1R which plays a key role in its activation and that mutations at this site in the human MC1R shows an almost complete loss of cAMP activation and α-MSH binding (García-Borrón *et al.* 2005; Pérez Oliva *et al.* 2009) support that the *rufous* and *white MC1R* alleles in the barn owl may respond to α-MSH more and less efficiently, respectively.

We observed that *MC1R* genotypes also differed in the expression levels of melanocortin genes, particularly *MC1R* itself and its antagonist, *ASIP*, which were also highlighted by the pDF analysis because of their contribution to discriminate between genotypes. However, we found that the differences observed can be explained *via* allelic transmission only for *MC1R* expression, whereas for *ASIP*, the differences observed between *MC1R* genotypes are more likely due to population stratification. The observed upregulation of

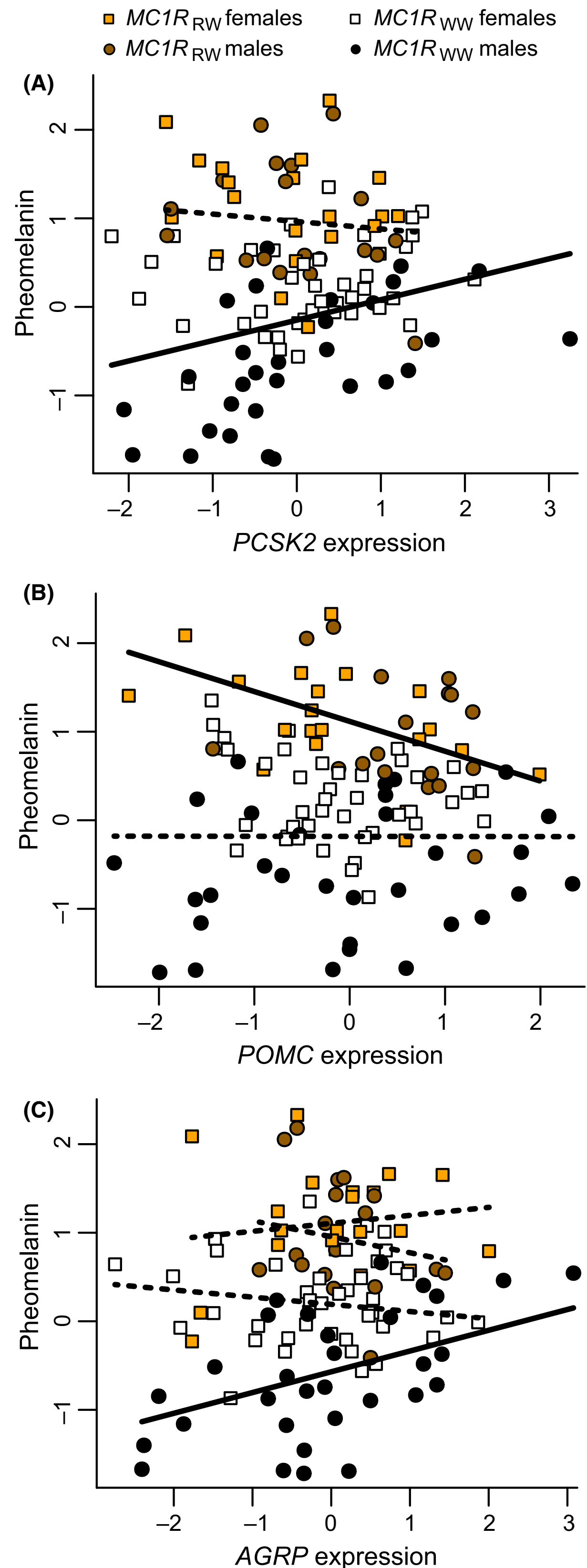

Fig. 5 Within-genotype variation in the relationship between feather pheomelanin content and the expression of melanocortin genes (A: *PCSK2*, B: *POMC* and C: *AGRP*) in nestling barn owls. Shown are the observed standardized values and the regression lines per genotype estimated from the linear mixed models (see text). In panel C, regression lines per sex and genotype are shown given the significant interaction between sex, genotype and *AGRP* expression (see text). Continuous and discontinuous lines denote regression lines significantly and nonsignificantly different from zero, respectively.

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MC1R expression in $MC1R_{RW}$ individuals (Fig. 2) may occur because MC1R activity (expected to be higher in this group) can promote the transactivation of the *MC1R* gene through a positive feedback loop via *MITF* (Aoki & Moro 2002). It is surprising that between-genotypes differences in *ASIP* expression could arise from population stratification associated with *MC1R* genotypes, given that assortative pairing with respect to *MC1R* genotype is rare (Ducret *et al.* 2016) and the absence of genetic differentiation between *MC1R* genotypes in the barn owl (L.M. San-Jose, unpublished data; Burri *et al.* 2016). Interestingly, because *ASIP* expression can downregulate melanocytes differentiation in birds, as suggested by Lin *et al.* (2013a,b), the higher expression of ASIP in $MC1R_{WW}$ individuals could contribute to produce their whiter coloration and, contrarily, the lower expression of ASIP in $MC1R_{RW}$ individuals could contribute to their redder appearance. The association between *ASIP* expression and *MC1R* alleles could be explained by selection, which could be acting on the *MC1R* and a putative regulator of *ASIP* expression, promoting the association of alleles inducing colour changes in the same direction. Unravelling why *MC1R* genotypes differ in *ASIP* expression in the barn owl, as also observed in beach mice, *Peromyscus polionotus* (Steiner *et al.* 2007), is of large interest given the potentially predominant role of these two genes in the evolution of vertebrate colour patterns (Mills & Patterson 2009; Olsson *et al.* 2013; Roulin & Ducrest 2013) and the potential role of *ASIP* in regulating other traits than coloration, such as energy expenditure and food intake (Yabuuchi *et al.* 2010; Agulleiro *et al.* 2014; Ito *et al.* 2015).

Differences in gene coexpression and in the association between melanocortin genes and melanin synthesis

Previous studies showed that melanocortin genes are coexpressed with melanogenic-related genes (Emaresi *et al.* 2013) and associated with variation in melanin-based coloration in vertebrates (Emaresi *et al.* 2013; Monti *et al.* 2013; Ducrest *et al.* 2014; Poelstra *et al.* 2015). Here, we observed that the coexpression of melanocortin genes with melanogenic-related and other melanocortin genes slightly differs between genotypes and sexes and that the association between feather pheomelanin content and the expression of melanocortin genes can differ between genotypes and in some cases within sexes of the same genotype (Fig. 5). This suggests that the regulatory role of the melanocortin genes might be somehow altered as a direct or indirect consequence of variation at the *MC1R*.

In line with previous findings in growing feathers of tawny owls (Emaresi *et al.* 2013), we showed that *PCSK2* expression is related to pheomelanin content. However, our study showed that in barn owls, this association is dependent on the existing variation at the *MC1R*. Whereas *PCSK2* was positively related with pheomelanin content in $MC1R_{WW}$ individuals, no significant association was found for individuals carrying the *rufous* allele. Two mechanisms could explain why *PCSK2* expression is associated with coloration in $MC1R_{WW}$ but not in $MC1R_{RW}$ individuals. The proprotein convertases, such as the one encoded by *PCSK2*, PC2, cleave the POMC prohormone at dibasic sites to produce functional peptides (ACTH and MSH peptides). PC2 further cleaves ACTH to release α-MSH in specific tissues including the skin (Mazurkiewicz *et al.* 2000; Kauser *et al.* 2005). Finally, α-MSH activates MC1R, inducing melanin synthesis (García-Borrón *et al.* 2005). If, as discussed above, the *rufous* allele encodes for a *MC1R* variant that binds α-MSH more efficiently or has a more efficient cAMP-coupling activity than the variant encoded by the *white* allele, we could expect that the same amount of α-MSH will result in a larger amount of melanin synthesized in individuals carrying the *rufous* allele than in individuals carrying the *white* allele. However, increasing concentrations of α-MSH will have no or little effect on melanin synthesis if the *rufous* variant of the *MC1R* reaches its maximum activity at low physiological α-MSH concentrations, or if melanin synthesis has a saturation point due to limited resources for melanin synthesis (e.g. cysteine, tyrosine intracellular levels) (Más *et al.* 2003). Alternatively, we could expect an association only in $MC1R_{WW}$ individuals if the *rufous* allele encodes for a constitutively active MC1R variant that no longer requires α-MSH binding to promote the synthesis of melanin (Robbins *et al.* 1993). However, this later explanation seems less likely because under such a scenario, we would have expected that *PCSK2* is differently coexpressed between groups and not, as suggested by our data, to have a more similar coexpression pattern among groups (Fig. 3).

We also observed that the expression of *AGRP* and *POMC* was differently associated with pheomelanin content in the different groups (Fig. 5), although these findings must be cautiously considered. On the one hand, the qPCR primers and probes for *AGRP* amplified pre-mRNA of *AGRP* (see Materials and methods), which indirectly reflects the mature, functional mRNA levels of *AGRP*. In fact, the expression levels of the mature *AGRP* transcript appear to be lower than the expression of its pre-mRNA (A.-L. Ducrest & A. Roulin, unpublished data), which could be due to post-transcriptional regulation involving mRNA stability. On the other hand, the majority of the detected *POMC* transcripts consisted of mRNA with a transcription start site located in the exon 3 (see Materials and methods). This transcript may not be functional because it might be

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translated in a protein that lacks the signalling peptides that are important for POMC traffic through the secretory granules, where the POMC prohormone will be processed into mature peptides (Clark *et al.* 1990). Moreover, the effect of *POMC* on pheomelanin content was not detected when also considering in the same model the effect of *PCSK2* (see Results). Nevertheless, the association between the expression of *AGRP* and *POMC* genes and pheomelanin content is worth noting given the little knowledge about the function of *AGRP* in the integument, where it is nevertheless largely expressed (Takeuchi *et al.* 2000; Kurokawa *et al.* 2006; Murashita *et al.* 2009), and the unknown function of the truncated *POMC*, which has been repeatedly detected in different tissues and in different species, including humans (Lacaze-Masmonteil *et al.* 1987; Zapletal *et al.* 2013). We observed that *AGRP* expression was positively related to pheomelanin content in $MC1R_{WW}$ males but not in $MC1R_{WW}$ females or in $MC1R_{RW}$ individuals. This is congruent with the pattern of coexpression of *AGRP* in $MC1R_{WW}$ males, where, contrarily to the other groups, *AGRP* expression was positively correlated to most melanogenic-related genes. A previous study in chickens, *Gallus gallus*, suggested an antagonistic role of *AGRP* on *MC1R* (Takeuchi *et al.* 2000; Li *et al.* 2011). However, we found little support for this hypothesis given that if *AGRP* binds antagonistically to MC1R, as *ASIP* does, we would have expected *AGRP* expression to be negatively and not positively associated with most melanogenic-related genes. The expression of *POMC*, the main actor of the melanocortin system (Krude & Grüters 2000), was negatively associated with pheomelanin content in $MC1R_{RW}$ individuals. Although the functionality of this truncated form is still not known, a previous study showed that the expression of the truncated *POMC* results in a reduced secretion of ACTH (Clark *et al.* 1990; Rees *et al*. 2002). This could actually explain the observed negative association between colour and *POMC* expression, supporting the regulatory role of truncated *POMC* expression over functional *POMC* expression suggested by some authors (Rees *et al.* 2002).

MC1R and pleiotropy in the melanocortin system

The fact that the melanocortin system pleiotropically regulates different phenotypic traits (Ducrest *et al.* 2008; Roulin & Ducrest 2011; Reissmann & Ludwig 2013) and that it is highly preserved in vertebrates (Cortés *et al.* 2014) offers a parsimonious general explanation to the associations that have been repeatedly observed in different vertebrates between coloration and distinct behavioural, physiological and morphological traits (Ducrest *et al.* 2008; L.M. San-Jose & A. Roulin, unpublished data). This hypothesis predicts that genes within the melanocortin system having more pleiotropic effects (i.e. those coding for the hormonal agonist and antagonists: *POMC*, *PCSK1/3*, *PCSK2*, *ASIP* and *AGRP*) will have a major influence in explaining colour differences between individuals, given that these genes can also influence the activity of other melanocortin receptors and thereby the association between coloration and other traits (Ducrest *et al.* 2008, 2014; Emaresi *et al.* 2013). In contrast to these genes, *MC1R* has fewer pleiotropic effects (but see Mogil *et al.* 2003; Gangoso *et al.* 2011) and it is therefore expected to have a minor influence in driving colour-trait associations. Current data indicate that the *MC1R* has nevertheless a major effect in coloration and its general importance in mediating adaptive colour variation in vertebrates is well acknowledged (Mundy 2005; Manceau *et al.* 2010). Its general importance might have been overestimated owing to the use of the candidate approach in QTL studies of coloration and, thus, less biased approaches (e.g. whole-genome sequencing, Poelstra *et al.* 2014) are still needed to have a more accurate measurement of the importance of *MC1R* relative to other genes within and outside the melanocortin system. However, regardless of its relative importance, the fact that *MC1R* has been shown to largely impact coloration in several species highlights the importance of hypothesizing how variation at the *MC1R* coexists with the hypothesis of 'pleiotropy in the melanocortin system' and the interesting avenues of research that this offers.

As our results suggest, *MC1R* mutations may block the relationship existing between coloration and the expression of melanocortin genes. This might decouple coloration from its association with other traits and thereby alter the potential adaptive value of such associations. In this scenario, we could therefore expect that colour response to selection will depend on the interaction between the selective forces acting at the *MC1R* gene, favouring large colour changes to adapt for instance to local environmental conditions (Rosenblum *et al.* 2004; Burri *et al.* 2016), and the putative selective forces that could explain the observed association between colour and other phenotypes (e.g. Emaresi *et al.* 2014). If selection strongly acts on new *MC1R* variants promoting adaptation to local conditions, previous existing associations between coloration and other traits could be hindered (like our data suggest for the *rufous* variant). If, contrarily, selective forces promoting associations between colour and other phenotypes are relatively stronger, colour variation will be expected to result from variation at the melanocortin genes that can pleiotropically drive such associations (Ducrest *et al.* 2008). In this scenario, *MC1R* mutations that hinder the associations between coloration and other traits will be deselected and variants that are more sensitive to the control of the

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melanocortin system (like, for instance, our data suggest for the *white* variant) could be expected to be favoured by selection. The most interesting scenarios would be those where the different selective forces are balanced. For instance, colour-trait associations seem to have evolved in a communication context (e.g. to communicate social status, resource holding capacity: Santos *et al.* 2011). In this scenario, *MC1R* mutants may appear dishonest signallers, because their coloration will be dissociated from the quality or message conveyed. If *MC1R* mutants are not strongly favoured by selection (as it seems to occur in our population where individuals of different genotypes coexist and interbreed), the occurrence of dishonest signallers will jeopardize the evolutionary stability of coloration as a signal (Bradbury & Vehrencamp 2011), promoting signal receivers to disregard coloration as a signal or the evolution of mechanisms reinforcing signal honesty (e.g. through multiple signalling: Candolin 2003).

To the best of our knowledge, the present study is the first effort conducted to unravel how variation at the *MC1R* interacts with the genes of the melanocortin system. The fact that distinct *MC1R* genotypes differ in the expression of other melanocortin genes, like observed here for *ASIP*, and in the association between important melanocortin genes (*PCSK2*) and coloration highlights the relevance of future studies investigating whether *MC1R* mutants also differ in other traits than coloration and/or in the association between coloration and other phenotypes. It would be also of great interest to conduct similar studies to this one in species with *MC1R* mutations at different nucleotide sites, given that different mutations and different genetic backgrounds can have different consequences for the functioning of the MC1R (García-Borrón *et al.* 2005). These studies would help to further understand the genetic architecture of melanin-based colorations, and the potential interactions and constraints among the genes that regulate such a widespread trait in animals.

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A.R. obtained the funding; A.R. and A.-L.D. designed the study. A.-L.D., C.S., V.D. and H.R. conducted the analyses on gene expression and the genotyping at the MC1R gene; K.W. conducted feather pigment analyses; L.M.S.-J. conducted the statistical analyses; and L.M.S.-J., A.-L.D. and A.R. wrote the manuscript. All the authors read and provided input on the manuscript.

Data accessibility

Gene expression data and phenotypic data are available from the Dryad repository: http://dx.doi.org/10.5061/dryad.c18hh. Sequences are deposited in GenBank (see Accession nos in Table S1, Supporting information).

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Materials and methods.

Table S1 Summary of the sequenced genes used for qPCR.

Table S2 Quantitative primers and probes.

Fig. S1 *ASIP* 5′ splice variants detected in growing breast feathers.

Appendix S2 Results.

Table S3 Results from the transmission disequilibrium tests.

Table S4 Results from linear models on the relationship between pheomelanin content and the expression of melanocortin genes.

Table S5 Relationship between the expression of melanocortin genes and feather eumelanin content in nestling barn owls of different *MC1R* genotype.

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